Microbial AHL signalling modulates plant resistance to insects

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Abstract

Insect infestation of crops represents a major threat to agriculture and food supply worldwide. Counteracting pesticides are often hazardous for consumer's health and the environment. As a more sustainable solution, beneficial rhizobacteria can induce plant resistance to insects. The targeted selection of these microbes and their efficient application in the field though requires a deep understanding of functional interaction mechanisms. It is largely unresolved which microbial compounds are involved in microbe-plant-insect interaction. Microbial *N*-acyl-homoserine lactone (AHL) signalling molecules might play a key role in interkingdom communication. However, it is unknown if AHL can modulate plant resistance to herbivorous insects.

The aim of this thesis was to understand whether microbial AHL signalling modulates plant insect suppression. This question was addressed using the rhizobacteria *Acidovorax radicis* N35e and *Rhizobium radiobacter* F4, the model crop barley (*Hordeum vulgare*) and English grain aphids (*Sitobion avenae*). Upon root inoculation, aphid biocontrol effect of the AHL-producing test strains was compared to the respective mutants impaired in AHL sensing and signalling across different barley cultivars and cultivation conditions. Complementation of AHL mutants with synthetic OH-C10-HSL and C6-HSL should subsequently disentangle direct effects of AHL molecules from indirect AHL-dependent self-regulation effects. Additionally, indirect AHL effects on plant aphid suppression via the rhizosphere microbiome were examined.

Inoculation of OH-C10-HSL-producing *A. radicis* N35e consistently increased plant aphid suppression across different barley cultivars. The test strain *R. radiobacter* F4, producing a mix of AHLs, showed inconsistent biocontrol effects and was therefore not further analyzed. Unexpectedly, when AHL synthesis was impaired in an *A. radicis* N35e mutant, aphids were even more suppressed compared to the AHL-producing strain in soil and as a tendency also under axenic cultivation conditions. This aphid suppression increase could be traced back to a significantly higher root colonization by the AHL synthesis mutant compared to the AHL-producing strain. Simultaneously, activation of *PR* plant immune response genes in barley cultivar Barke and suppression of *PR* genes in cultivar Scarlett indicated that the *A. radicis* N35e AHL synthesis mutant was perceived differently by the plant than the AHL-producing

strain. The different immune pathway stimulation in both barley cultivars suggested that the plant genotype strongly influenced the plant response towards colonization of the different *A. radicis* strains. Application of synthetic OH-C10-HSL tended to have a positive effect on plant aphid suppression. As a positive stimulus, AHL might have directly mitigated the plant response towards *A. radicis* N35e facilitating a beneficial interaction. In addition, an AHL-mediated change in bacterial gene expression might have caused enhanced colonization capacity, possibly alongside with an increase in other beneficial traits. The rhizosphere microbiome was significantly altered by AHL synthesis mutant inoculation, likely due to its increased colonization. However, no other microbial genus could be causally linked to aphid suppression changes. Instead, upregulation of flavonoid biosynthesis in barley leaves upon AHL synthesis mutant colonization might mechanistically explain the altered aphid suppression. Complementation of AHL sensing and signalling mutants with OH-C10-HSL and C6-HSL confirmed that overlapping direct and indirect AHL effects of different directions most probably contributed to the complex microbe-plant-insect interactions. All direct and indirect AHL effects were strongly determined by the sensitivity of the barley cultivar and the cultivation system. These findings underline the complexity of AHL effects in interkingdom communication.

Overall, this thesis confirms that microbial AHL signalling can modulate the interplay between microbes, plants and insects. Hence, this work highlights the necessity to better understand the multiple factors involved in microbially-induced insect biocontrol for its successful application in a more sustainable agriculture.

Zusammenfassung

Insektenbefall von Getreide stellt die Landwirtschaft und Nahrungsmittelproduktion weltweit vor ein großes Problem. Chemische Pestizide, die dem Befall entgegenwirken sollen, sind schädlich für die Gesundheit von Konsument*innen und die Umwelt. Eine nachhaltigere Lösung können nützliche Wurzelbakterien sein, die Pflanzen resistenter gegenüber Insekten machen. Für die gezielte Auswahl solcher Mikroorganismen und deren effizienten Einsatz auf dem Feld braucht es jedoch ein tiefgehendes Verständnis funktioneller Interaktionsmechanismen. Welche mikrobiellen Moleküle in die Mikroben-Pflanzen-Insekten-Interaktion eingebunden sind, ist dabei weitgehend ungeklärt. Mikrobielle *N*-Acyl-Homoserine-Lactone-Signalmoleküle (AHL) könnten bei der artübergreifenden Kommunikation eine Schlüsselrolle spielen. Allerdings ist unbekannt, ob AHL die Pflanzenresistenz gegenüber pflanzenfressenden Insekten verändern kann.

Das Ziel dieser Doktorarbeit war es, zu verstehen, inwiefern Signalweiterleitung durch mikrobielles AHL die pflanzliche Insektenabwehr modulieren kann. Um sich dieser Frage zu nähern, wurden die Wurzelbakterien *Acidovorax radicis* N35e und *Rhizobium radiobacter* F4, der Modellorganismus Gerste (*Hordeum vulgare*) und die Große Getreideblattlaus (*Sitobion avenae*) verwendet. Im Zuge von Wurzelinokulationen wurde der Schädlingsbekämpfungseffekt der AHL-produzierenden Teststämme mit jenem der entsprechenden Mutanten mit defekter AHL-Produktion und AHL-Detektion verglichen. Die Supplementierung der AHL-Mutanten mit synthetischem OH-C10-HSL und C6-HSL sollte anschließend direkte Effekte der AHL-Moleküle gegenüber indirekten Effekten durch die AHL-abhängige Selbstregulierung aufzeigen. Zusätzlich wurden indirekte AHL-Effekte, die sich über das Rhizosphärenmikrobiom auf die pflanzliche Blattlausbekämpfung auswirken, untersucht.

Die Inokulierung von OH-C10-HSL-produzierendem *A. radicis* N35e erhöhte die pflanzliche Blattlausabwehr durchwegs über verschiedene Gerstenkultivare hinweg. Der Teststamm *R. radiobacter* F4, der eine Mischung aus AHLs produziert, zeigte inkonsistente Abwehreffekte und wurde daher nicht weiter analysiert. Unerwarteterweise wurden Blattläuse sogar noch stärker unterdrückt, wenn in *A. radicis* N35e die AHL-Synthese defekt war im Vergleich zum AHL-produzierenden Stamm. Dies erfolgte unter Kultivierungsbedingungen in Erde, teils aber auch unter sterilen Bedingungen. Diese erhöhte Blattlausabwehr konnte auf eine signifikant

höhere Wurzelkolonisierung durch die AHL-Synthesemutante verglichen mit dem AHL-produzierenden Stamm zurückgeführt werden. Zugleich wurden *PR*-Gene der pflanzlichen Immunantwort durch die *A. radicis* N35e AHL-Synthesesmutante im Gerstenkultivar Barke aktiviert und im Kultivar Scarlett supprimiert, was darauf schließen lässt, dass die Pflanze die AHL-Mutante auf andere Weise wahrnahm als den AHL-produzierenden Stamm. Die Stimulierung unterschiedlicher Immunwege in beiden Gerstenkultivaren lässt dabei vermuten, dass die Reaktion der Pflanze auf die Kolonisierung der verschiedenen *A. radicis*-Stämme entscheidend vom pflanzlichen Genotyp abhängt. Die Zugabe von synthetischem OH-C10-HSL zeigte einen tendenziell positiven Effekt auf die Blattlausabwehr der Pflanze. Als positiver Stimulus könnte AHL die Immunantwort der Pflanze gegenüber *A. radicis* N35e direkt abgeschwächt und eine Interaktion begünstigt haben. Zusätzlich verbesserten AHL-bedingte Veränderungen in der bakteriellen Genexpression deren Kolonisierungsfähigkeit, zusammen mit womöglich anderen nützlichen Eigenschaften. Das Rhizosphärenmikrobiom veränderte sich durch die Inokulation der AHL-Synthesemutante signifikant, wahrscheinlich aufgrund der verstärkten Kolonisierung durch die Mutante. Allerdings konnte kein anderer mikrobieller Genus kausal mit den Veränderungen in der Blattlausabwehr in Verbindung gebracht werden. Stattdessen könnte eine Hochregulierung der Flavonoid-Biosynthese in Gerstenblättern als Reaktion auf die Kolonisierung der AHL-Synthesemutante die veränderte Blattlausabwehr mechanistisch erklären. Supplementierung der AHL-Mutanten mit OH-C10-HSL and C6-HSL bestätigte, dass überlappende direkte und indirekte AHL-Effekte höchstwahrscheinlich zu den komplexen Mikroben-Pflanzen-Insekten-Interaktionen beigetragen haben. Alle direkten und indirekten AHL-Effekte wurden dabei stark durch das Kultivierungssystem und die Sensitivität der Gerstenkultivare bestimmt. Diese Ergebnisse unterstreichen damit die Komplexität von AHL-Effekten in der Kommunikation über Artgrenzen hinweg. Insgesamt bestätigt diese Dissertation, dass mikrobielle AHL-Signalweiterleitung das Zusammenspiel zwischen Mikroorganismen, Pflanzen und Insekten modulieren kann. Damit hebt diese Arbeit die Notwendigkeit hervor, die zahlreichen Faktoren besser zu verstehen, die bei der mikrobiell-induzierten Schädlingsbekämpfung involviert sind, um diese schonende Bekämpfungsstrategie in einer nachhaltigeren Landwirtschaft erfolgreich einzusetzen.

List of publications

Sanchez-Mahecha, O., **Klink, S.**, Rothballer, M., Sturm, S., Weisser, W. W., Zytynska, S., & Heinen, R. (2023). Microbe-induced plant resistance against insect pests depends on timing of inoculation but is consistent across climatic conditions. *Functional Ecology*.

Sanchez-Mahecha, O.*, **Klink, S.***, Heinen, R., Rothballer, M., & Zytynska, S. (2022). Impaired microbial *N*-acyl homoserine lactone signalling increases plant resistance to aphids across variable abiotic and biotic environments. *Plant, Cell & Environment, 45*(10), 3052-3069.

Hartmann, A., **Klink, S.**, & Rothballer, M. (2021). Importance of *N*-acyl-homoserine lactone-based quorum sensing and quorum quenching in pathogen control and plant growth promotion. *Pathogens*, *10*(12), 1561.

Hartmann, A., **Klink, S.**, & Rothballer, M. (2021). Plant growth promotion and induction of systemic tolerance to drought and salt stress of plants by quorum sensing auto-inducers of the *N*-acyl-homoserine lactone type: recent developments. *Frontiers in Plant Science, 12*, 683546.

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List of poster and oral presentations

2019/03	Poster presentation at the Annual Meeting 2019 of the Association for General and Applied Microbiology (VAAM) in Mainz, Germany Titel: The functional relevance of microbe-plant-insect interaction in a cereal crop system
2019/08	Oral presentation at the DFH Summer School on Functions of Microbial Communities in Soil, Neuherberg, Germany Titel: How does microbial AHL signalling influence the plant‘s response to insect attack?
2021/12	Flash poster presentation at the IS-MPMI eSymposia on Plant-Microbe Interactions in the Environment, online Titel: Influence of microbial AHL signalling on plant resistance to insects

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Abbreviations

AE1-AE6	axenic experiments
AHL	*N*-acyl-homoserine lactone
Amp^R	ampicillin resistance
ArN35e	*Acidovorax radicis* N35e initial test strain
ArI_{neg}	*Acidovorax radicis* N35e *araI::tet* (AHL signalling mutant)
ArR_{neg}	*Acidovorax radicis* N35e *araR::gen* (AHL sensing mutant)
$ArR_{neg}I_{neg}$	*Acidovorax radicis* N35e *araR::gen*, *araI::tet* (AHL sensing and signalling mutant)
C6-HSL	C6-homoserine lactone
CFU	colony forming unit
CLSM	confocal laser scanning microscopy
cm	centimeter
DMSO	dimethyl sulfoxid
dpi	days post inoculation
EtOH	ethanol
FISH	fluorescence *in situ* hybridization
gen	gentamicin resistance gene
GFP	green fluorescent protein
Gm^R	gentamicin resistance
HMGU	Helmholtz Zentrum München für Gesundheit und Umwelt
HSL	homoserine lactone
INET	Institute of Network Biology, Helmholtz Zentrum München
ISR	induced systemic resistance
Kan^R	kanamycin resistance
L	liter
MDS	multidimensional scaling
MilliQ	deionized water
min	minutes
mL	milliliter

µL	microliter
µM	micromolar
NB	nutrient broth
NFW	nuclease-free water
nm	nanometer
nM	nanomolar
OD	optical density
OH-C10-HSL	*N*-3-hydroxy-C10-homoserine lactone
PBS	phosphate buffer solution
PCR	polymerase chain reaction
PFA	paraformaldehyde
pH	potential of hydrogen
QS	quorum sensing
rpm	revolutions per minute
RrF4	*Rhizobium radiobacter* F4 initial test strain
RrI_{neg}	*Rhizobium radiobacter* F4 lactonase expressing strain (AHL signalling mutant)
SAR	systemic aquired resistance
SE1-SE6	soil experiments
sec	seconds
$Spec^R$	spectinomycin resistance
$Tell^R$	tellurite resistance
Tc^R	tetracycline resistance
tet	tetracycline resistance gene
TUMmesa	climate chamber facility in Weihenstephan, Germany

Introduction

1.1 Beneficial rhizobacteria as pesticide alternative

In times of increasing food demand, pest insects represent a threat to agriculture that requires urgent solutions (Culliney 2014, Sharma *et al.* 2017). Anually, up to 20 % of crop yields are lost because of insect feeding (Dhaliwal *et al.* 2015). While foraging, insects often transmit plant diseases which lead to additional global yield damage and climate warming is expected to aggravate insect productivity and geographical distribution even more (Lehmann *et al.* 2020, Skendžić *et al.* 2021). Conventionally, chemical pesticides are applied to control insect outbreaks. However, more and more insects turn resistant to these compounds (Sharma *et al.* 2019). Chemical compounds with insecticidal activity are often hazardous for the environment, contribute to species extinction and are also harmful for human health (Mahmood *et al.* 2016, Rani *et al.* 2021). In order to secure food production on a long-term scale and to improve food security for the consumer, more sustainable alternatives for pest management are crucially needed (Douglas 2018).

Biological pest control represents an effective strategy to counteract yield losses owed to insects. Apart from diverse other approaches including manual removal, insect traps or natural predators, soil microoganisms are used as biocontrol agents that naturally occur in the plant vicinity and do not menace ecosystem functions (Riaz *et al.* 2021). Plant growth promoting bacteria (PGPRs, Kloepper 1978) have already been applied to agricultural fields for many years and effectively increased plant health and resistance (Bender *et al.* 2016, Parray *et al.* 2016). Typical PGPR effects include biofertilization, phytostimulation, abiotic stress control and the control of biotic menaces like phytopathogens (Glick 2012). Also, for biocontrol of pest insects, soil microorganisms and their beneficial interactions with plants bear a promising potential that can be exploited in agricultural practice (Disi *et al.* 2019). However, at this juncture, the successful application of bacterial biocontrol agents requires a more profound understanding of microbe-plant-insect interaction in general.

1.2 Microbe-plant interaction

In the early 20th century, it was postulated by Lorenz Hiltern that the plant's well-being highly depends on the microorganisms in the rhizosphere (Hiltner 1904). Within a dynamic ecological network, plants and soil microorganisms live in mutualistic relationships that can benefit plant nutrition and fitness. Mutual microorganisms commonly colonize the close proximity of roots influenced by root exudates (the "rhizosphere" as defined in Hartmann *et al.* 2008), attach to the root surface and sometimes protrude into the spaces between root cortex cells or even live intracellularly (Gray & Smith 2005, Compant *et al.* 2021). In these physical and ecological niches, microorganisms retrieve energy-rich hydrocarbons, secondary metabolites and protection from the host plant. In turn, the plant profits from bacterial metabolic functions, higher soil moisture and mineralized organic matter (Turner *et al.* 2013). Especially under stressful conditions, plants can actively modulate their microbiome composition and make use of distinct bacterial properties, as formulated in the "cry for help" hypothesis (Berendsen *et al.* 2018). To achieve this, plants release exudates from the root tips and recruit beneficial bacteria from the surrounding bulk soil (Berg *et al.* 2014, Huang *et al.* 2014). It is said that plants thus invest a large proportion – up to 30 % – of their fixed carbon into the rhizosphere again (Kuzyakov & Domanski 2000). This way, individual plants tailor their below-ground microbiome which is low in diversity compared to bulk soil communities but rich in genetic potential. Because of this potential, the rhizosphere microbiome is considered as the plant's second genome (Berendsen *et al.* 2012).

1.2.1 Plant growth promotion effects of beneficial bacteria

It has been shown multiple times that plant growth can be enhanced by plant growth promoting bacteria (PGPR) in the rhizosphere. Common PGPRs belong to the genera *Azospirillum, Bacillus, Bradyrhizobium, Burkholderia, Pseudomonas, Rhizobium, Serratia* and *Streptomyces* which are part of numerous commercial biofertilizers (Basu *et al.* 2021a). When inoculated to the root compartment, these strains can modulate shoot and root biomass. For instance, *Bacillus* strains often enhance shoot fresh weight in important crops, like shown for barley (Canbolat *et al.* 2006) and wheat (Çakmakçi *et al.* 2007). *Rhizobium* strains strongly promoted shoot and root growth in barley (Glaeser *et al.* 2016, Kumar *et al.* 2021). The well-known beneficial strain

Pseudomonas simiae WCS417r strongly increased *Arabidopsis* root biomass and plasticity (Zamioudis *et al.* 2013, Wintermans *et al.* 2016) while *Azospirillum* was efficiently inoculated on numerous crops, like maize and millet (Fallik & Okon 1996). Beneficial bacteria often also accelerate flower onset, germination of seeds and increase crop yields (Panke-Buisse *et al.* 2017, Gholami *et al.* 2009). Chlorophyll content can be enhanced as well which has again implications for the plant's overall fitness, like demonstrated in inoculated wheat (Sarkar *et al.* 2018, Singh & Jha 2017). Most commonly, the root development is stimulated by beneficial bacteria leading to a more branched root architecture with more lateral root hairs. For example, different *Bacillus* strains modulated the root architecture in *Arabidopsis* (Gutiérrez-Luna *et al.* 2010), tomato (Batista *et al.* 2021) and wheat (Talboys *et al.* 2014). This morphological change enlarges the root surface which in turn allows more nutrient and water uptake (Kumar *et al.* 2019), a better ion homeostasis (Salas-González *et al.* 2021) and provides a larger contact interface for microbe-plant interactions.

Typically, PGPRs exhibit the described plant growth promoting effect by biofertilization, phytostimulation, abiotic stress control or the antagonistic control of phytopathogens that often constrain plant growth (Glick 2012).

During biofertilization, PGPRs provide the host plant with nitrogen, phosphorus and other valuable nutrients (Verma *et al.* 2001, Vessey 2003). Similar to the legume-rhizobia symbiosis in root nodules, several bacteria are able to fix nitrogen in the form of ammonia (Zheng *et al.* 2022). Since atmospheric nitrogen is naturally not available for the plant, only bacterial nitrogen fixation enables and supports plant growth (Dixon & Kahn 2004). Analogously, unsoluble phosphorus is hardly accessible for plants so that bacteria contribute to plant growth by producing small molecules for phosphor solubilization, like gluconic acid (Rodriguez *et al.* 2004) and phosphatases (Rodríguez *et al.* 2006). Moreover, many rhizobacteria produce small iron-binding siderophores that can transport iron into the plant cells and can improve the nutrient availability in iron-deficient environments. In recent studies, siderophore-like pyroverdines from *Pseudomonas simiae* WCS417 improved the growth of *Arabidopsis* (Pieterse *et al.* 2021) and siderophore production of *Streptomyces spp.* has been reported to promote growth in rice (Rungin *et al.* 2012).

During phytostimulation, bacterial strains use additional tools that modulate root and shoot development (Parray *et al.* 2016). Many microorganisms produce hormones and secondary metabolites that can directly interefere with the plant's hormone level e.g., cytokinin, ethylene and gibberellin (Maheshwari *et al.* 2015). Auxin-like indole-3-acetic acid (IAA) stimulates the release of plant exudates from root cells. IAA produced from *Variovorax sp*. balanced the auxin level in root growth inhibited *Arabidopsis* (Finkel *et al.* 2020) and 1-aminocyclopropane-1-carboxylate (ACC) deaminase production of microorgansisms interferes with ethylene signalling in plants (Saleem *et al.* 2007, Saraf *et al.* 2011). For instance, *Hartmannibacter diazotrophicus* enhanced ACC deaminase activity in barley (Suarez *et al.* 2015). Finally, also volatile organic compounds (VOCs) produced by bacteria have been shown to influence plant growth (Ryu *et al.* 2003, Tahir *et al.* 2017).

Apart from biofertilization and phytostimulation, beneficial rhizobacteria can help with abiotic stress control, balance water uptake and ion homeostasis and in this way improve plant growth. Numerous studies have shown that beneficial bacteria enhance salinity tolerance in crops including barley, rice and maize (Bal *et al.* 2013, Suarez *et al.* 2015, Ali *et al.* 2022) and support plant growth and survival under drought conditions like in wheat, tomato and barley (Kasim *et al.* 2013, Gowtham *et al.* 2020, Slimani *et al.* 2023).

Moreover, PGPRs are able to control biotic stress and antagonistically act on other microbiome members which are eventually pathogenic (Beneduzi *et al.* 2012, Durán *et al.* 2018). An infection with pathogenic bacteria or fungi often goes along with reduced growth so that pathogen suppression by beneficial bacteria can indirectly facilitate plant growth (Beneduzi *et al.* 2012).
Beneficial microbes might interfere with pathogens by competing for space and nutrients (Vannier *et al.* 2019, Zelezniak *et al.* 2015) or by interrupting signals of microbial quorum sensing (see chapter 1.4). In addition, many PGPRs produce antimicrobial compounds that might counteract plant pathogens (Kumar *et al.* 2014, Zhou *et al.* 2021). In this context, it has been shown that especially *Bacillus* and *Pseudomonas* strains act as antagonistic agents against phytopathogens and fungi like *Ralstonia solanacearum* and *Fusarium oxysporum* (Cao *et al.* 2018, Ali *et al.* 2020). They secreted antimicrobial compounds like surfactin, iturin or fengycin and facilitated the normal growth of tomato, potato, banana and other crops (Cao *et al.* 2018,

Ghadamgahi *et al.* 2022). If pathogenic pressure and the hereby triggered plant defense mechanisms are reduced, the plant can invest more energy into growth.
Unceasingly, plants face this trade-off between growth and defense which is well-described (He *et al.* 2022). Increased plant growth uses-up resources that are no longer available for plant immunity and vice versa. The plant's state can therefore be best approximated when evaluating growth and immune system parameters together.

1.2.2 Plant immune system stimulation by rhizobacteria

Apart from modulating plant growth, microorganisms in the rhizosphere also interfere with the plant immune system. Since the beginning of their coevolution with microbes, plants evolved a multitude of barriers and innate defense lines against potential invaders (Han & Tsuda 2022). Upon contact in the apoplast, the plant perceives microbial metabolites, signalling compounds and surface molecules like microbe associated molecular patterns (MAMPs) which automatically elicit a so-called pattern triggered immunity (PTI) (Jones & Dangl 2006, Boller & Felix 2009, Zhang & Zhou 2010). Typical MAMPs are the conserved flagellin peptide flg22 on bacterial flagella, peptidoglycan, lipopolysaccharides in the bacterial cell wall or chitin in the case of fungi (Felix *et al.* 1999, Erbs & Newman 2012, Sanchez-Vallet *et al.* 2015). Most MAMPs are bound by plasma-membrane-located pattern recognition receptors (PRRs) which activate a downstream MAP kinase signalling cascade, induce immune gene expression and plant hormone signalling (Beck *et al.* 2012). For example, when bacterial flg22 is perceived by the receptor kinase FLS2, it complexes with another receptor-like kinase BAK1, initiates a downstream phosphorylation cascade which stimulates the WRKY transcription factor family (Chinchilla *et al.* 2007, Heese *et al.* 2007). These well-studied transcription factors, including WRKY22, induce the expression of early defense-related genes (Muthamilarasan & Prasad 2013). Among others, plant defense genes comprise namely *PR1* to *PR17* which encode for pathogen-related (PR) proteins with antimicrobial properties. In barley, multiple classes of PR proteins have been identified so far (Muthukrishnan *et al.* 2001, Christensen *et al.* 2002). The *PR1* gene was found to encode for an antifungal protein which inhibited a powdery mildew infection in barley which is caused by the fungus *Blumeria graminis* (Schultheiss *et al.* 2003). In healthy tissues, *PR* genes are not active or only transcribed at low levels. However, under

pathogen attack, PR proteins usually accumulate in the plant tissue to high concentrations (van Loon *et al.* 2006).

1.2.3 Immune dampening by pathogens

In the course of evolution, pathogenic microorganisms invented multiple mechanisms to infect tissues despite PTI. Many pathogens release effector molecules through a type III secretion system (T3SS) into the host cells that directly inhibit PTI components (Block *et al.* 2008). For example, the phytopathogen *Pseudomonas syringae* DC3000 was found to suppress PTI by multiple effector molecules that interfere with PRR recognition, MAP kinase and plant hormone signalling simultaneously (Göhre *et al.* 2008, Xiang *et al.* 2008). An effector of pathogenic *Acidovorax citrulli* suppressed reactive oxygen species (ROS) burst, WRKY6 and salicylic acid (SA) activation while causing bacterial fruit blotch in watermelon (Zhang *et al.* 2020a).

Other microorganisms escape from host recognition by preventing MAMP production, release or perception (Buscaill & van der Hoorn 2021, Sanguankiattichai *et al.* 2022). For example, pathogenic *Pseudomonas ssp.* degrade flagellin into monomers preventing immune recognition during *Arabidopsis* colonization (Pel *et al.* 2014). Other pathogens shield their MAMPs with glycans. The pathogenic *Acidovorax avenae* K1 strain avoided flagellin recognition with a different glycosylation pattern compared to the non-pathogenic strain *A. avenae* N1141 which still elicited an immune response in rice (Hirai *et al.* 2011).

However, in the evolutionary arms race, plants evolved strategies again to identify effector molecules and to overcome the immune dampening attempts of persistent pathogens. When the plant encounters effector molecules, it produces nucleotide-binding leucine-rich repeat proteins (NLRs, often also abbreviated as NB-LRRs) and activates an even stronger form of PTI, the effector triggered immunity (ETI) (Nguyen *et al.* 2021). This hypersensitive reaction is often followed by programmed cell death to restrict local pathogen invasion (Greenberg & Yao 2004).

1.2.4 Immune dampening by beneficial microorganisms

Similar to pathogens, beneficial bacteria have to dampen the plant immune response to colonize and establish a successful interaction (Zamioudis & Pieterse 2012, Yu *et al.* 2019a). Researchers agree that both beneficial and pathogenic microorganisms can trigger plant immune responses by MAMPs and target the MAPK cascade persumably with the same molecular mechanisms (Zhou *et al.* 2020).

In barley for example, the endophytic basidiomycet *Serendipita indica* (syn. *Piriformospora indica*) inhibits the MAMP-induced ROS burst and lowers the expression of plant immune hormones (Hilbert *et al.* 2013, Akum *et al*. 2015). Additionally, *S. indica* produces fungal effectors to disrupt plant perception of chitin and flg22 (Wawra *et al.* 2016). Flagellin polymorphism assists immune escape of the crown-gall-causing pathogen *Agrobacterium tumefaciens* (syn. *Rhizobium radiobacter)* as well as the closely related beneficial strain *Ensifer meliloti* (Felix *et al.* 1999, Gómez-Gómez & Boller 2002). Beneficial *Pseudomonas spp*. just lower the environmental pH to inhibit MAMP recognition by the plant (Yu *et al.* 2019b) while beneficial bacteria from the genus *Bradyrhizobium* produce nodulation (Nod) factors to abolish a MAMP-triggered immunity (Liang *et al.* 2013). This illustrates that beneficial microbes escape from host recognition in a similar manner as pathogens.

How plants distinguish between friend and foe is still unknown but on the verge of being revealed (Zhang *et al.* 2020b, Zhou *et al.* 2020, Li *et al.* 2021). Especially in the early phase of interaction, beneficial bacteria have been shown to actively suppress plant immunity (Xu *et al*. 2019). But also, the plant lowers its defense lines avoiding over-reactions towards harmless microorganisms (Li *et al*. 2021). A recent study revealed that *Arabidopsis* normally expresses MAMP receptors to an extremely small extent in roots, so that root cells are not sensitive to the residuing microbiome (Zhou *et al.* 2020). Only when additional pathogen-related signals appear, like damaged plant cell wall particles, immune receptors are quickly produced. This mechanism specifically activates plant immune responses upon invasion while tolerating mutual interactions at the same time (Zhou *et al.* 2020).

1.2.5 Induced systemic resistance

On the systemic level, plants acquired two different forms of immunity which confers resistance to pathogens and other biotic and abiotic threads: systemic aquired resistance (SAR) and induced systemic resistance (ISR). Although these immunity pathways have been proven to be highly interconnected, both terms are currently still in use to highlight stimulus origin and hormone dependency (Pieterse *et al.* 2014).

In response to local infections by biotrophic pathogens, the plant often mediates resistance also to distal tissues so that uninfected plant parts are strengthened and prepared for future attack (Pieterse *et al.* 2014). This systemic acquired resistance (SAR) is mainly conveyed by salycilic acid (SA), respectively its methylated equivalent (MeSA), through the phloem what leads to a characteristic increase in overall SA levels (Park *et al.* 2007). Besides, other molecules have been found to be involved in SAR long-distance signalling like glycerolipids (Jung *et al.* 2009, Chanda *et al.* 2011) and pipecolic acids (Pieterse *et al.* 2014). In distal tissues, SA typically interacts with the co-activator NPR1 and other transcription factors, like from the WRKY family, that induce the expression of *PR* immune response genes (Pieterse *et al.* 2009). Especially *PR1* is widely used as SAR marker gene in *Arabidopsis* and tobacco (Yalpani *et al.* 1991, Maleck *et al.* 2000).

In contrast to SAR, induced system resistance (ISR) is mainly triggered by beneficial microorganisms that inhabit the rhizosphere and often also promote plant growth like the prominent PGPR *Pseudomonas simiae* WCS417r (van der Ent *et al.* 2009, Beneduzi *et al.* 2012, Pieterse *et al.* 2021). During ISR, beneficial microorganisms systemically stimulate immune response genes, callose deposition and stomatal closure which provide broad-spectrum protection against pathogens. For example, *P. simiae* WCS417r induced ISR in *Arabidopsis* which showed increased callose deposition and prevented the entry of pathogenic *Hyaloperonospora arabidopsidis* (van der Ent *et al.* 2009). Similarly, *Bacillus subtilis* FB17 triggered ISR in uninfected *Arabidopsis* leaves and accelerated stomata closure when attacked by the pathogen *P. syringae* (Kumar *et al.* 2012). Especially cell wall strengthening by callose deposition can finally result in long-lasting plant resistance ("priming", see chapter 1.2.6). Furthermore, ISR can be elicited by lipopolysaccharides, flagella, siderophores, VOCs and

N-acyl-homoserine lactones (Pieterse *et al.* 2014, 2021).

While SAR is mainly governed by SA signalling, ISR rather depends on jasmonic acid (JA) and ethylene (ET) signalling pathways. However, already several studies discovered an ISR plant state that was related to the SA signalling pathway but not to JA/ET (Petti *et al.* 2010, van de Mortel *et al.* 2012, Takishita 2018). Moreover, it was commonly thought that ISR typically functions without NPR1-mediated *PR* gene activation (Mathys *et al.* 2012). By now, different pools of NPR1 are assumed to modulate a complex crosstalk of immune signalling pathways (Pieterse *et al.* 2014, Nie *et al.* 2017). Increasing evidence suggests that the SA and JA/ET signalling pathways can either act synergistically or antagonistically, depending on the specific stimulus, pathogen, plant species and hormone concentration (Mur *et al.* 2006). In addition, the immune signalling network is modulated by gibberellins, auxins, cytokinins and brassinosteroids (Navarro *et al.* 2008, Kazan & Manners 2009, Giron *et al.* 2013, Nakashita *et al.* 2003). With this inter-linked system, plants can fine-tune their protection when facing multiple stressors at the same time, as it is mostly the case in natural environments (Zhang *et al.* 2020b, Jiao *et al.* 2021).

1.2.6 Priming

During the systemic defense responses of SAR and ISR, plants can develop additional priming on a long-term scale. Primed plants respond in a faster and more robust manner towards low levels of abiotic stimuli as well as beneficial microorganisms than unprimed plants (Conrath *et al.* 2006). Priming is characterized by a sensitization of plant tissues to future challenges without direct activation of defense genes (Mauch-Mani *et al.* 2017). For example, it was shown that *Arabidopsis* plants, primed with *P. simiae* WCS417r, first showed no transcriptional change in the leaves but later an accelerated expression of defense-related genes when challenged with *P. syringae pv. tomato* (Verhagen *et al.* 2004). Persumably, this defense strategy is less cost-intensive than a direct immune system activation. Mechanistically, priming involves an accumulation of dormant transcription factors, inactive proteins and signalling kinases which are not used until the next challenge (Beckers *et al.* 2009, Pastor *et al.* 2013). In addition, epigenetic modifications on chromatin, DNA methylation and histone level might determine how quickly defense genes are accessed for transcription (Jaskiewicz *et al.* 2011,

Luna *et al.* 2012, Conrath *et al.* 2015). These pre-transcriptional changes facilitate a stronger and quicker response, to memorize stressful situations and immunize also next generations against subsequent pathogen or herbivore attacks (Pastor *et al.* 2013, Dutilloy *et al.* 2022).

1.3 Microbe-plant-insect interaction

1.3.1 Plant defense against insect herbivores

Beneficial rhizosphere microorganisms cannot only induce resistance to pathogens but also help the plant to deal with herbivores (Pineda *et al.* 2013). Insects, plants and microorganisms interact since approx. 350 million years with each other (Gatehouse 2002). Unsurprisingly, complex interactions arose with positive and negative effects on the partners, from pollination to insect feeding. Herbivorous insects attack above- and belowground plant parts and represent a major threat for vascular plants. Caterpillars, beetles and other chewing insects rip up leave parts and cause physiological damage (Pineda *et al.* 2010). Sap-sucking insects, like aphids, stick their stylet mouthpart between plant cells and forage on phloem sugar while avoiding direct cell damage (Powell *et al.* 2006). They retrieve nutrients, disturb water and ion homeostasis and transmit phytoviruses and other diseases (Cabrera *et al.* 1995, Pineda *et al.* 2010, Fingu-Mabola & Francis 2021).

Plants developed sophisticated strategies to cope with pest insects (Fürstenberg-Hägg *et al.* 2013). When plants face herbivore attack, similar defense mechanisms are stimulated compared to the basal plant immune response to pathogenic and beneficial microorganisms (Noman *et al.* 2020, see chapter 1.2.2). Herbivore-associated molecular patterns (HAMPs), like saliva proteins, metabolites in the honey dew or chitin particles, function as general cues that have been demonstrated to result in a PTI-like immune response in *Arabidopsis* (de Vos & Jander 2009, Prince *et al.* 2014, Losvik 2018). Like in PTI, different *R* genes were activated in the course of aphid feeding in tomato (Rossi *et al.* 1998), melon (Dogimont *et al.* 2014) and lettuce (Wroblewski *et al.* 2007). Insect feeding elicited ROS production (Jaouannet *et al.* 2015) while damage-associated molecular patterns (DAMPs), like wounded plant cell walls, have been found to stimulate JA biosynthesis (Morkunas *et al.* 2011, Pieterse *et al.* 2012). In general, chewing herbivores induce a JA-dependent plant response involving the MYC

transcription factor family. Sap-sucking insects, like aphids, rather stimulate the SA-dependent signalling pathway. Ethylene and abscisic acid (ABA) modulate the plant response additionally (Erb *et al.* 2012, Verma *et al.* 2016).

Plants directly defend themselve against herbivores with leaf surface wax, a thick cuticula and trichomes as physical barrier (War *et al.* 2012, Nalam *et al.* 2019). In addition, secondary metabolites and toxins are produced for chemical defense e.g., glucosinolates, thionins and flavonoids (Pangesti *et al.* 2013). Flavonoid production is induced by JA and MYC signalling and exhibits insecticidal activity (Pangesti *et al.* 2013). Moreover, the plant physiology is strengthened during plant defense. Lignin and callose deposition at sieve element sites stops the phloem flow so that insect feeding is hindered (Vanholme *et al.* 2010, Nalam *et al.* 2019). In addition, many plants produce VOCs and other secondary metabolites that attract natural enemies of the insect invader in an indirect defense strategy (Price *et al.* 1980, Turlings & Erb 2018).

1.3.2 Plant microbiome modulation upon insect infestation

Plant hormones and secondary metabolites cannot only attract insect enemies, but also influence root exudates which consequently affects plant-microbe interaction in the rhizosphere (Carvalhais *et al.* 2015, Eichmann *et al.* 2021). Root exudates with elevated flavonoid and auxin levels might recruit soilborne microorganisms with insect suppressive capacity (Zamioudis & Pieterse 2012, Park & Rhyu 2021). Thus, plants actively shape their rhizosphere microbiome in response to biotic stress (Ourry *et al.* 2018, Friman *et al.* 2021). Indeed, recent studies reported that the microbiome composition was significantly altered if plants were attacked by herbivores. Aphid infestation changed the rhizosphere microbiome of tomato plants (French *et al.* 2021) and English grain aphids *Sitobion avenae* influenced the rhizosphere microbiome of barley (Zytynska *et al.* 2020). Herbivory by various chewing and phloem-feeding insects differentially affected the belowground microbiome of potato (Malacrinò *et al.* 2021a, 2021b) and cabbage (Friman *et al.* 2021). Whitefly infestation increased *Bacillus* and *Pseudomonas* abundances in the rhizosphere of pepper (Lee *et al.* 2012, Kong *et al.* 2016) and especially attracted Gram-positive bacteria (Yang *et al.* 2011). However, in other trials like from O'Brien and colleagues (2018), aphid herbivory had no impact on the

rhizosphere microbiome of cabbage *Brassica oleracea*.

Taken together, these observations illustrate the close link between plant microbiome composition and insect presence. Since rhizobacteria and herbivores compete for the same resources, it can be suggested that recruited bacteria exhibit biocontrol activity with a positive effect on plant insect suppression.

1.3.3 Plant insect suppression effect of beneficial bacteria

Numerous studies have demonstrated that beneficial rhizobacteria can induce plant resistance against herbivores. The most studied bacteria in this context belong to the genera *Pseudomonas* and *Bacillus* (Kloepper *et al.* 2004, Sivasakthi *et al.* 2014). For instance, *Pseudomonas* members, which contain insecticidal gene clusters (Flury *et al.* 2016), successfully exhibited insect-killing functions in whitefly-infested pepper plants (Kong *et al.* 2016). *Bacillus subtilis* treatment retarded whitefly development on tomato (Valenzuela-Soto *et al.* 2010). Different *Bacillus* species reduced aphids on cabbage and even had the highest effect in mixed inoculation assays suggesting that microbe-microbe interaction played a role in effect generation (Gadhave *et al.* 2016a). *Pseudomonas* and *Bacillus* also suppressed green peach aphid *Myzus persicae* infestation in pepper (Pappas *et al.* 2021). Beneficial microorganisms can help to increase nutrition, balance water maintenance (Pineda *et al.* 2012) and produce VOCs like 2,3-butanediol (Ryu *et al.* 2004). These VOCs and other microbe-derived compounds can again have a direct effect on the infesting herbivore but also indirectly attract natural enemies of a higher trophic level (Kupferschmid *et al.* 2013). Aphid parasitoid *Aphidius ervi* was attracted in response to a symbiotic fungus in tomato (Guerrieri *et al.* 2004). Zytynska *et al.* (2010) demonstrated that the fitness of parasitoid wasp *Aphidius ropalosiphi* depended on beneficial *Pseudomonas aeruginosa* 7NSK2, strongly modulated by barley and aphid genotypes. The interactions between *Bacillus amyloliquefaciens*, arugula plants and herbivores affected the behaviour of predatory earwings (Bell *et al.* 2020). Similarly, the presence of *Pseudomonas fluoresence* in rice enhanced the performance of a predator of the rice leaf folder (Saravanakumar *et al.* 2008). However, the mechanisms behind these complex tetrapartite interactions are not yet understood.

Beside all positive effects on plant insect suppression, multiple studies revealed that beneficial microbe-plant-insect interaction is not straightforward (Disi *et al.* 2019). To the same extent, rhizobacteria can have a negative effect on plant insect suppression by enhancing insect development and performance (Pineda *et al.* 2013, Gadhave *et al.* 2016b). For example, *P. fluorescens* inoculation increased whitefly survival (Shavit *et al.* 2013) while *P. syringae* reduced *Arabidopsis* resistance to cabbage looper (Groen *et al.* 2013). Blubaugh *et al.* (2018) found that *Pseudomonas spp.* and *Bacillus spp.* inoculation correlated with higher aphid growth and also had an advert effect on aphid parasitism rate. Frequently, the effect direction differed between insect species. For instance, ISR induced by *P. simiae* WCS417r decreased plant infestation with generalist lepidopteran caterpillars *Mamestra brassicae* (Pangesti *et al.* 2016), *Spodoptera exigua* (van Oosten *et al.* 2008) and *Trichoplusia ni* (Haney *et al.* 2018) but increased the susceptibility to the aphid *Myzus persicae* (Pineda *et al.* 2012).

These different outcomes of microbe-plant-insect interaction can be rationalized by complex feedback loops (Mahdavi-Arab *et al.* 2014). Some beneficial microorganisms do not only activate the plant immune system, but promote plant growth at the same time. Enhanced plant growth leads to a better nutritional status of the plant which ultimately benefits insect feeders (Schoonhoven *et al.* 2005). Thus, a positive immune stimulation effect often overlays with other negative effects. On the same note, soil-borne bacteria can suppress the production of plant volatiles so that natural enemies are not recruited (Blubaugh *et al.* 2018). Total microbial effects on herbivore performance therefore needs to be evaluated as a balance between improved plant growth and increased resistance (Pineda *et al.* 2013).

As described above, the outcome of microbe-plant-insect interaction is highly context-dependent (Pineda *et al.* 2013). Tripartite interactions regularly depend on nitrogen availability (Dean *et al.* 2014), soil legacy (Pineda *et al.* 2020), insect species (Stewart *et al.* 2016) and the specific plant cultivar (Zytynska & Weisser 2016). Abiotic stressors like drought and climate change also need to be considered as modulating factors (de Bobadilla *et al.* 2017, Zytynska *et al.* 2020). Under stressful conditions, positive as well as negative effects of microbes on herbivores have been shown to be strengthened, probably because of increasing signalling crosstalk (Pineda *et al.* 2013).

The mechanisms of microbe-plant-insect interactions are still scarcely understood. It is unclear which compounds play the key role in turning plant resistant to herbivorous insects. Apart from structural MAMPs, microbe-derived lipopeptides, camalexin, glucosinolates and other secondary metabolites have been shown to induce plant defenses against insects (Ongena & Jacques 2008, Clay *et al.* 2009). Additionally, it appears very likely that also other signals like the autoinducer family of *N*-acyl-homoserine lactones (AHLs) are integrated by the plant as valuable information for a targeted response (Hartmann *et al.* 2014). AHLs are crucial for bacterial communication in the rhizosphere and are connected to cell density, pathogenicity and biofilm formation. Therefore, it appears plausible that AHLs might also play a decisive role in interkingdom communication.

1.4 Microbial AHL signalling in microbe-plant interaction

1.4.1 Bacterial communication by AHL signalling

N-acyl-homoserine lactones (AHLs) are the most studied signalling compounds in microbial cell-to-cell communication (Baltenneck *et al.* 2021, Kumar *et al.* 2022). Many Gram-negative bacteria use AHLs to communicate with each other and to coordinate their social behaviour in the process of quorum sensing (QS, Fuqua *et al.* 1994). During QS, individual bacteria produce and release small-sized AHL signalling molecules into the environment. With increasing cell density, also the AHL concentration rises. Once the critical cell density threshold (“quorum”) is reached, AHLs bind to a receptor protein in the bacterial cytoplasma which alters the expression of associated genes including the AHL synthase gene (Fuqua *et al.* 1996, Miller & Bassler 2001). Thus, AHL recognition also stimulates the production of more AHLs in a positive feedback loop. With this biochemical communication system, bacteria can synchronize their activity based on population dynamics, like the production of virulence factors, pigments and antibiotics, motility and biofilm formation (Eberl 1999, Fuqua & Greenberg 2002, Mukherjee & Bassler 2019, Liu *et al.* 2022). This behavioural orchestration guarantees a flexible reaction to stress and changing environmental conditions (Fekete *et al.* 2010, Buddrus-Schiemann *et al.* 2014). Since AHLs have a low molecular weight, they can be inexpensively produced to test the environments diffusion properties before synthesizing more

cost-intensive molecules ("efficiency sensing", Hense *et al.* 2007). With this, QS not only serves as a measure for cell density but also allows bacteria to estimate the diffusion space and spatial distribution of neighbouring cells. In combination, this system assures a fine-tuned and economic reaction of the individual cells to its surrounding (Hense *et al.* 2007).

Quorum sensing was first discovered in the marine bacterium *Aliivibrio fisheri* (formerly *Vibrio fischeri*) which lives in the light organ of the Hawaiian bobtail squid *Euprymna scolopes* (Ruby & Nealson 1976, Kaplan & Greenberg 1985). As part of its symbiotic relationship, *Aliivibrio fischeri* regulates its bioluminescence in a QS-dependent manner with a LuxI/LuxR system (Visick *et al.* 2000, Nyholm & McFall-Ngai 2014). In this system, a *luxI* gene encodes for the LuxI AHL synthase and a *luxR* gene for the LuxR receptor which forms a complex with AHL and acts as transcriptional regulator (Fuqua & Winans 1994). A homologuous two-component system exists in many Gram-negative bacteria. For example, the TraI/TraR system of *Agrobacterium tumefaciens* regulates the transfer of oncogenic genes from its Ti plasmid to the host plant (Fuqua & Winans 1994, Hwang *et al.* 1995). Quorum sensing is a very ancient ability and therefore widespread in the bacterial kingdom (Lerat & Moran 2004). Up to date, QS-dependent communication is described for more than 500 bacterial species (Babenko *et al.* 2021) of which at least 90 include AHL signalling (Kumar *et al.* 2022).

AHL molecules consist of a homoserine lactone (HSL) ring and an acyl chain, ranging in length from 4 to 18 carbon atoms. Depending on the bacterial species, the acyl side chain can be modified by unsaturated double bonds or hydroxy- or oxo-substitutions at the third carbon atom (Prescott & Decho 2020). These structural differences ensure the specific recognition of own and heterogenous AHLs (Papenfort & Bassler 2016). Most bacterial species produce more than one type of signalling molecule. While AHLs can only be found in Gram-negative bacteria, Gram-positives mainly harbour peptides as QS signals (Williams 2007). Other QS molecules include furanosyl borate ester, hydroxy-palmitic acid methyl ester (PAME), diffusible signalling factor (DSF), quinolone signals, etc. (Whiteley *et al.* 2017). This biochemical diversity suggests that signalling molecules mediate intraspecies as well as interspecies communication. Especially the alternative autoinducer AI-2 was found to be produced by both Gram-negative and Gram-positive bacteria and is therefore likely to function as "universal language" (Schauder *et al.* 2001, Pereira *et al.* 2013).

Recently, it was postulated that AHL signalling might even play a key role in interkingdom communication (Hartmann & Schikora 2012, Hartmann *et al.* 2014). Signalling molecules are ubiquitous in the environment and often regulate genes associated with pathogenicity or symbiosis. Therefore, it is not surprising that also eukaryotic cells have adapted to perceive bacterial signalling (Hartmann *et al.* 2014). At least, most bacteria with QS capacity are able to establish pathogenic or symbiotic relationships with animals or plant hosts (Marketon & Gonzalez 2002). For plant-associated bacteria a high diversity of AHLs and other signalling molecules was reported, suggesting that AHLs also mediate plant-microbe interactions (Cha *et al.* 1998).

1.4.2 Interkingdom communication in the rhizosphere

In the last two decades, evidence accumulated that plants respond to microbial and synthetic AHL signalling molecules (Mathesius *et al.* 2003; Ortiz-Castro *et al.* 2008). As positive effects, AHLs mainly enhanced plant shoot and root growth (for more details see 1.4.5) and increased plant resistance towards pathogens (see 1.4.6). Besides, AHLs differentially regulated phytohormones, stomata conductance, production of lactonase, phenolic toxins and callose deposition in treated plants (Babenko *et al.* 2021). In general, short-chain AHLs (C4-HSL to C6-HSL) rather promoted plant growth while long-chain AHLs (C10-HSL to C16-HSL) primarily affected plant immunity. In this course, the respective AHL effect strongly depends on the targeted plant, substitutions in the acyl side chain and environmental conditions. Nevertheless, AHL-containing biofertilizers are already developed and successfully applied for plant growth and health stimulation (Gupta *et al.* 2019, Gahoi *et al.* 2021). In order to exploit AHLs in future agriculture most efficiently, a better understanding about the AHL effect origin would be indispensable.

1.4.3 Direct AHL interaction with plants

The effect of AHL signalling on plants can be of direct or indirect nature (Moshynets *et al.* 2019). Repeatedly, it has been demonstrated that AHLs can be directly taken up by plants which might lead to an immediate reaction in the respective root or shoot tissue. Small lipid-

permeable AHLs can passively diffuse through membranes while large AHLs (> C8-HSL) need to be transported in and out of cells via active pumps. In barley, C8- and C10-AHLs were taken up by ATP-driven carriers and transported in the phloem to distal shoot tissues (Sieper *et al.* 2014). In contrast, longer lipophilic AHLs were not taken up into barley and *Arabidopsis* (Götz *et al.* 2007, von Rad *et al.* 2008). In the distal tissues, a direct AHL transmission into feeding insects is conceivable.

Direct interference with microbial AHL molecules can also arise from the plant's side (Gao *et al.* 2003, Bauer & Mathesius 2004). Many plants developed lactonases which degrade QS signals by opening the lactone ring so that microbial signalling and molecule uptake is hindered ("quorum quenching", QQ). AHL-degrading lactonase activity has been found in *Arabidopsis* and several legumes like *Lotus corniculatus* (Delalande *et al.* 2005, Götz *et al.* 2007) but have not been discovered for barley and yam bean (Götz *et al.* 2007). Other plants like rice and bean produce AHL-mimicking substances to interfere with microbial AHL signalling (Teplitski *et al.* 2000, Pérez-Montaño *et al.* 2013), including rosmarinic acid or flavonoids (Vandeputte *et al.* 2010, Corral-Lugo *et al.* 2016).

Certainly, these direct effects need to be evaluated in respect to the environmental setting. The rhizosphere is a spacially complex environment where AHL concentration can vary locally to a large extent. In acidic soils, AHLs are considered to be relatively stable if not degraded by lactonases (Wang & Leadbetter 2005). In alkaline environments, they abiotically degrade by opening of the lactone ring. In this hydrolyzed form, AHLs are no longer biologically active (Dong *et al.* 2001, Decho *et al.* 2011).

Which molecular mechanisms are directly activated by AHL, is not yet completely understood. AHLs are thought to be transported from bacteria to plants by transmembrane vesicles (Toyofuku 2019). Even the question if plants possess specific receptors for direct AHL recognition, just begins to be resolved (Götz-Rösch *et al.* 2015, Schikora *et al.* 2016). Only recently, a potential AHL receptor was proposed for *Arabidopsis*; the plant glucuronokinase AHL-priming protein 1 (ALI1) mediated the plant reaction to oxo-C14-HSL (Shrestha *et al.* 2022). Further AHL-interacting proteins were identified and need to be investigated for their AHL-binding ability (Shrestha *et al.* 2016).

1.4.4 Indirect AHL interaction with plants

Apart from direct effects, AHLs can also indirectly influence microbe-plant interactions via the bacterial behaviour (Hartmann *et al.* 2014). The expression of many bacterial traits is coupled to AHL sensing and signalling which can make a difference for colonization in the rhizosphere. Quorum sensing classically regulates the formation of cell accumulations and biofilms on surfaces like plant roots (Davies *et al.* 1998). AHL signalling mutants have been shown to colonize less which might also be associated with a less beneficial interaction (Wei & Zhang 2006, Zúñiga *et al.* 2013, Han *et al.* 2016, Rondeau *et al.* 2019). Reduced colonization might be additionally caused by a reduced tolerance against ROS release during early plant defense (Alquéres *et al.* 2013). For instance, AHL mutants of *P. aeroginosa* showed lower production of catalase and superoxide dismutase to cope with plant-derived ROS (Hassett *et al.* 1999). Also plant growth promoting traits of beneficial bacteria can be positively or negatively regulated by QS. An example for a negative AHL mediated regulation can be found in Müller *et al.* (2009). In this publication, the authors describe how microbial gene expression for IAA production was upregulated in *Serratia plymuthica* if the bacterium could not accumulate AHLs, leading to increased oil seed rape growth when inoculated.
The presence or absence of AHL signals might also influence the abundance and functions of other soil-borne microorganisms. Beneficial or pathogenic bacteria from the rhizosphere might enhance or quench the QS signal or degrade AHL molecules as nutrient source for their metabolism. Eventual changes in the surrounding microbial community, directly mediated by AHL, can again indirectly affect plant growth and health.

1.4.5 AHL effect on plant growth

Two decades ago, it was demonstrated for the first time that plants react on synthetic AHL application with a specific physiological response (Joseph & Philips 2003). By now, a plethora of studies have shown that microbially-derived as well as commercially synthesized AHLs majorly provoke enhanced shoot and root growth, increased biomass (Götz *et al.* 2007, von Rad *et al.* 2008, Barriuso *et al.* 2008, Shrestha *et al.* 2020), an elongated primary root (Bai *et al.* 2012, Liu *et al.* 2012, Zhao *et al.* 2015) and altered root architecture with more adventitious root hairs (Pazarlar *et al.* 2020). Only a few studies reported a negative effect, no plant growth

promotion or only small effect sizes that appeared not significant (Schikora *et al.* 2011, Götz-Rösch *et al.* 2015). Most studies focused on model organisms and important crops comprising *Arabidopsis*, *Medicago*, barley, tomato, yam bean and wheat (von Rad *et al.* 2008, Veliz-Vallejos *et al*. 2014, Götz-Rösch *et al*. 2015, Schikora *et al.* 2011, Moshynets *et al*. 2019). The constructions of transgenic plants expressing AHL-related genes further shed light on the direct molecular action of AHLs. For instance, transgenic AHL-producing tomato plants altered the effect of applied PGPR and increased salt tolerance (Barriuso *et al.* 2008). Furthermore, AHLs have been proven to play a key role for nodulation in the symbiosis between legumes and nitrogen-fixing rhizobia like *Rhizobium* (Calatrava-Morales *et al*. 2018). An overview of AHL effects on plant growth is presented below in Table 1.1.

Table 1.1 Effect of microbial and synthetic AHLs on plant growth and physiology.

AHL	**AHL source**	**plant**	**plant growth response**	**reference**
C4-HSL	synthetic	*Arabidopsis thaliana*	increased plant growth	von Rad *et al.* 2008
	Burkholderia graminis	*Lycopersicon esculentum*	increased plant growth	Barriuso *et al.* 2008
	synthetic	*Cicer arietinum*	increased plant growth	Gupta *et al.* 2019
OH-C4-HSL	synthetic	*A. thaliana*	elongated primary root	von Rad *et al.* 2008
C6-HSL	*Serratia liquefaciens*	*H. vulgare*	increased plant growth	Götz *et al.* 2007
	synthetic	*A. thaliana*	increased plant growth	von Rad *et al.* 2008
	synthetic	*A. thaliana*	increased root and shoot growth	Schikora *et al.* 2011
	synthetic	*A. thaliana*	increased plant growth	Schenk *et al.* 2012
	synthetic	*Hordeum vulgare*	no plant growth promotion	Götz-Rösch *et al.* 2015
	synthetic	*Pachyrhizus erosus*	reduced plant biomass	Götz-Rösch *et al.* 2015
	synthetic	*T. aestivum*	increased plant growth, germination, yield	Moshynets *et al.* 2019
	synethtic	*T. aestivum*	increased chlorophyll, cell wall thickness	Kosakivska *et al.* 2020
	synthetic	*Cucumis sativus*	increased shoot biomass and leave surface	Pazarlar *et al.* 2020
	synthetic	*Quercus robur, Q. rubra*	increased plant growth, lateral root formation	Kosakivska *et al.* 2022
oxo-C6-HSL	synthetic	*A. thaliana*	elongated primary root	Liu *et al.* 2012
	synthetic	*A. thaliana*	elongated primary root	Zhao *et al.* 2015, 2016
	synthetic	*A. thaliana*	elongated primary root, increased biomass	Shrestha *et al.* 2020
C8-HSL	synthetic	*H. vulgare*	increased plant growth	Götz *et al.* 2007
	B. graminis	*L. esculentum*	increased plant growth	Barriuso *et al.* 2008
	synthetic	*H. vulgare, P. erosus*	increased shoot growth (not significant), reduced biomass, AHL not detected in shoots	Götz-Rösch *et al.* 2015
	synthetic	*H. vulgare*	increased root growth and biomass, lateral root formation and elongation	Rankl *et al.* 2016

C8-HSL	synthetic	*Panax ginseng*	reduced shoot growth	Ibal *et al.* 2021
oxo-C8-HSL	synthetic	*A. thaliana*	elongated primary root	Liu *et al.* 2012
	synthetic	*A. thaliana*	modified primary root growth	Palmer *et al.* 2014
C10-HSL	synthetic	*H. vulgare*	increased plant growth	Götz *et al.* 2007
	B. graminis	*L. esculentum*	increased plant growth and salt tolerance	Barriuso *et al.* 2008
	synthetic	*A. thaliana*	reduced primary root growth, modified lateral root formation	Oriz-Castro *et al.* 2008
	synthetic	*A. thaliana*	increased lateral root formation	Bai *et al.* 2012
	synthetic	*A. thaliana*	modified root development	Schenk *et al.* 2012
	synthetic	*H. vulgare*	increased shoot growth (not significant)	Götz-Rösch *et al.* 2015
	synthetic	*P. erosus*	increased biomass	Götz-Rösch *et al.* 2015
	synthetic	*C. sativus*	modified root architecture	Pazarlar *et al.* 2020
	synthetic	*P. ginseng*	increased plant growth	Ibal *et al.* 2021
	synthetic	*A. thaliana*	reduced primary root growth, increased lateral root formation	Cao *et al.* 2022
oxo-C10-HSL	synthetic	*Vigna radiata*	increased lateral root formation	Bai *et al.* 2012
	synthetic	*A. thaliana*	specific plant growth responses	Schenk *et al.* 2014
	synthetic	*C. sativus*	increased shoot growth and leave area, modified root architecture	Pazarlar *et al.* 2020
OH-C10-HSL	synthetic	*V. radiata*	increased lateral root formation	Bai *et al.* 2012
	Acidovorax radicis	*H. vulgare*	increased dry weight	Li *et al.* 2012
	Acidovorax radicis	*H. vulgare*	increased fresh weight (only in soil after 2 months)	Han *et al.* 2016
	synthetic	*Zea mays*, *Glycine max*, *Arachis hypogaea*	reduced shoot and root growth	Lucero *et al.* 2022
C12-HSL	synthetic	*A. thaliana*	modified root growth and lateral root formation, root shortening and thickening	Oriz-Castro *et al.* 2008
	synthetic	*A. thaliana*	modified root development	Schenk *et al.* 2012
	synthetic	*H. vulgare*	increased root and shoot biomass (not significant), lateral root formation	Rankl *et al.* 2016
oxo-C12-HSL	*Pseudomonas aeruginosa*	*A. thaliana*	modified primary root growth and lateral root formation	Oriz-Castro *et al.* 2011
	synthetic	*A. thaliana*	no plant growth promotion	Schenk *et al.* 2012
	synthetic	*A. thaliana*	increased plant biomass	Shrestha *et al.* 2020
C14-HSL	*B. graminis*	*L. esculentum*	increased plant growth and salt tolerance	Barriuso *et al.* 2008
oxo-C14-HSL	synthetic	*A. thaliana*	no plant growth promotion	Schikora *et al.* 2011
	synthetic	*A. thaliana*	no plant growth promotion	Schenk *et al.* 2012
	Ensifer meliloti	*Medicago truncatula*	increased nodule formation	Veliz-Vallejos *et al.* 2014
	synthetic	*C. sativus*	no plant growth promotion	Pazarlar *et al.* 2020
AHL mix	*Serratia sp.*	various crops	increased plant growth, lateral root formation	Hanif *et al.* 2020
	Aeromonas	*T. aestivum*	increased plant growth, chlorophyll content	Nawaz *et al.* 2020
	Rhizobium radiobacter	*A. thaliana*, *T. aestivum*	increased root growth	Alabid *et al.* 2020
	Serratia sp.	*Z. mays*, *G. max*, *A. hypogaea*	increased root and shoot growth	Lucero *et al.* 2022
	Pseudomonas sp.	*Z. mays*	increased plant growth	Singh & Chauhan 2022

1.4.6 AHL effect on plant resistance to pathogens

Apart from enhanced plant growth, AHLs can also stimulate plant immunity. It was demonstrated in 2003 for the first time that *Medicago truncatula* plants react on application of synthetic AHL with a specific plant immune response (Mathesius *et al.* 2003). Since then, especially long-chain AHLs have been demonstrated to enhance plant resistance towards pathogens causing powdery mildew (Schikora *et al.* 2011), leaf spot disease (Schuhegger *et al.* 2006) or gray mold (Pang *et al.* 2009). Plant defense and detoxification genes have been found upregulated in AHL-treated model plants like *Arabidopsis*, *Medicago*, tomato, barley and yam bean (von Rad *et al.* 2008, Mathesius *et al.* 2003, Schuhegger *et al.* 2006, Han *et al.* 2016, Götz-Rösch *et al.* 2015). Moreover, lactonase induction suggested active quorum quenching of yam beans in response to the AHL stimulus (Götz *et al.* 2007). Recent studies on plant immunity upon AHL treatment are summerized in Table 1.2.

Table 1.2 Effect of microbial and synthetic AHLs on plant defense against pathogens and insects.

AHL	**AHL source**	**plant**	**plant immune response**	**reference**
C4-HSL	*Serratia liquefaciens*	*Lycopersicon esculentum*	upregulated defense genes, resistance to *Alternaria alternata*	Schuhegger *et al.* 2006
	synthetic	*Arabidopsis thaliana*	upregulated defense genes (auxin and cytokinin), others not upregulated	von Rad *et al.* 2008
	synthetic	*Cicer arietinum*	resistance to *Fusarium oxysporum*	Gupta *et al.* 2019
C6-HSL	*S.liquefaciens*	*L. esculentum*	upregulated defense genes, resistance to *A. alternata*	Schuhegger *et al.* 2006
	S.liquefaciens	*P. erosus*	lactonase induction	Götz *et al.* 2007
	synthetic	*A. thaliana*	upregulated defense genes (auxin and cytokinin), others not upregulated	von Rad *et al.* 2008
	synthetic	*Nicotiana attenuata*	increased herbivory of larvae *Manduca sexta*, modulated JA-defense	Heidel *et al.* 2010
	synthetic	*Hordeum vulgare*, *Pachyrizus erosus*	upregulated detox genes	Götz-Rösch *et al.* 2015
oxo-C6-HSL	*Pseudomonas putida*	*L. esculentum*	resistance to *A. alternata*	Schuhegger *et al.* 2006
	Serratia plymuthica	*Cucumis sativus*	resistance to *Pythium aphanidermatum*	Pang *et al.* 2009
	S. plymuthica	*L. esculentum*	resistance to *Botrytis cinerea*	Pang *et al.* 2009
	S. plymuthica	*Brassica napus*	resistance to *Verticillium dahliae*	Müller *et al.* 2009
C8-HSL	synthetic	*H. vulgare*, *P. erosus*	upregulated detox genes	Götz-Rösch *et al.* 2015
oxo-C8-HSL	*P. putida*	*L. esculentum*	resistance to *A. alternata*	Schuhegger *et al.* 2006

oxo-C8-HSL	*synthetic*	*A. thaliana*	resistance to *Pseudomonas syringae*, SA-dependent	Liu *et al.* 2020
C10-HSL	synthetic	*H. vulgare, P. erosus*	upregulated detox genes	Götz-Rösch *et al.* 2015
	synthetic	*L. esculentum*	resistance against *B. cinerea*, JA-dependent	Hu *et al.* 2018
	synthetic	*A. thaliana*	upregulated defense genes	Cao *et al.* 2022
OH-C10-HSL	*Acidovorax radicis*	H. vulgare	upregulated flavonoid-producing gene, slightly induced early defense	Han *et al.* 2016
oxo-C12-HSL	synthetic	*Medicago truncatula*	upregulated defense and stress managment genes, auxin-dependent	Mathesius *et al.* 2003
	synthetic	*A. thaliana*	slight resistance	Schikora *et al.* 2011
oxo-C14-HSL	synthetic	*H. vulgare*	resistance to *Blumeria graminis*	Schikora *et al.* 2011
	synthetic	*A. thaliana*	resistance to *Pst* and *Golovinomyces orontii*, upregulated WRKY, PR1, enhanced MAPK activity, callose deposition, no ROS accumulation	Schikora *et al.* 2011
	synthetic	*A. thaliana*	resistance against *Pst*	Schenk *et al.* 2012
	Ensifer meliloti	*A. thaliana*	resistance against pathogen	Zarkani *et al.* 2013
	synthetic	*A. thaliana*	resistance to pathogen, cell wall reinforcement, SA/oxylipin-dependent	Schenk *et al.* 2014
	E. meliloti, synthetic	*A. thaliana, H. vulgare, L. esculentum, T. aestivum*	resistance against pathogen	Hérnandez-Reyes *et al.* 2014
	E. meliloti, synthetic	*H. vulgare*	resistance to *B. graminis*	Shrestha *et al.* 2019
	synthetic	*C. sativus*	resistance to *Pseudoperonospora* and *Pseudomonas syringae pt. lachrymans*	Pazarlar *et al.* 2020
OH-C14-HSL	synthetic	*A. thaliana*	slight resistance to *Pst*	Schikora *et al.* 2011
oxo-C16	synthetic	*M. truncatula*	upregulated defense and stress managment genes, auxin-dependent	Mathesius *et al.* 2003
AHL mix	synthetic	*A. thaliana*	upregulated defense genes, resistance to *Pst*	Shrestha *et al.* 2020
	Rhizobium radiobacter	*A. thaliana, T. aestivum*	upregulated defense genes, resistance against *Xanthomonas translucens* and *Pst*	Alabid *et al.* 2020
	synthetic	*A. thaliana*	upregulated defense genes when challenged with flg22, JA-dependent	Duan *et al.* 2023

All these examples indicate that microbial AHLs play an important role in conveying plant resistance to pathogens. Hence, it appears likely that microbial AHLs can also help the plant to better cope with other biotic menaces like herbivores. Herbivorous insects can be successfully suppressed by beneficial plant-associated microbes and their stimulating metabolites (see 1.2.4). Microbial signalling molecules here appear as obvious compounds to be potentially involved in microbe-plant-insect interaction. Nevertheless, it remains largely unknown if AHLs can also modulate plant resistance to herbivorous insects.

1.4.7 AHL effect on plant resistance to insects

Microbial AHLs are considered to play a key role in interkingdom communication and have been shown to improve plant resistance in general. But the effect of AHLs on plant insect suppression is barely studied. This research gap will be tackled in the present thesis.

To our knowledge, only two studies investigated the connection between AHLs and pest insects so far (Table 1.2). Heidel and colleagues (2010) examined the effect of 10 µM synthetic C6-HSL to tobacco plants infested with leave-chewing larvae of the tobacco hawk moth *Manduca sexta*. The researchers found a 4-fold mass gain of the insect larvae when C6-HSL was applied compared to samples without added AHL. The AHL effect on plant-insect interaction appeared to be JA-dependent (Heidel *et al.* 2010). This result demonstrates that AHL can impact plant insect resistance. However, in this case, the AHL stimulus rather decreased plant resistance. It also remains unclear whether the AHL effect is of direct or indirect origin (Heidel *et al.* 2010).

In a second study, Wehner *et al.* (2021) investigated the effect of oxo-C14-HSL-producing legume symbiont *Ensifer meliloti* on the aphid *Rhopalosiphum padi* feeding on barley. With an electrical penetration graph technique, they showed that aphids fed less on plants treated with the AHL-producing strain *E. meliloti* expR^{+}ch in comparison to the lactonase expressing strain *E. meliloti* attM where AHLs did not accumulate. In addition, AHL-producing *E. meliloti* expR^{+}ch treatments showed reduced aphid biomass but unchanged aphid fecundity (Wehner *et al.* 2021). At the same time, plant biomass increased but only when plant growth was weakened by aphid infestation. Importantly, all these effects were only present in an AHL-sensitive barley genotype Morex while in another AHL-insensitive genotype BCC1415 the effects often pointed in the reverse direction (Wehner *et al.* 2021). These outcomes illustrate that AHL-producing bacteria can have a positive effect on plant aphid suppression in contrast to AHL signalling mutants. Also in that study, direct or indirect AHL effects could not be discriminated. It remains to be further explored which role AHL signalling molecules play during microbe-plant-insect interaction.

This thesis aims to shed more light on the question if microbial AHL signalling can modulate plant resistance to insects. For this investigation, two AHL-producing well-described rhizobacteria (*Acidovorax radicis* N35e and *Rhizobium radiobacter* F4) were selected and tested on the model plant barley (*Hordeum vulgare*) infested with English grain aphids (*Sitobion avenae*) under axenic and soil conditions.

1.5 The rhizobacterium *Acidovorax radicis* N35e

Acidovorax radicis N35e is a soil-borne β-Proteobacterium from the family *Comamonadaceae* (Burkholderiales) which was first isolated from wheat roots (Li *et al.* 2011, Willems & Gillis 2015). The aerobic, rod-shaped cells are motile due to usually one polar flagellum. The Gram-negative bacterium colonizes the root surface as well as the plant endosphere and has been shown to promote plant growth (Li *et al*. 2011, Willems & Gillis 2015, Han *et al.* 2016). In barley, *A. radicis* N35e inoculation increased plant dry weight and shoot biomass up to 40 % compared to the untreated control (Li *et al.* 2012). An irreversible phase switch was discovered for *A. radicis* to the phenotypic variant N35v, which showed only one mutation in the *mutL* gene but a substantially different transcriptional activity compared to the wildtype form N35e (Li *et al*. 2012). Individually, N35e and N35v showed no apparent differences in root colonization but when co-inoculated the variant N35v was outcompeted by N35e (Li *et al.* 2012). AHL production was not affected by the phase switch. Both *A. radicis* N35e and N35v produce 3-hydroxy-decanoyl-homoserine lactone (OH-C10-HSL) which is coupled to an *araI*/*araR* two component system; no other AHL or QS system could be identified unambiguously so far (Fekete *et al.* 2007). An *araI* mutant deficient in AHL synthesis was generated by directed insertion mutagenesis in strain N35e (*A. radicis araI::tet*, further also abbreviated as ArI_{neg}, Li 2011). This AHL-negative mutant showed no differences in swarming and siderophore production compared to the AHL-producing strain but colonized less on barley roots (Li 2011). The *A. radicis* wildtype formed microcolonies while the *araI* mutant rather attached as single cells (Han *et al.* 2016). Both *A. radicis* N35e wildtype and *araI* signalling mutant increased barley fresh weight after two months in soil, but not after two weeks in soil or under axenic cultivation conditions (Han *et al.* 2016). Surprisingly, differential gene expression analysis revealed that the *A. radicis* N35e *araI* mutant stimulated a stronger immune response in barley than the AHL-producing strain (Han *et al*. 2016). Especially flavonoid-related (UGT) genes were upregulated in *araI* mutant treated plants which also showed elevated flavonoid levels in leaves (Han *et al.* 2016). This suggests that AHL signalling of *A. radicis* contributes to plant stress resistance during microbe-plant interaction.

1.6 The rhizobacterium *Rhizobium radiobacter* F4

The α-Proteobacterium *Rhizobium radiobacter* F4 (form. *Agrobacterium tumefaciens*) has been isolated as fungal endosymbiont from the well-known basidiomycot *Serendipita indica* (form. *Piriformospora indica*, Varma *et al.* 1999, Sharma *et al.* 2008). The rod-shaped, aerobic cells build dense biofilms on a variety of host plants. Even though *R. radiobacter* F4 is closely related to crown gall causing *A. tumefaciens* (syn. *Rhizobium radiobacter*), the strain F4 has a beneficial effect on plants (Glaeser *et al.* 2016). Actually, *R. radiobacter* is thought to be responsible for many beneficial traits which have been attributed to its well-studied host fungus (Guo *et al.* 2017). Like its fungal host, *R. radiobacter* increased plant biomass and yield in several crop plants and induced resistance against powdery mildew in barley (Sharma *et al.* 2008). *Rhizobium radiobacter* strains promoted growth in barley (Humphry *et al.* 2007, Glaeser *et al.* 2016, Guo *et al.* 2017, Hadide *et al.* 2019, Kumar *et al.* 2021), maize (Singh *et al.* 2020a), brokkoli (Atal *et al.* 2019), bean and arugula (de Souza *et al.* 2016) and improved nutrient content of lettuce (Verma *et al.* 2020). Classically, nitrogen-fixing *Rhizobium* members form beneficial associations with mycorrhizal fungi and legumes in which AHL signalling plays an important role (Calatrava-Morales *et al.* 2018). Preferably, *R. radiobacter* F4 produces several C8-HSL, C10-HSL, C12-HSL and C14-HSL as well as derivates with C3-hydroxyl- or oxo-substitution (Li 2011, Alabid *et al.* 2020). The Ti plasmid of *R. radiobacter* F4 possesses two AHL-based *traI*/*traR* operons (Glaeser *et al.* 2016, Alabid *et al.* 2020). To inhibit AHL signalling, an AHL-negative strain of *R. radiobacter* named F4 NM13 (in this work also abbreviated as RrI_{neg}) has been generated by introducing the lactonase expressing plasmid pMLBAD-*aiiA* which hinders AHL accumulation (Alabid *et al.* 2020). This AHL mutant did no longer induce plant resistance against the bacterial pathogen *Xanthomonas translucens pv. translucens* (*Xtt*) in wheat (Glaeser *et al.* 2016, Alabid *et al.* 2020) and *Arabidopsis* (Alabid *et al.* 2020). The *R. radiobacter* wildtype induced a JA/ET-dependent immune response against *P. syringae* pv. *tomato* DC3000 (*Pst*) while the AHL inhibited mutant induced less resistance in *Arabidopsis* seedlings (Alabid *et al.* 2020). Also root colonization appeared compromised in the *R. radiobacter* AHL mutant (Alabid *et al.* 2020). These previous studies suggest that AHL signalling in *R. radiobacter* maintains the mutualistic relationship between the interacting microbe and its host.

1.7 Barley (*Hordeum vulgare*) as model plant

Barley (*Hordeum vulgare* L.) is a vascular plant from the family *Poaceae* and one of the most widely cultivated cereals worldwide (Dutilloy *et al.* 2022). The monocot plant is prone to several diseases and herbivores. Insect feeding causes up to 20 % yield loss in major crops these days (Deutsch *et al.* 2018). To face insect outbreaks, million tons of pesticides are required on barley fields annually what underlines the need for more sustainable biocontrol solutions (Dutilloy *et al.* 2022). Biostimulation with plant growth promoting and resistance inducing rhizobacteria has been studied extensively in this model crop (Canbolat *et al.* 2006, Çakmakçi *et al.* 2007, Wehner *et al.* 2019, Dutilloy *et al.* 2022). However, the beneficial interaction outcomes as well as insect susceptibility differs strongly between barley cultivars (Tétard-Jones *et al.* 2007, Zytynska *et al.* 2014, Shrestha *et al.* 2019).

Among the countless accessions, the modern barley cultivar Scarlett is widely grown but lacks resistance to foliar diseases (Hickey *et al.* 2017). In comparison, barley cultivar Chevallier is regarded as historic landrace with robust resistance and favorable characteristics for modern breeding programs (Goddard *et al.* 2019). Barke and Grace are moderately resistent cultivars and commonly used models for plant-pathogen interactions and plant genomics (Hofer *et al.* 2016, Thomas *et al.* 2018, Jayakodi *et al.* 2020).

Barley plants have been shown to be sensitive to microbial AHL signalling (Götz *et al.* 2007, Hérnandez-Reyes *et al.* 2014, Han *et al.* 2016) and synthetic AHL molecules (Schikora *et al.* 2011, Götz-Rösch *et al.* 2015, Rankl *et al.* 2016), although, a putative AHL receptor remains to be identified (Shrestha *et al.* 2020). Uptake and transport of AHL molecules into barley shoots has been described (Sieper *et al.* 2014) but quorum quenching by lactonases or explicit mimetic signal production was not discovered so far (Götz *et al.* 2007). Regarding the rich literature on inoculation experiments and our expanding knowledge about its defense gene regulation, barley represents one of the best plant models for multitrophic interaction studies with a short route for translational application in agriculture.

1.8 English grain aphids (*Sitobion avenae*) as pest insects

The English grain aphid (*Sitobion avenae* L.) is a pest insect from the family *Aphididae* (Hemiptera) which infests many economically important cereals (Blackman & Eastop 2000). Infested plants suffer from water loss through phloem-feeding, wilting and virus transmission which causes severe yield loss with aggravating tendency under climate change (Lehmann *et al.* 2020, Skendžić *et al.* 2021). *Sitobion avenae* feeds plant sap with a specialized stylet (Powell *et al.* 2006). The aphid body colour varies from green or black (e.g., genotype Fescue) to orange or pink (e.g., genotype Sickte) (Jenkins *et al.* 1999). Typically, the green legs bear a black stripe across femora and tarsi. Grain aphids mainly reproduce by parthenogenesis creating identical female clones. After four nymph stages, viviparous winged or unwinged adults usually produce up to three nymphs per day over a life span of approx. 30 days (Helden & Dixon 2002, Dai *et al.* 2015). A single sexual generation occurs in autumn triggered by lower temperatures and shorter photoperiods (Jaouannet *et al.* 2014). Thanks to these homogenous population properties, *S. avenae* represents a valuable insect model for interaction and biocontrol studies.

Thriving for a more sustainable pest management, it has been shown that aphids can be efficiently suppressed by plant stimulation with beneficial rhizobacteria (Pineda *et al.* 2012, Naeem *et al.* 2018, Zytynska *et al.* 2020, Gadhave & Gange 2022). The outcome of these interactions as well as the aphid's resistance towards plant defenses here strongly depend on the aphid genotype (Zytynska *et al.* 2016). In tetrapartite interactions, aphids are controlled by natural predators and parasitoid wasps like *Aphidius ervi* or *Aphidius rhopalosiphi* which can be attracted by soil microorganisms to regulate aphid populations (González-Mas *et al.* 2019, Bell *et al.* 2020, Gadhave & Gange 2022). These complex microbe-plant-insect interactions require a more profound understanding before being exploited in the field.

1.9 Aim of this study

The objective of this study is to understand whether microbial AHL signalling modulates plant resistance to pest insects. This knowledge gap will be addressed using the two AHL-producing rhizobacteria *Acidovorax radicis* N35e and *Rhizobium radiobacter* F4, the model crop barley (*Hordeum vulgare*) and English grain aphids (*Sitobion avenae*). Plant resistance to aphid feeding upon the respective AHL sensing and signalling mutant inoculation are compared to the effect of synthetic AHL molecules (OH-C10-HSL and C6-HSL) on the model system under axenic and soil conditions and across different barley cultivars.

The aim of this thesis is (1) to test if the rhizobacteria *A. radicis* N35e and *R. radiobacter* F4 successfully increase aphid suppression in the selected model system, (2) to explore if AHL signalling has an impact on plant aphid suppression and subsequently, (3) to disentangle the direct effect of synthetic AHL molecules from eventual indirect AHL effects that might be exhibited via a microbial rhizosphere community or via bacterial AHL-dependent self-regulation.

Results

2.1 Strategy of investigating AHL effects

In order to investigate the role of microbial AHL signalling on plant aphid suppression, a descriptive approach was used and narrowed down to a more and more functional analysis. In the following chapters, the AHL-producing and well-described rhizobacteria *Acidovorax radicis* N35e and *Rhizobium radiobacter* F4 were tested for their aphid suppression capacity in barley plants (*Hordeum vulgare*) under natural soil and sterile axenic conditions (chapter 2.2). The observed aphid suppression effects were traced upon their connection to plant growth and bacterial colonization. Hereafter, the effect of these test strains on aphid suppression, plant growth and immune response was compared to the effect of the respective bacterial mutant strains with impaired AHL synthesis (chapter 2.3). Afterwards, direct and indirect AHL effects are further disentangled. It was investigated to which extent the rhizosphere microbiome contributes to aphid suppression (chapter 2.4). Subsequently, direct AHL effects were explored by synthetic AHL molecules in a reduced axenic plant cultivation system (chapter 2.5). Bacterial mutant strains of *A. radicis* N35e impaired in AHL sensing by an *araR* gene knock-out were newly generated to compare the bacterial self-regulation effect when complementing the respective AHL sensing and signalling mutants with synthetic AHL (chapter 2.6).

The studied bacterial strains, as well as the plant and insect model organisms, were selected based on promising pre-results and tested systematically (Glaeser *et al.* 2016, Zytynska *et al.* 2020). As exemplary synthetic AHLs, the long-chain OH-C10-HSL was selected due to its occurrence in *A. radicis* N35e (Fekete *et al.* 2007) while C6-HSL represents the best studied short-chain AHL (Schuhegger *et al.* 2006, Schikora *et al.* 2011, Götz-Rösch *et al.* 2015). Experiments were conducted in climate chambers. Soil substrate here approximated natural conditions and provided information on interactions of bacterial AHL signalling effects with multiple biotic and abiotic factors, e.g., the rhizosphere microbiome, earthworms or nutrient availability. Bacteria-earthworm interactions and the influence of low nutrient concentration on impaired AHL signalling were the subject of our project partners; information on this can

be found in the published article Sanchez-Mahecha *et al.* (2022).

Despite the aforementioned advantages, soil systems do not allow a straight-forward analysis of AHL concentration that can vary locally to a vast extent due to the complexity of substrate niches. Addition of synthetic AHL molecules to natural soil is not suitable as they can be rapidly degraded by lactonase-producing microorganisms (Wang & Leadbetter 2005). Therefore, the more functional analysis of AHL effects was performed solely under axenic conditions in liquid plant growth medium (Hoagland's solution) where AHLs persist longer and can distribute more equally. An overview of the investigation system is given in Figure 2.1. Experimental details can be found in chapter 6.4.

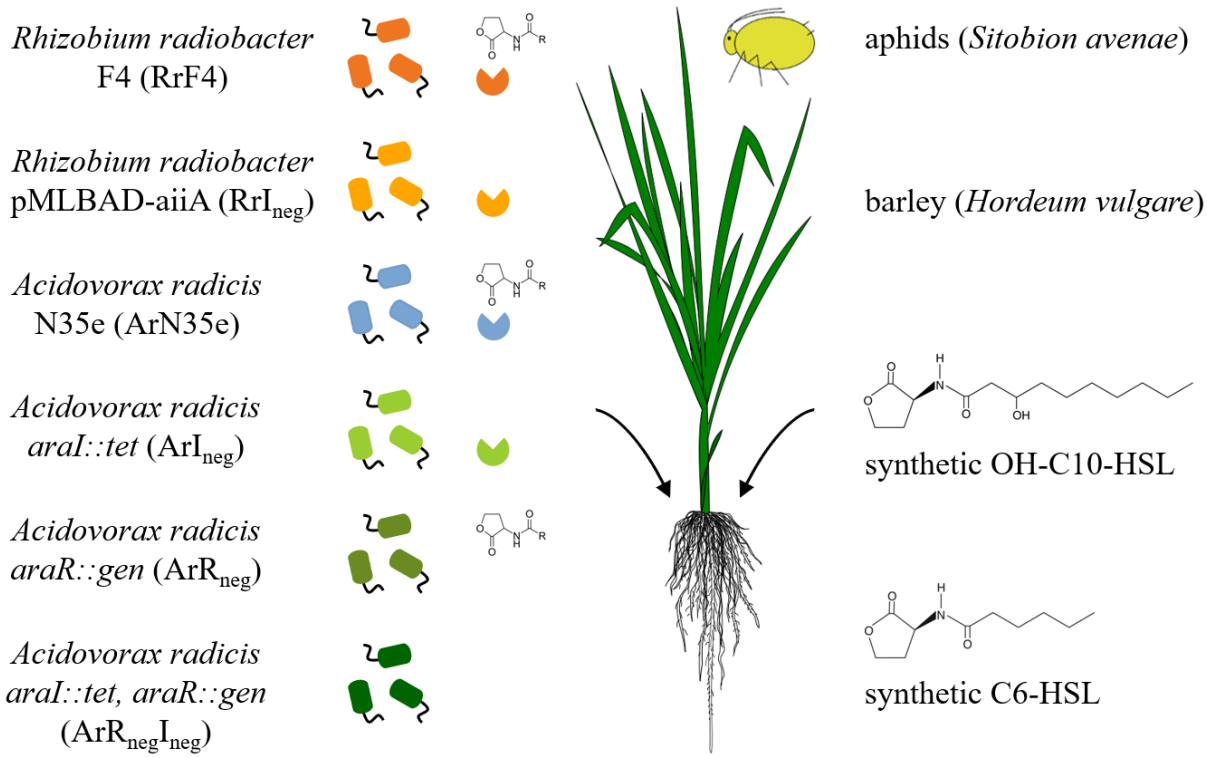

Figure 2.1 Overview of the experimental system investigating AHL effects.
The model crop barley (*Hordeum vulgare*, middle) was root inoculated with *Acidovorax radicis* N35e (left, cells depicted in blue) and *Rhizobium radiobacter* F4 (orange). These two rhizobacteria were fully functional in sensing and producing AHLs while their AHL mutants (in yellow and green) were impaired in AHL synthesis (*araI* gene knockout or introduced *aiiA*-encoded lactonase, no AHL molecule depicted) and/or in AHL sensing (*araR* gene knockout, no AHL receptor depicted). Aphids of the genotype Sickte (*Sitobion avenae*, scematically depicted on the right) served as insect model infesting barley leaves. In axenic complementation assays, the synthetic AHLs 3-*N*-OH-C10-homoserine lactone (OH-C10-HSL) or C6-homoserine lactone (C6-HSL) were added to barley roots.

2.2 Effect of bacterial test strains

2.2.1 ArN35e inoculation effect on plant aphid suppression

For a first test on aphid suppression in barley, the rhizobacteria *A. radicis* (ArN35e) and *R. radiobacter* (RrF4) were selected based on promising pre-results (Glaeser *et al.* 2016, Zytynska *et al.* 2020). The bacterial test strains were inoculated to barley seedlings under variable soil and axenic conditions. Aphid suppression was measured by counting the total aphid number (normalized to cm shoot length) 21 days post inoculation (dpi). For description of experimental setup and data collection see chapter 6.4.

The test strain ArN35e repeatedly increased aphid suppression under axenic conditions as well as in soil (Figure 2.2 a, b). In soil (Figure 2.2 b), ArN35e reduced aphid loads slightly in barley cultivar Scarlett (ArN35e – control: $p = 0.10$, ArN35e – RrF4: $p = 0.09$.) and Barke ($p = 0.02$ *) but not in Chevallier ($p = 0.92$). On average, each shoot centimeter was infested by 0.5 to 1 aphid less compared to uninoculated plants. This is equivalent to minus 15 – 25 % of the entire aphid load. Both aphid genotype Sickte and Fescue responded to the same extent upon ArN35e inoculation (see Supplementary Figure S2). The aphid suppression effect appeared to be barley cultivar dependent since aphid loads were only visibly decreased in barley cultivar Scarlett and Barke, but not in Chevallier (see Supplementary Figure S1 for absolute and relative soil data). Aphid suppression upon ArN35e treatment could be observed across diverse biotic and abiotic treatments and at different experimental sites (HMGU, TUMmesa, University of Liverpool). Therefore, the observed aphid reduction was considered to be a reproducible effect of biological relevance.

Under axenic conditions, the same aphid suppression tendency upon ArN35e inoculation was observed in four independent experiments AE1-AE4 (Figure 2.2 a, bacterial main effect $p = 0.193$, experiment effect $p = 0.021$ *). Yet, this tendency was not significant in all four trials. Power analysis revealed that small effect sizes – to reject the null hypothesis with a power of 80 % and a two-sided significant level of $\alpha = 0.05$ – would require several hundreds of true biological plant replicates what was not viable in the frame of this work (compare for a power analysis example Supplementary Table S1).

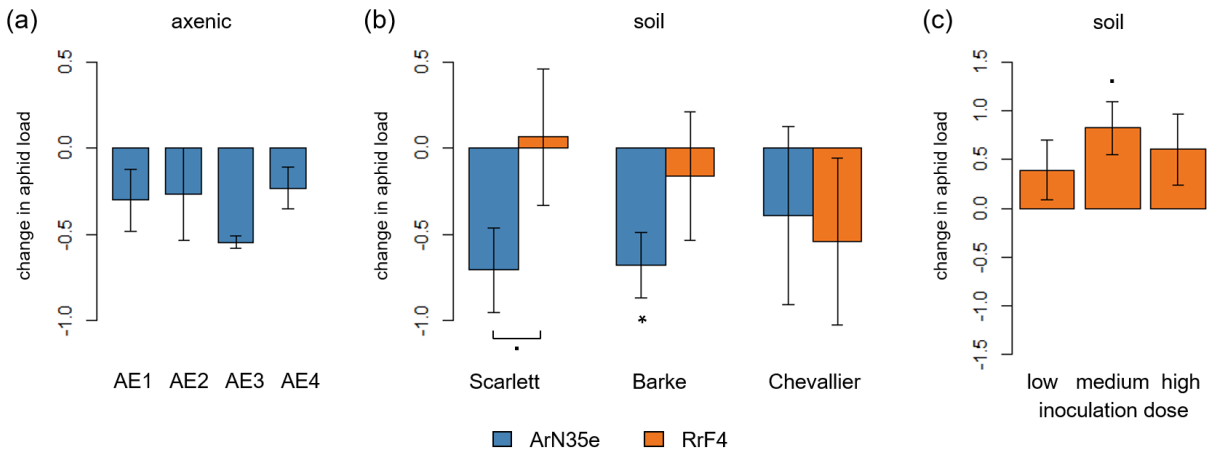

Figure 2.2 Effect of bacterial test strains on aphid suppression.
Acidovorax radicis N35e repeatedly reduced aphid load (a) in four experiments AE1-AE4 under axenic conditions (barley cultivar Scarlett, aphid genotype Sickte) and (b) in two experiments SE1 and SE2 across barely cultivar Scarlett, Barke and Chevallier (aphid genotypes Sickte and Fescue combined, results normalized and combined) under soil conditions. *Rhizobium radiobacter* F4 had no visible effect on aphid suppression in SE1 (b) and (c) rather increased aphid loads in soil experiment SE4 upon low (OD_{600} = 0.15), medium (OD_{600} = 0.5) and high (OD_{600} = 1.0) bacterial inoculation doses (barley cultivar Barke, aphid genotype Sickte). Depicted is the change in aphid number per cm shoot length normalized by control plants within each experiment on day 21 post inoculation. Error bars ± 1 SE, a) n = 4-21. b) n = 12-21. c) n = 8. Significance level p = 0.01 – 0.05 *, 0.05 – 0.1 . visualized with asterisk or point. Brackets: pairwise comparison, no brackets: significance compared to the baseline control.

2.2.2 RrF4 inoculation effect on plant aphid suppression

Test strain RrF4 showed no aphid suppression effect in soil (SE1, Figure 2.2 b). In comparison to control and ArN35e treated plants, the aphid load per cm shoot length did not change for barley cultivar Scarlett and Barke plants with RrF4 inoculation. Also in Chevallier, no aphid suppression could be observed similar to the ArN35e treatment. Based on this result, the possibility needed to be excluded that the relatively high inoculation dose had an unwanted detrimental effect on the microbe-plant interaction. Therefore, a separate soil experiment was conducted with lower RrF4 inoculation doses to screen for the optimal bacterial concentration (Figure 2.2 c). However, in all concentrations, the RrF4 inoculum rather increased aphid loads instead of decreasing them (bacterial main effect across inoculation doses p = 0.104, medium inoculation dosis p = 0.063 .). Therefore, this study focuses on *A. radicis* N35e AHL signalling in the following while *R. radiobacter* F4 was further used as comparison.

Based on the so far described aphid suppression effect of *A. radicis* N35e, this bacterium was considered as most promising candidate to study to which extent its AHL signalling contributes to the observed interaction outcome. Moreover, barley cultivar Scarlett and Barke were selected for further in-depth analysis since aphid suppression was most pronounced in these cultivars. Both bacterial inocula decreased aphids of the genotype Sickte in soil while genotype Fescue was only decreased in ArN35e treatments (see Supplementary Figure S2). Therefore, aphid genotype Sickte was chosen for further axenic experiments.

Aphid suppression requires a considerable amount of plant energy. As a consequence, this often affects plant growth in the well-known trade-off between plant growth and defense (He *et al.* 2022). The observed aphid suppression upon ArN35e treatment might thus cause decreased plant shoot and root growth. This way, plant growth can indirectly indicate if the plant immune system is stimulated. On the same note, plant growth can be inhibited via direct aphid damage or when the plant invests more energy in suppressing aphids than in plant growth. Shoot and root length and shoot and root fresh biomass were therefore determined 21 dpi, complementing the aphid suppression data.

2.2.3 ArN35e inoculation effect on plant growth

Barley plants inoculated with ArN35e showed no unambiguous change in shoot and root growth compared to uninoculated plants. Under axenic conditions, the growth differences between ArN35e and the uninoculated control in barley cultivar Scarlett were in a range below 1 cm shoot and root length (Figure 2.3 a-b) and 0.1 g shoot and root biomass (Figure 2.3 c-d) and thus not significant for all parameters. Also in soil, growth differences were not substantial upon ArN35e treatment and tendencies pointed into different direction between barley cultivars (Figure 2.3 e-h). Only in Barke, plants increased in shoot length (Figure 2.3 e: $p = 0.092$.) and root biomass (h: $p = 0.022$ *) upon ArN35e treatments compared to uninoculated plants.

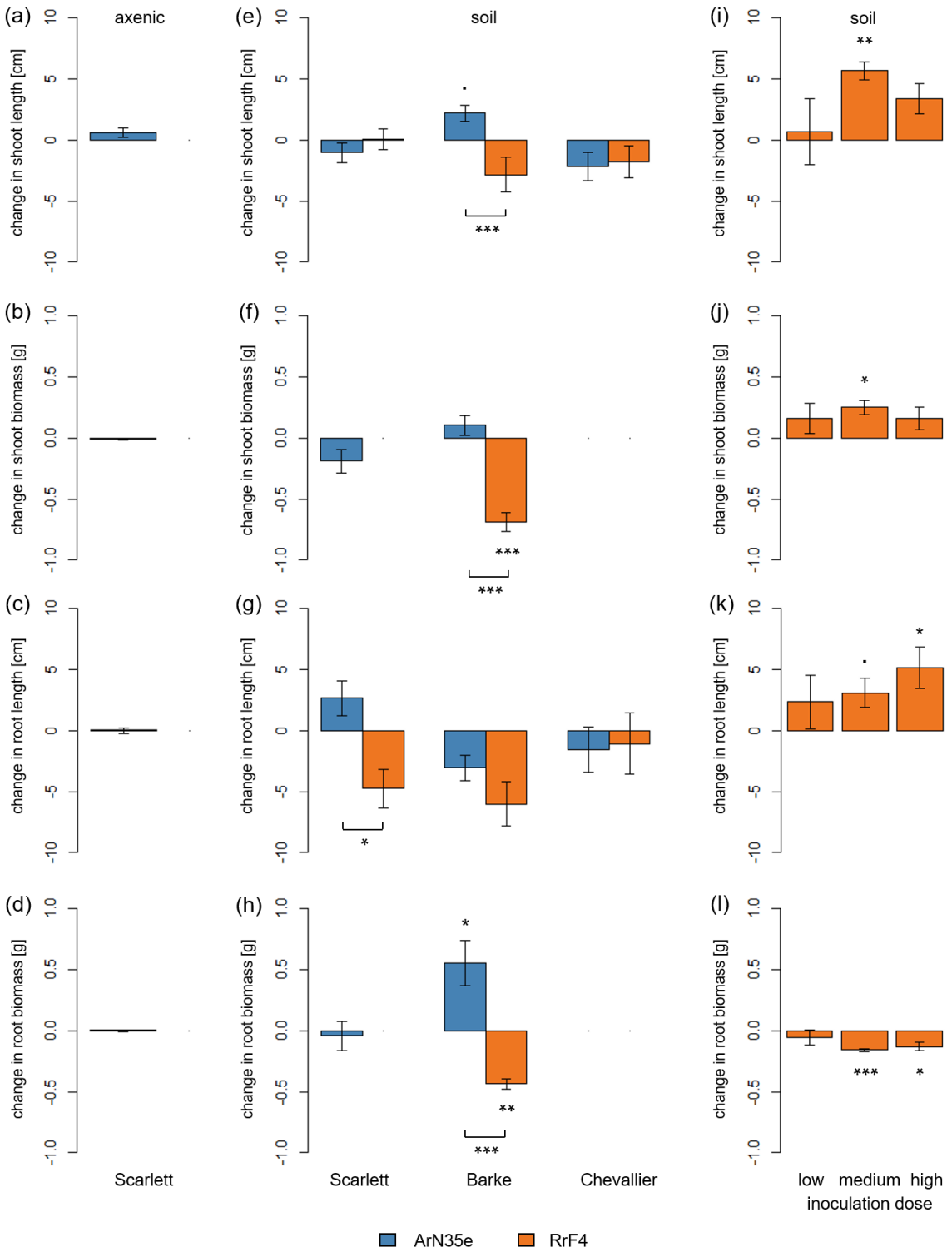

Figure 2.3 Effect of bacterial test strains on plant growth.
Acidovorax radicis N35e (a-d) did not change plant growth under axenic conditions (AE1, AE2, AE3, AE4 combined and normalized, barley cv. Scarlett), (e-h) had a small and variable effect on different barley cultivars Scarlett, Barke and Chevallier in soil experiments (SE1, SE2, SE3 combined and normalized, aphids excluded). *Rhizobium radiobacter* F4 (e-h) mainly decreased plant growth in soil

experiments SE2 and SE3, (i-l) but mainly increased plant growth in soil experiments SE4 when bacteria were inoculated in low, medium and high doses (barley cv. Barke). Depicted is the respective growth parameter change normalized by uninoculated control plants within each experiment on 21 dpi. Error bars ± 1 SE, a-d) n = 76, e-h) = 8-32, i-l) n = 16. Significance level p < 0.001 ***, 0.001 – 0.01 **, 0.01 – 0.05 *, 0.05 – 0.1 . visualized with asterisks or point. Brackets: pairwise comparison, no brackets: significance compared to the baseline control. Significant p-values are listed in Table S2.

2.2.4 RrF4 inoculation effect on plant growth

In contrast to ArN35e treatments, the effect of RrF4 on plant growth was significantly stronger. In the combined soil experiments SE1 and SE3 (Figure 2.3 e-h), barley plants treated with RrF4 showed mostly a negative growth tendency. The reduced shoot and root biomass was especially pronounced in Barke plants where the growth difference was significant between RrF4 and the control baseline (Figure 2.3 f: p = 9.65e-05 *** and h: p = 0.0049 **). Also, the growth difference between RrF4 and ArN35e appeared repeatedly significant (Figure 2.3 e: p = 0.0001 ***, f: p = 0.0001 ***, g: p = 0.0106 *, h: p = 0.0001 ***).
In turn, in an additional experiment SE4 with barley cultivar Barke, RrF4 was associated with a consistent increase in shoot growth and root length across inoculation doses (Figure 2.3 i-k, i: p = 0.003 **, j: p = 0.043 *, k: p = 0.083 . and p = 0.0221 *). Only root biomass was repeatedly decreased (Figure 2.3 l: p = 0.0006 *** and p = 0.019 *, all p-values are listed in Table S2).

Reduced plant growth in combination with increased aphid suppression would have given a first hint that a beneficial interaction is taking place between the plant and microbial interaction partners. However, this trait combination was not observed. This gives rise to the question to which extent the inoculated bacteria were actually able to develop and persist in the rhizosphere which is considered as an important prerequisite for a successful interaction.

2.2.5 ArN35e and RrF4 colonization of plant roots

Plant-microbe interaction happens at the root surface and in close proximity to the plant roots. Therefore, it can be insightful to track how successful the respective test bacteria established in the rhizosphere. The density and mode of bacterial colonization can cast light on the strength and quality of interaction, e.g., if a close contact is established which favours signal exchange

on a long-term basis. On one hand, only weak colonization might indicate that bacteria are fended off by the plant or outcompeted by other microorganisms in the rhizosphere. On the other hand, a very dense colonization can be of detrimental outcome for the plant.
In order to explore bacterial colonization, inoculated barley plants were harvested and fixed for microscopic analysis with Confocal Laser Scanning Microscopy (CLSM). All bacterial strains used in the presented experiments were available with GFP-labelling which did not require additional staining.

Both bacterial test strains, ArN35e and RrF4, could be detected in the rhizosphere of barley grown in soil and axenic systems. ArN35e was present 4 dpi in large accumulations but cells became undetectable after this time point (Figure 2.4 a, see also chapter 2.3.4 for time series micrographs). RrF4 was visible after 7 dpi in moderate quantities and closely attached to the root surface (Figure 2.4 b).

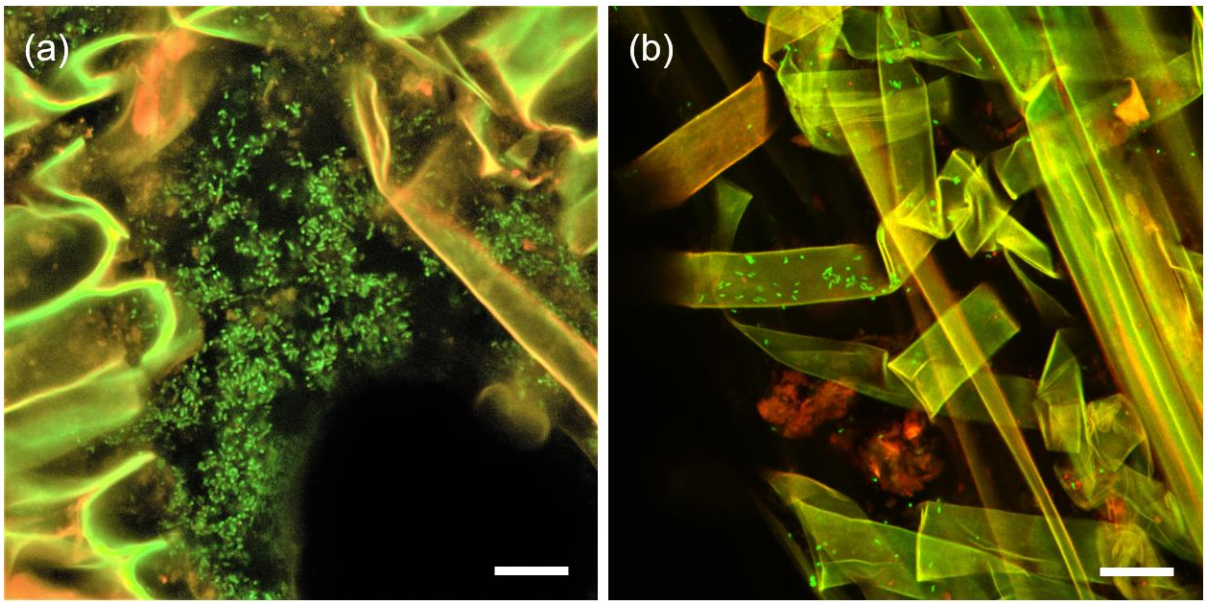

Figure 2.4 Root colonization of inoculated test bacteria in soil.
(a) *Acidovorax radicis* N35e and (b) *Rhizobium radiobacter* F4 were detectable by Confocal Laser Scanning Microscopy 4 dpi in soil. Both bacterial strains were GFP labelled and are visible in green, root background in yellow, soil particles in red. No additional staining performed. Micrographs were taken from (a) SE6 and (b) SE3. Scale bar = 10 µm.

Microscopic analysis revealed the successful establishment of both test bacteria at least until day 4, with a higher colonization density by ArN35e compared to RrF4.

To investigate the effect of bacterial inoculation on the rhizosphere more deeply, a microbiome analysis was additionally performed based on 16S rRNA Illumina sequencing (for Material and methods see 6.5). The microbial community composition was slightly but significantly affected by ArN35e treatment (uncorrected p = 0.015 *) while RrF4 had no significant effect on the microbial profiles (p = 0.162) (see Supplementary Figure S4). Based on 16S rRNA gene sequences, this analysis detected the genera *Acidovorax* and *Rhizobium* in the rhizosphere 21 dpi (data presented in the Supplementary Figure S3). Both genera were relatively low abundant with 0.002 % for *Acidovorax* and 0.5 % for *Rhizobium*. However, the abundance of both genera was not elevated when inoculated with the respective strain compared to uninoculated control samples. Therefore, the detected sequences rather belong to other genus members already prevailing in the sampled soil. These observations corroborate the impression that inoculated ArN35e and RrF4 vanished from the rhizosphere on a long-term basis but still had the potential to exhibit long-lasting effects.

2.3 Effect of bacterial inoculation with impaired AHL synthesis

In a first descriptive approach, the aphid suppression effect of *A. radicis* impaired in AHL synthesis (ArI_{neg}) was compared to the observed effect of the initial strain ArN35e. Since AHL is considered as a positive signal for the plant, the missing signal should lead to less aphid suppression compared to the inoculation effect of AHL-producing ArN35e.

2.3.1 ArI_{neg} inoculation effect on plant aphid suppression

Surprisingly, the AHL mutant ArI_{neg} showed a significantly stronger aphid suppression effect than ArN35e in soil (Figure 2.5 a, p = 0.012 *). Scarlett plants were infested with approx. 15 aphids less per g shoot biomass – equivalent to ca. minus 15 % – when treated with ArI_{neg} compared to ArN35e. In comparison to control treatments, the ArI_{neg} treatment reduced aphids with high significance – nearly 40 aphids less per g shoot biomass i.e., minus 50 % – when

calculated based on load per g biomass (p = 0.0004 ***, Figure 2.5). When calculated based on aphid load per cm shoot length, the difference between ArI_{neg} and control was still robustly significant (p = 0.018 *, Supplementary Figure S5) while the difference between ArN35e and ArI_{neg} appeared less pronounced (p = 0.28, Figure S5). Since correlation analysis revealed a significant interaction between aphid number and shoot biomass in soil (for correlation plot see Supplementary Figure S7), it was preferred to calculate aphid loads in respect to plant biomass in this case. The stronger aphid suppression effect of ArI_{neg} compared to ArN35e could only be found in barley cultivar Scarlett (bacterial main effect p = 0.001 **). For other cultivars and absolute data see Supplementary Figure S5.

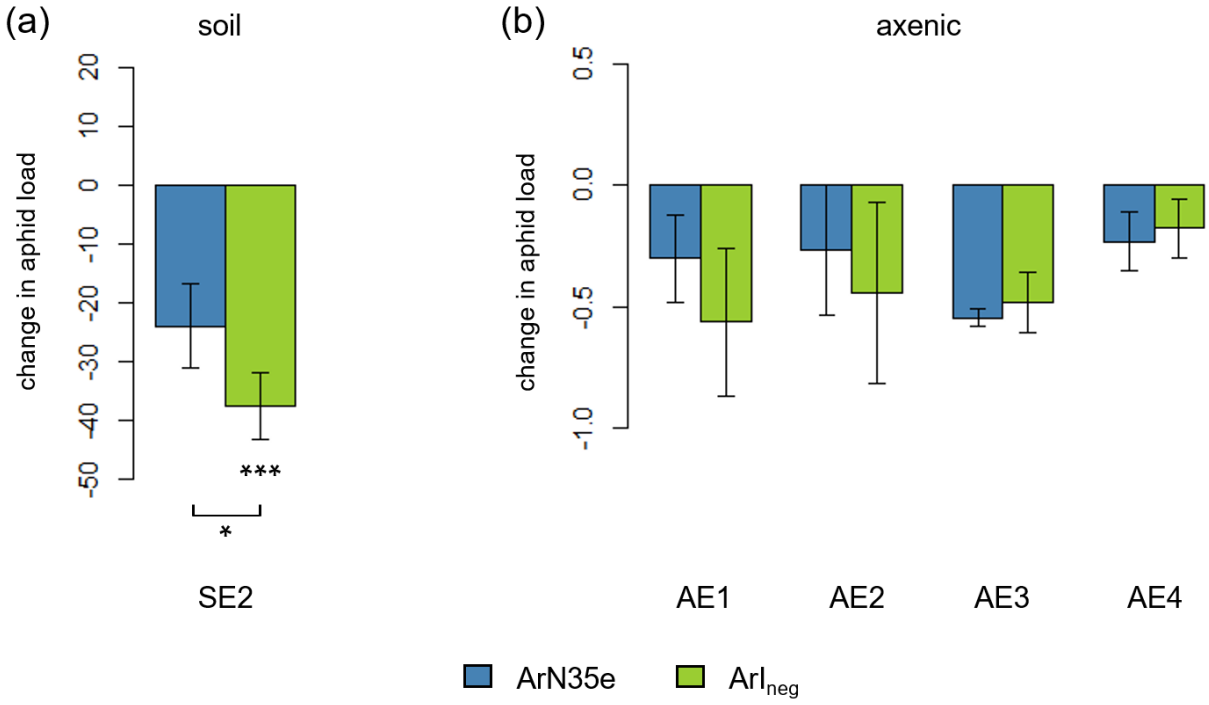

Figure 2.5 Impaired AHL signalling effect on aphid suppression.
Acidovorax radicis AHL synthesis mutant (ArI_{neg}) decreased aphid loads stronger compared to the AHL-producing strain *A. radicis* (ArN35e) in barley cultivar Scarlett in (a) soil experiment SE2 (aphid genotype Fescue) and (b) in four axenic experiments AE1-AE4 (aphid genotype Sickte, synthetic AHL treatments excluded). Depicted is the bacterial effect size on aphid load (a) per g shoot biomass or (b) by cm shoot length normalized by control plants within each experiment on 21 dpi. Error bars ± 1 SE. a) n = 21-24. b) n = 4-24. Significance level p < 0.001 ***, 0.01 – 0.05 * visualized with asterisks. Brackets: pairwise comparison, no brackets: significance compared to the baseline control.

The same tendency towards reduced aphid loads upon ArI_{neg} inoculation could be observed also under axenic conditions (Figure 2.5 b). However, this difference was not significant (AE1: $p = 0.51$, AE2: $p = 0.56$) and could not be repeated in follow-up experiments AE3 and AE4. In these trials, both bacterial treatments ArN35e and ArI_{neg} were associated with a reduction in aphid load approximately to the same extent (AE3: $p = 0.86$, AE4: $p = 0.76$). Apart from the cultivation system (discussed below), replicate numbers might have contributed to the statistical differences here (see comment on statistical power in 2.2.1 as well as 3.11 and sample size calculation example in Supplementary Table S1). Absolute data on aphid load under axenic conditions are depicted in the supplement (Figure S5 and S6).

Since AHLs are considered to contribute positively to microbe-mediated plant resistance, a missing AHL signal was expected to lead to less aphid suppression. Therefore, it was surprising to observe an increasing aphid suppression effect of *A. radicis* upon impaired AHL signalling (ArI_{neg}) compared to the initial strain ArN35e in soil. In contrast, under axenic conditions, this stronger aphid suppression effect of ArI_{neg} disappeared. It is conceivable that, in soil, the versatile functions of a complex rhizosphere microbiome might have intensified the inoculum effect. Therefore, a possible modulating role of the microbial community, indirectly mediated by missing AHL, was explored as shown further below (see 2.4). Additionally, the described increase of aphid suppression upon ArI_{neg} inoculation could likely be attributed to a stronger stimulation of the plant immune system. Therefore, it was investigated in a next step if the plant immune system was activated differently by ArI_{neg} and ArN35e inoculation.

2.3.2 ArI_{neg} inoculation effect on plant immune response

In order to study the effect of impaired AHL signalling on the plant immune response, a qPCR analysis targeting well-known plant immune response genes was performed on selected samples from soil and axenic inoculation experiments. Five genes were chosen based on common literature including 1) one ethylene responsive gene (*ERF-like*), 2) two salycilic acid-mediated pathogenesis-related genes (*PR1* and *PR17b*), 3) the trancription factor *WRKY22* and 4) a precursor gene essential for the production of UDP-glucuronosyltranferase (UTG) which is involved in the production of insecticidal flavonoids in plants.

Plant defense gene expression in the soil cultivation system (SE2) was analyzed by Oriana Sanchez-Mahecha. An additional analysis from the axenic system (AE2) was generated based in this protocol. In order to allow a comparison of the plant immune response under different conditions, both datasets were re-visualized for this thesis and depicted in Figure 2.6.

In soil, inoculation of ArI_{neg} was associated with a visible change in plant immune gene expression compared to the uninoculated control (Figure 2.6 a-j). In Barke, *PR1*, *PR17b* and *WRKY22* were upregulated up to two times when AHL signalling was impaired in ArI_{neg} treatments (Figure 2.6 b: p = 0.026 *, c: p = 0.045 *, d: p = 0.003 **). Comparing bacterial treatments ArN35e and ArI_{neg}, the gene expression only differed nearly significantly for *WRKY22* (d: p = 0.08 .).

In Scarlett under soil conditions, *PR1* and *PR17b* were significantly downregulated in ArI_{neg} compared to the uninoculated control (g: p = 0.008 **, h: p = 0.029 *). The expression differences between ArN35e and ArI_{neg} were significant in the *ERF-like* transcription factor (f: p = 0.047 *) and *PR1* (g: p = 0.034 *). The expression of *WRKY22* remained unchanged in Scarlett which is in contrast to the significant effect in Barke. In general, the barley cultivar highly influenced the gene expression of *PR1*, *PR17b* and *WRKY22* in soil (p < 0.0001 ***, respectively).

In Scarlett under axenic conditions, ArN35e treatment showed a significant downregulation effect in *PR1* (Figure 2.6 l: p = 0.007 **) and *PR17b* (m: p = 0.0008 ***), approximatley two times stronger than ArI_{neg} (l: p = 0.09 .) and five times stronger than the baseline control. This pattern was in remarkable contrast to the same analysis in soil where ArN35e provoqued no significant downregulation of these genes. In Scarlett, when cultivated in soil as well as under axenic conditions, *PR1* and *PR17b* were two times downregulated when ArI_{neg} was inoculated. This difference was significant in soil trials but appeared not significant under axenic conditions – probably due to less data points. The same gene regulation pattern of *ERF-like* and *WRKY22* from Scarlett in soil could be confirmed for axenic cultivation.

Independent of barley cultivar and cultivation system, a small upregulation could be observed for the UGT-related gene when ArI_{neg} was inoculated. This tendency appeared significant in axenically grown Scarlett compared to the uninoculated control (o: p = 0.031 *) and nearly significant compared to ArN35e (o: p = 0.088 .).

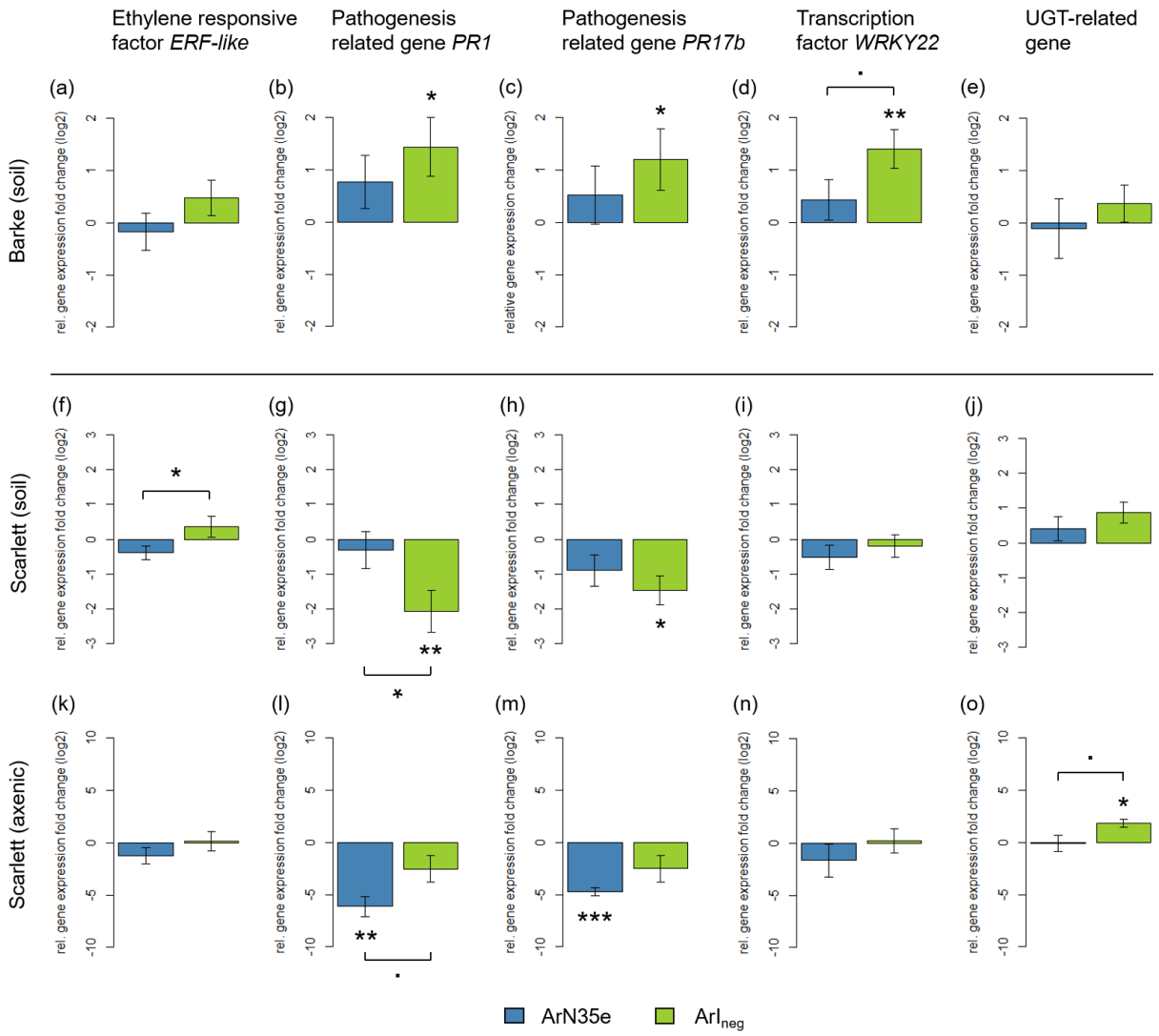

Figure 2.6 Effect of impaired AHL signalling on relative immune response gene expression of barley plants.

Figure adapted after Sanchez-Mahecha *et al.* (2022). Data from SE2 were generated by Oriana Sanchez-Mahecha and re-visualized for this study. A qPCR analysis was performed targeting plant immune response genes selected based on common literature for barley plants. The increase or decrease in gene expression was depicted for (a-e) barley cv. Barke in soil, (f-j) barley cv. Scarlett in soil and (k-o) barley cv. Scarlett in axenic systems. Samples were independently processed from soil experiment SE2 (a-j) and axenic experiment AE2 (k-o) following the same protocol. Values are given as the normalized log2 fold change (2^-ΔΔCt). Ct values from genes were normalized to the housekeeping gene *EF1α*. ΔΔCt values were calculated from the respective bacterial treatments normalized to control plants. (a-j) NoAphid and Aphid treatments and (k-o) NoAphid treatments. Error bars ± 1 SE. n = 3-12. True replicate number n = 3 while each replicate is a pool of two biological replicates. Significance level p < 0.001 ***, 0.001 – 0.01 **, 0.01 – 0.05 *, 0.05 – 0.1 . visualized with asterisks or point. Brackets: pairwise comparison, no brackets: significance compared to the baseline control. Significant p-values are listed in Table S3.

In total, plant immune response genes were observed to be differently regulated upon intact and impaired microbial AHL synthesis. The strength and direction of gene expression changed strongly depended on barley cultivar and cultivation system. In all analyzed genes, the direction of expression change was constant when comparing ArN35e and ArI_{neg} but with different strength. Interestingly, the expression change was more pronounced in ArI_{neg} treatments when cultivated in soil and less pronounced compared to ArN35e when cultivated under axenic conditions. This result is corresponding to the actual aphid suppression pattern (compare to 2.3.1) and represents a hint that the bacterial treatments are connected to plant resistance. As mentioned earlier, a modulated plant immune state can again have a significant impact on the plant performance including growth. Because of the known trade-off between plant growth and defense, complementing plant growth data can be insightful to better judge the plant's immune response. Plant growth behaviour upon impaired AHL signalling was therefore analyzed and compared to the plant immune response as presented in the following paragraph.

2.3.3 ArI_{neg} and RrI_{neg} inoculation effect on plant growth

When the plant immune system is activated by inoculated microbes with impaired AHL synthesis, it is conceivable that this energy is no longer invested in shoot and root growth. It has been revealed previously, that inoculation of *A. radicis* N35e had no significant plant growth promotion effect, despite a repeated aphid suppression effect. In contrast, inoculated bacteria with impaired AHL synthesis were associated with even stronger aphid suppression in soil (see 2.3.1). Therefore, it was expected that ArI_{neg} inoculation would lead to less plant growth compared to ArN35e inoculation and untreated control plants.
The AHL-producing strain *R. radiobacter* F4, which was used as a comparison, previously showed a rather negative effect on plant growth (see 2.2.4). Therefore, an even more negative growth effect could be expected upon impaired AHL accumulation in RrI_{neg}.

Despite changes in the plant immune response, plants inoculated with bacteria impaired in AHL signalling showed no unambiguous trend concerning growth (Figure 2.7). Shoot and root length and biomass remained the same upon ArN35e and ArI_{neg} treatments under axenic conditions (Figure 2.7 a-d) and fluctuated across barley cultivars in soil (Figure 2.7 e-h).

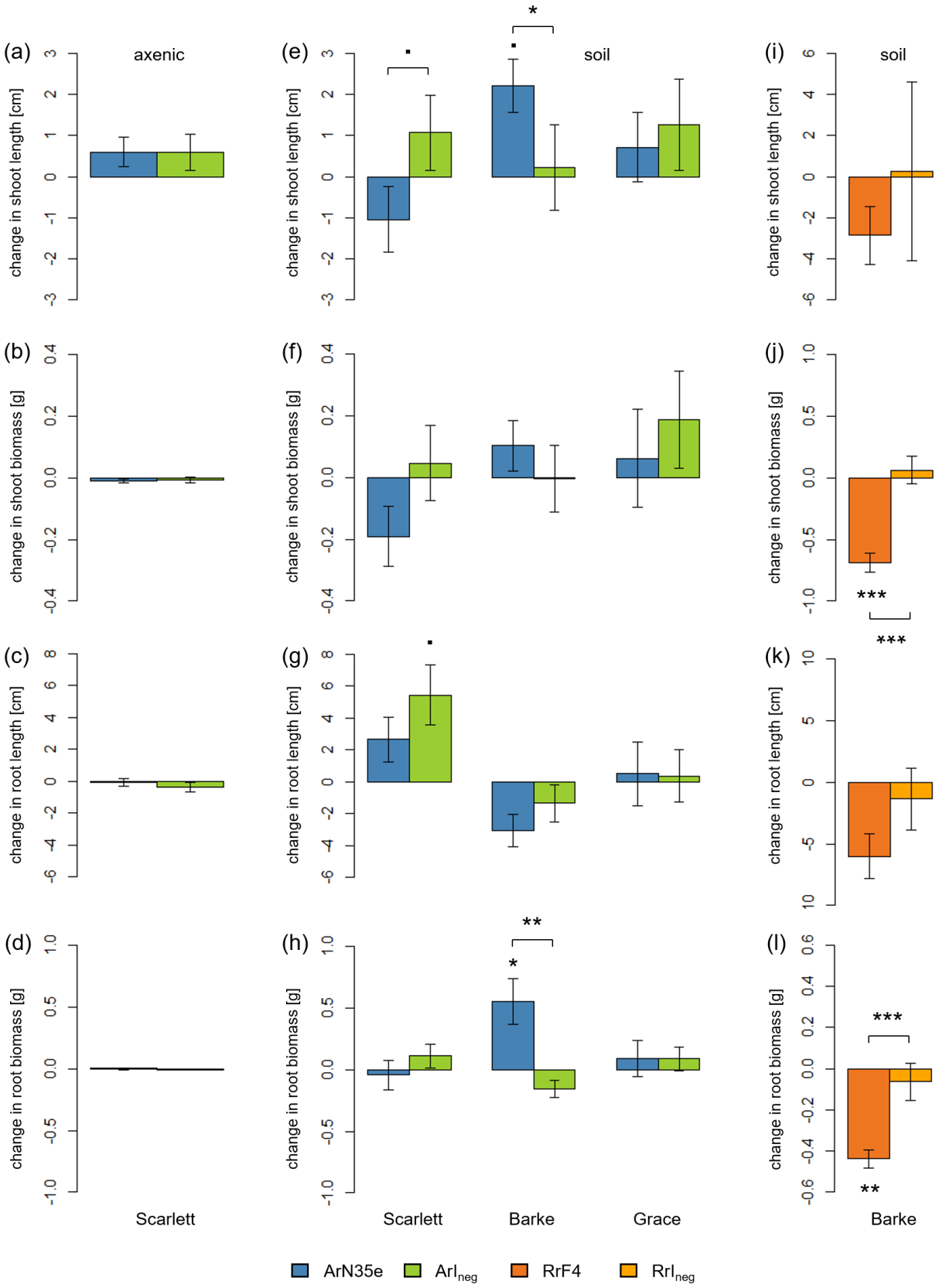
(a)
axenic
(e)
soil
(i)
soil
change in shoot length [cm]
(b)
(f)
(j)
change in shoot biomass [g]
(c)
(g)
(k)
change in root length [cm]
(d)
(h)
(l)
change in root biomass [g]
Scarlett
Scarlett
Barke
Grace
Barke
ArN35e
Arl$_{neg}$
RrF4
Rrl$_{neg}$

Figure 2.7 Impaired AHL signalling effect on plant growth.
Acidovorax radicis AHL synthesis mutant (ArI_{neg}) had only minor effects on plant growth, similar to the AHL-producing *A. radicis* strain (ArN35e) (a-d) in axenic experiments (AE1, AE2, AE3, AE4 combined and normalized, barley cv. Scarlett) and (e-h) soil experiments (SE1, SE2, SE3 combined and normalized, aphids excluded for better comparison). (i-l) *Rhizobium radiobacter* AHL signalling mutant (RrI_{neg}) showed a less negative plant growth effect compared to the AHL-producing *R. radiobacter* strain (RrF4) in soil experiment SE3 (barley cv. Barke). Depicted is the change in the respective plant parameter normalized by uninoculated control plants within each experiment on 21 dpi. Error bars ± 1 SE- a-d) n = 69-76, e-h) n = 12-36, i-l) n = 8-23. Significance level p < 0.001 ***, 0.001 – 0.01 **, 0.01 – 0.05 *, 0.05 – 0.1 . visualized with asterisks or point. Brackets: pairwise comparison, no brackets: significance compared to the baseline control. Significant p-values are listed in Table S4.

In Scarlett (soil), ArI_{neg} treatment slightly increased shoot length compared to ArN35e (Figure 2.7 e: p = 0.078 .) and root length in comparison to the control baseline (Figure 2.7 e: p = 0.087 .). In Barke, the positive growth trend of ArN35e treated plants was significantly diminished with missing AHL (ArI_{neg}) (Figure 2.7 e: p = 0.026 * and h: p = 0.001 **).
In addition, impaired AHL signalling in *R. radiobacter* (RrI_{neg}) showed a less negative growth effect compared to RrF4 in all parameters (Figure 2.7 i-l). While RrF4 showed a significant reduction compared to the baseline control (j: p = 0.0001 *** and l: p = 0.0013 **), shoot growth and root biomass were significantly less reduced upon RrI_{neg} inoculation (Figure 2.7 j: p = 1.42e-05 *** and l: p = 0.0002 ***). This result was unexpected since an even more pronounced growth reduction was assumed when RrI_{neg} stimulated the plant immune system.

In summary, plant growth was hardly influenced by present or absent AHL signalling. Again, the differences were more pronounced in soil than under axenic conditions. The expected growth reduction upon a stimulated immune system could not be confirmed. Instead, a small increase in plant growth upon ArI_{neg} treatments was observed in Scarlett under soil conditions. In Barke, the respective AHL-producing strains ArN35e and RrF4 had comparably strong effects on plant growth while in both cases the effect vanished when AHL signalling was impaired in the ArI_{neg} and RrI_{neg} treatments.
The small variations observed in plant growth effect between inoculation of the AHL-producing strain and the AHL mutant could originate from a different colonization capacity between these strains. Such colonization differences are very likely since AHL signalling influences surface attachment and biofilm formation (Davies *et al.* 1998). If bacterial root colonization differs between the *A. radicis* strains is investigated in the following.

2.3.4 ArI_{neg} colonization of plant roots

Colonization depends on biofilm formation, the production of an exopolysaccharid matrix and other important traits involved in cell attachment that naturally depend on AHL-mediated quorum sensing (Davies *et al.* 1998). Therefore, it is likely that impaired AHL synthesis can influence the colonization behaviour of the respective mutant strain. In order to track the colonization capacity of ArN35e and ArI_{neg} over the first days post inoculation, root samples were analyzed via CLSM.

The AHL synthesis mutant ArI_{neg} colonized in higher quantaties and persisted longer in detectable amounts on barley roots compared to ArN35e (Figure 2.8). This colonization pattern was verified in two independent time series experiments (SE5 and SE6) conducted in soil. By microscopic means, ArI_{neg} was detectable in the rhizosphere 4, 7 and 10 dpi (Figure 2.8 b, d, f) before it mostly disappeared from day 10 onwards. In contrast to this, ArN35e was only in some cases visible 4 dpi in soil – but then in considerable accumulations (Figure 2.8 a) – before it was no longer detectable after 7 and 10 dpi (Figure 2.8 c, e).

In the axenic system, ArN35e was hardly detectable microscopically (depicted further below for better comparison with mutant strains in Figure 2.14 a). In contrast to this, ArI_{neg} colonized strongly on the sterile roots and built biofilm-like structures (Figure 2.14 c). This colonization difference could be confirmed by two additional analyses. A more quantitative screen revealed several hundred ArI_{neg} cells in 90 – 100 % of the examined microscopic fields while ArN35e cells were visible in 0 – 5 % of the examined images and only built small accumulations with not more than 150 cells per field of view (see Figure 2.15 further below). In a CFU counting across three independent axenic experiments, ArI_{neg} cells were up to four times more abundant than ArN35e per g root (Figure 2.16).
Also in the axenic system, the cellular abundance of both *A. radicis* strains was highest in the early colonization phase. Cell numbers and CFU decreased over time but ArI_{neg} cells were still detectable after 21 days in all cases.

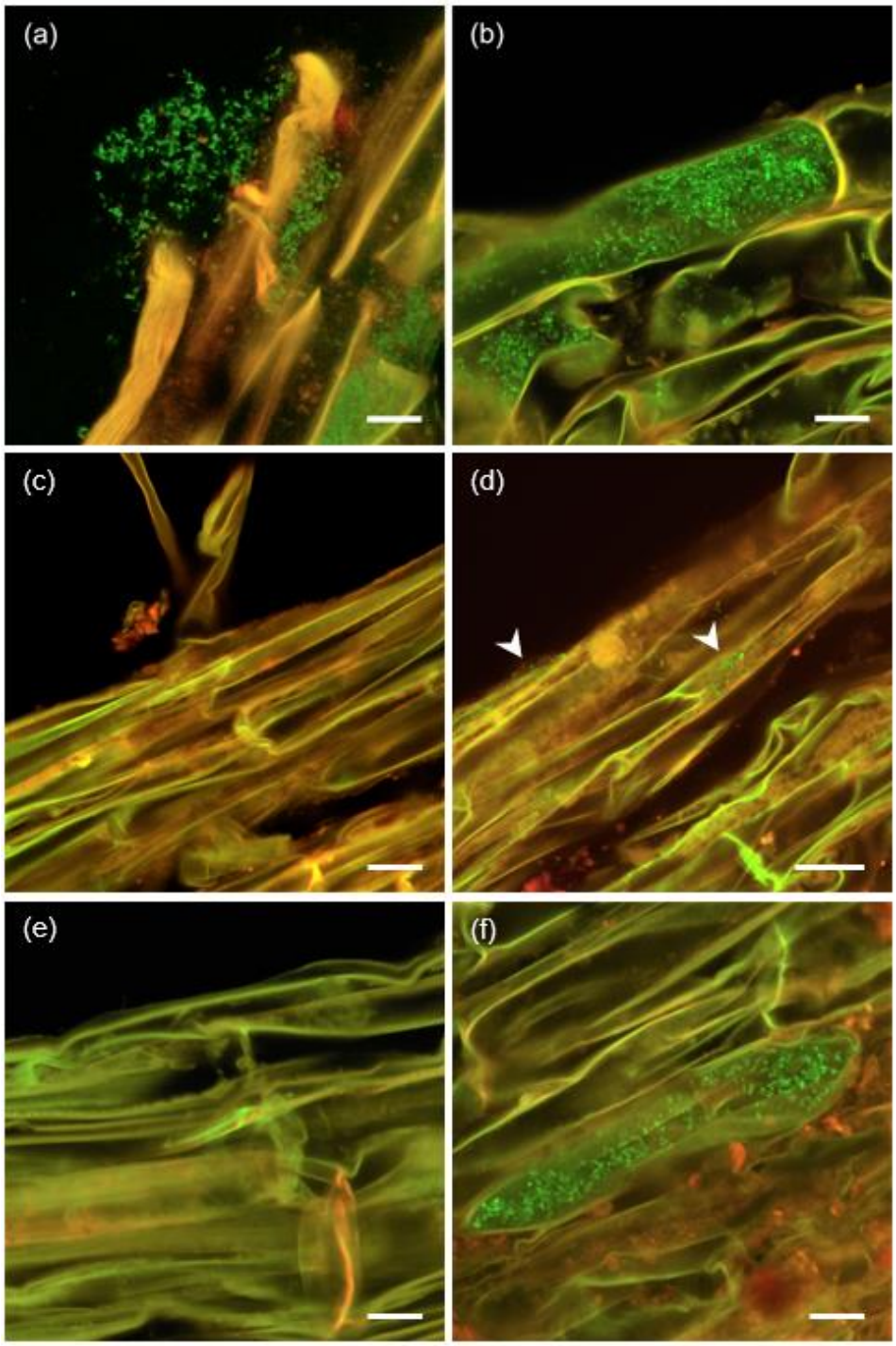

Figure 2.8 Root colonization of bacteria with impaired AHL synthesis over time in soil.
Figure adapted after Sanchez-Mahecha *et al*. (2022). (a) AHL-producing *A. radicis* (ArN35e) and (b) *A. radicis* AHL synthesis mutant (ArI_{neg}) were detectable by Confocal Laser Scanning Microscopy 4 dpi in soil. ArN35e was no longer visible at day 7 (c) and day 10 (e). ArI_{neg} was still detectable in low quantities at day 7 (d, arrowheads) and at distinct spots on day 10 (f) post inoculation. Compare also to the colonization pattern of ArI_{neg} in the axenic system in Figure 2.14. Both bacterial strains were GFP labelled and are visible in green, root background in yellow, soil particles in red. No additional staining performed. Micrographs were taken from SE6. Scale bar = 10 µm.

The additional quantitative analyses also surmounted any visibility bias that could have derived from the different GFP labelling of ArN35e and ArI_{neg}. In most analyzed samples, vector-labelled ArI_{neg} cells showed a comparably bright fluorescence, since the GFP gene was constitutively expressed, while ArN35e cells tended to be less bright due to chromosomal GFP labelling. This circumstance contributed to the impression of a different colonization pattern in a quick microscopic screen. Quantitative microscopic screen and CFU counting are described more in detail in chapter 2.6 including all AHL sensing and signalling mutants for the best comparison.

In summary, these analyses revealed a colonization difference between ArN35e and ArI_{neg}. Before, a stronger aphid suppression effects of ArI_{neg} compared to ArN35e was observed (see 2.3.1) while at the same time the plant immune response seemed to be stimulated in different ways (see 2.3.2). Plant growth was weakly affected by bacterial inoculation with impaired AHL synthesis while, in certain cases, it seemed to alleviate the effect of ArN35e (see 2.3.3). Together with the yet described differences in bacterial colonization (see 2.3.4), it is conceivable that different mechanisms connected to AHL signalling resulted in the different aphid suppression effect of ArN35e and ArI_{neg}. Together, direct and indirect AHL effects might fine-tune the plant response, as investigated in the following chapter.

2.4 Direct and indirect AHL effects: rhizosphere microbiome

Microbial AHL molecules can exhibit their effects on plant resistance in many direct and indirect ways. On the one hand, AHLs can be directly perceived, taken up into the plant tissue and transported to leaves and aphid guts. On the other hand, presence or absense of AHLs can indirectly change the colonization properties of the inoculated bacteria and thus the contact between beneficial interaction partners in the rhizosphere. At the same time, the expression of many AHL-dependent bacterial genes can be affected by the presence or absense of AHLs so that the bacterial behaviour can again indirectly alter the plant reaction. Furthermore, other factors in the plant surrounding might be modulated by intact or missing microbial AHL signalling. Especially other microorganisms in the rhizosphere can sense and react on AHLs, which might in turn have an indirect impact on the plant aphid suppression.

In order to disentangle these eventual direct and indirect AHL effects, the role of the microbiome in the context of impaired AHL synthesis (chapter 2.4.1 and 2.4.2), the effect of synthetic AHLs alone (chapter 2.5) and the bacterial self-regulation effect via AHL sensing (chapter 2.6) are more deeply analyzed in the ensuing paragraphs.

2.4.1 ArI_{neg} inoculation effect on the rhizosphere microbiome

In complex natural environments, the presence or absense of AHLs can modulate other biotic factors in the rhizosphere which can change the outcome of beneficial microbe-plant-insect interaction. Many Gram-negative bacteria are able to sense AHL molecules, they regulate their gene expression according to AHL concentrations or even degrade AHLs from inoculated bacteria by lactonase activity. This way, impaired AHL signalling could directly influence the abundance of other beneficial microorganisms in the rhizosphere which could indirectly affect plant aphid suppression. Given the additional case that the inoculated bacterial strains from this work do not persist permanently in the rhizosphere, as it was described above (see 2.3.4), the role of other microbial interaction partners might be even more important for plant immunity. This is why a deeper analysis of the indirect AHL-mediated effect via the rhizosphere microbiome might be of value for a more functional statement about the AHL mode of action. It can be hypothesized that ArN35e as well as ArI_{neg} inoculation has an impact on the rhizosphere microbial community composition. Identifying and correlating taxa to the results on aphid suppression could then provide insights about AHL effect ways.

For rhizosphere microbiome analysis, three root samples with only small, closely attached soil particles were selected across ArN35e, ArI_{neg}, aphid treatments, earthworms (data excluded from this work) and three barley cultivars from soil experiment SE2 (n = 108). Illumina sequencing of the 16S rRNA gene resulted in 20.7 million total paired-end reads and 12.4 million paired-end reads after quality filtering. After an initial cut-off threshold of 0.001 % was applied, 639 OTUs with 181 known and 105 unknown genera were retrieved. All hereafter described microbiome results were already published in Sanchez-Mahecha *et al.* (2022).

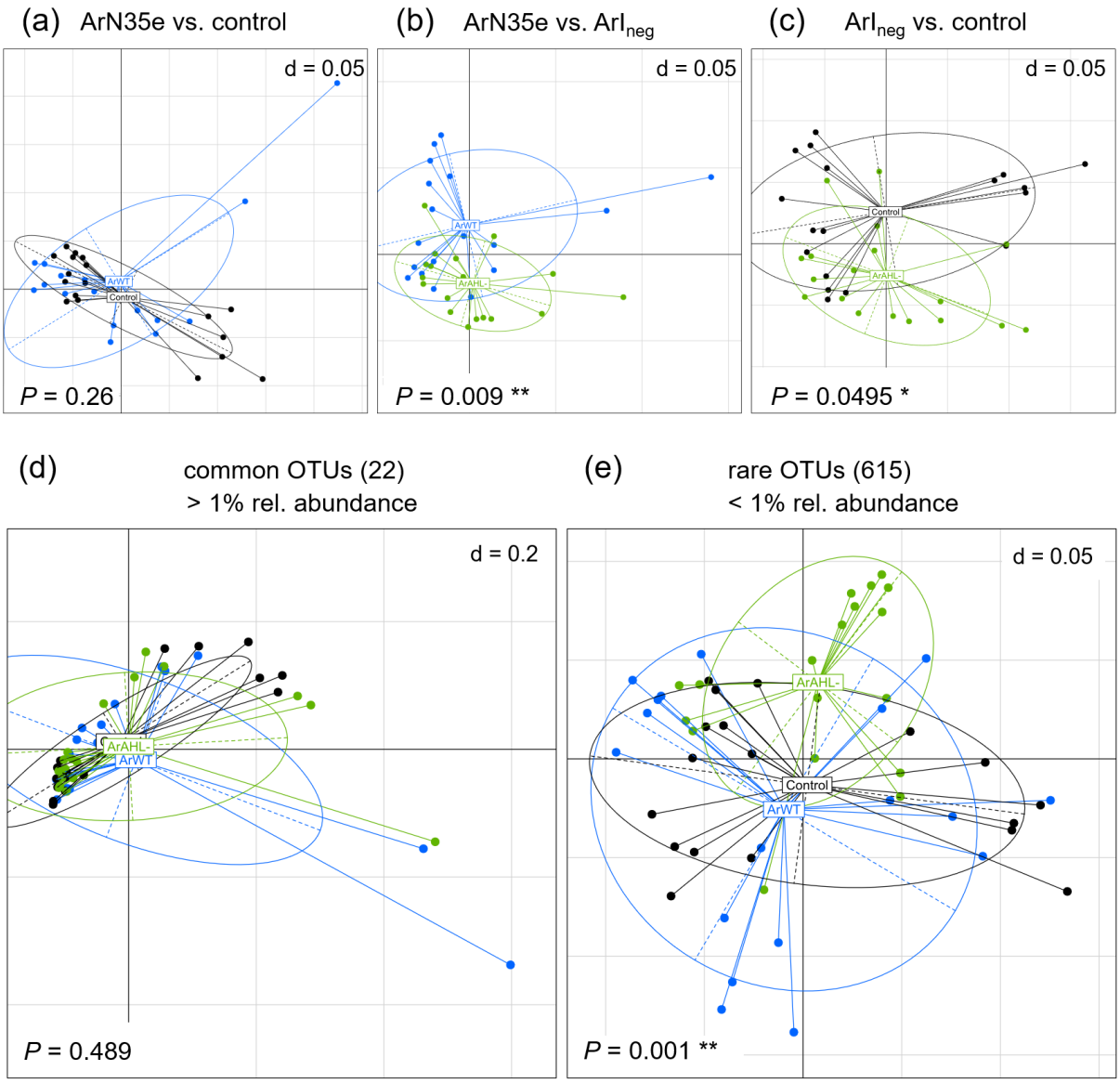

Figure 2.9 Effect of impaired AHL signalling on the microbiome profile of the barley rhizosphere. Figure adapted after Sanchez-Mahecha *et al.* (2022). Depicted are multi-dimensional scaling plots of the beta-diversity. Similarity of microbial profiles was calculated using a generalized UniFrac distance matrix. Permutational multivariate analysis of variance (vegan::adonis) was performed to determine significant separation of bacterial treatment groups. (a) Microbial groups treated with AHL-producing *A. radicis* (ArN35e, blue) did not separate significantly from not inoculated control (black). (b) Microbial groups treated with the *A. radicis* AHL synthesis mutant (ArI$_{neg}$, green) separated significantly from ArN35e (blue). (c) Microbial groups treated with ArI$_{neg}$ (green) separated significantly from the control (black). (d) Microbial profiles of the 22 most common OTUs did not separate significantly from each other. (e) Microbial profiles of rare OTUs separated significantly when inoculated with ArI$_{neg}$ (green) compared to ArN35e (blue) and the control (black). OTUs were classified as common or rare based on the relative abundance mean across all samples of one treatment. Significance level p = 0.001 – 0.01 **, 0.01 – 0.05 * visualized with asterisks.

Comparison of microbial profiles revealed that ArN35e had no significant impact on the rhizosphere microbial community ($p = 0.26$, Figure 2.9 a). In contrast to this, samples with ArI_{neg} treatments separated significantly from the control ($p = 0.009$ **, Figure 2.9 b) and from ArN35e ($p = 0.0495$ *, Figure 2.9 c). This separation was mainly driven by rare OTUs (rel. abundance <1 %, $p = 0.001$ **, Figure 2.9 e) while common OTUs (rel. abundance >1 %) did not contribute to this change ($p = 0.489$, Figure 2.9 d). Aphid treatments and barley cultivar did not change the microbial composition ($p = 0.769$ and $p = 0.974$ respectively, Figure S8 a, b). Species richness and evenness remained the same across all treatments (Figure S9).

2.4.2 Correlation of microbiome changes with plant aphid suppression

Genera with significantly changing relative abundance were identified across all bacterial treatments. Eighteen genera changed significantly when inoculated with either ArN35e or ArI_{neg} (bacterial main effect, $p < 0.05$). These genera are listed in Table 2.1 and comprised most prominently *Afipia*, *Bdellovibrio*, *Lacunisphaera*, *Limnobacter*, *Rhodanobacter* and *Sphingopyxis*. Sixteen of these genera also responded differently between ArN35e and ArI_{neg} treatments what might have been caused by missing AHL (Table 2.1, AHL effect). Eleven of the mentioned genera increased in their relative abundance when ArI_{neg} was inoculated (e.g., *Rhodanobacter*, *Pseudomonas*, *Microbacterium*, *Caulobacter*, *Granulicella*, *Pedobacter*) while only some genera decreased accordingly (*Lacunisphaera*, *Spirochaeta 2*). With ArN35e treatment, a few genera increased (*Opitutus*, *Bdellovibrio*) and decreased (*Limnobacter*) in relative abundance. Only three genera were significantly altered upon aphid treatment (*Dyadobacter*, *Hirschia* and *Stenotrophomonas*). Those were also selected as candidates for further investigation because of their eventual connection to aphid suppression (Table 2.1, Aphid effect). Most changing genera were rare community members while only *Opitutus*, *Pseudomonas* and *Rhodanobacter* belonged to the common OTUs. This finding fits to the aforementioned microbiome profiles (Figure 2.9 e) where separations were mainly driven by rare OTUs. The relative abundances of all changing genera are depicted in the supplement (Figure S10). An additional list with abundance values of all known genera is provided in Supplementary Table S5.

Table 2.1 Effect overview on significantly changing genera in the barley rhizosphere microbiome. Displayed are significant effects of *A. radicis* inoculation in general (ArN35e and ArI_{neg} together, bacterial effect), aphids and between ArN35e and ArI_{neg} (AHL effect) on other bacterial genera in the rhizosphere microbial community of barley plants. Eighteen genera changed significantly ($p < 0.05$) in their relative abundance at least upon one of the mentioned treatments. Genera are sorted by decreasing mean relative abundance (% of all known and unknown genera across all treatments). Significance level $p < 0.001$ ***, 0.001 – 0.01 **, 0.01 – 0.05 *, 0.05 – 0.1 . visualized with asterisks or point. n.s. = not significant.

Changing genera	**Mean rel. abundance** (%)	**Bacteria** (p-value)	**Aphids** (p-value)	**AHLs** (p-value)
Rhodanobacter	3.69	<0.001 ***	n.s.	<0.001 ***
Pseudomonas	1.46	0.031 *	n.s.	0.009 **
Opitutus	1.00	0.009 **	n.s.	0.003 **
Microbacterium	0.62	0.010 *	n.s.	0.006 **
Pedobacter	0.61	0.003 **	n.s.	<0.001 ***
Bdellovibrio	0.57	<0.001 ***	n.s.	0.001 **
Granulicella	0.54	0.002 **	n.s.	<0.001 ***
Caulobacter	0.52	<0.001 ***	n.s.	<0.001 ***
Afipia	0.26	<0.001 ***	n.s.	<0.001 ***
Lacunisphaera	0.24	<0.001 ***	n.s.	<0.001 ***
Spirochaeta 2	0.20	0.008 **	n.s.	0.005 **
Dyadobacter	0.17	0.007 **	0.022 *	n.s.
Sphingopyxis	0.17	<0.001 ***	n.s.	<0.001 ***
Stenotrophomonas	0.17	n.s.	0.047 *	n.s.
Hirschia	0.15	0.013 *	0.059 .	n.s.
Limnobacter	0.11	<0.001 ***	n.s.	<0.001 ***
Pajaroellobacter	0.09	0.014 *	n.s.	0.004 **
Cellulomonas	0.08	0.043 *	n.s.	0.049 *
Solimonas	0.05	0.005 **	n.s.	0.002 **

The identified community members responded differently to bacterial treatments with intact or impaired AHL synthesis. It was therefore explored to which extent the individual microbes contributed indirectly to the observed aphid suppression effect which might be mediated by the presence of AHL-producing ArN35e and the AHL synthesis mutant ArI_{neg}.

This question was answered by correlating the relative abundance of these genera with aphid loads. Most interesting here was to reveal negative associations, displayed in red in the correlation graph (Figure 2.10), because they indicate a potential connection between the presence of a bacterial genus and aphid suppression. A negative correlation in red here means that aphid loads were low while the bacterial abundance was high and the other way round.

However, these connections can be causal or coincidental and need to be evaluated in their respective context. If a genus is involved in aphid suppression, can be better judged by comparing the direction of correlation with the actual development of bacterial abundance like shown below (Table 2.2). For instance, aphids could be causally or coincidentally reduced when either a genus with decreasing relative abundance co-occurs with a positive correlation (blue) or when a genus with increasing relative abundance shows a negative correlation (red). In both possibilities, this genus could have contributed to a stronger plant aphid suppression as it was observed in ArI_{neg} treatments compared to ArN35e treatments.

Correlation analysis revealed small but specific correlations (Figure 2.10). The Pearson's coefficient of correlation was only significant in one association where the relative abundance of *Bdellovibrio* was associated with aphid numbers in a positive correlation (blue). When the AHL mutant was inoculated (ArI_{neg} vs. ArN35e treatment), the relative abundance of *Bdellovibrio* decreased (see Table 2.2).

All other correlations turned out to be rather weak. In ArN35e treated plants, correlations between the selected bacteria and aphid loads were principally more positive (darker blue) in comparison to the control. Only *Lacunisphaera* changed here from a positive correlation (blue) in control to a negative correlation (red) in ArN35e treated plants. ArN35e inoculation was associated with increasing relative abundance of *Lacunisphaera* while impaired AHL signalling decreased its relative abundance again (Table 2.2).

Most interestingly, *Hirschia, Sphingopyxis* and *Solimonas* turned from a positive correlation in ArN35e samples (blue) to a negative correlation in ArI_{neg} samples (red). In addition, *Spingopyxis* and *Solimonas* showed an increase in relative abundance with this negative correlation. The same pattern was revealed for *Limnobacter* (Table 2.2).

As an additional observation, correlations appeared to be less pronounced in ArI_{neg} treatments compared to ArN35e and the uninoculated control (e.g., in *Caulobacter*, *Cellulumonas*, *Opitutus* and *Rhodanobacter*). In general, correlations did not display any consistent effect pattern in all evaluated subsets. This underlines the variability of connections.

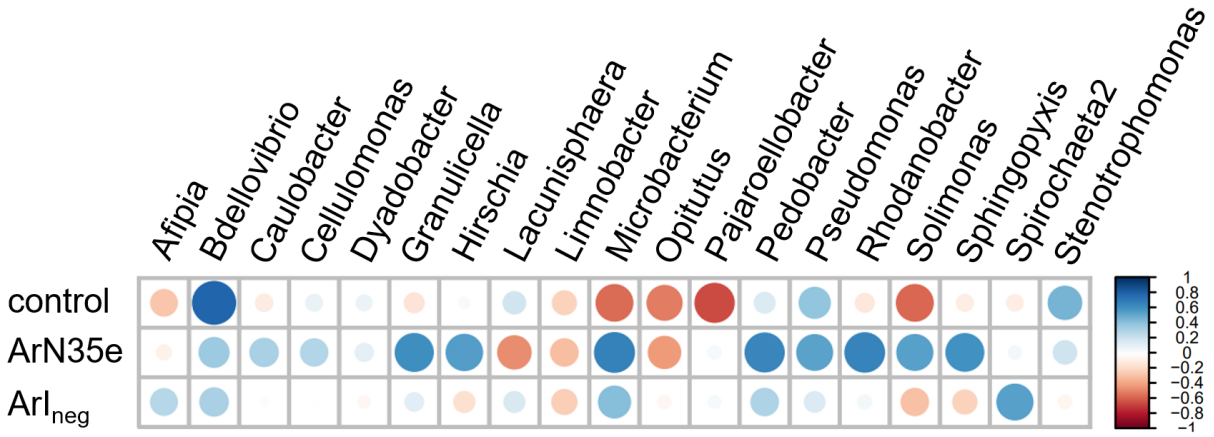

Figure 2.10 Correlations between changing genera and aphid load in the barley rhizosphere microbiome.

Figure adapted after Sanchez-Mahecha *et al.* (2022). Correlation plot showing positive (blue) and negative (red) correlations between aphid load per g shoot biomass and the relative abundance of genera, in dependence of the respective bacterial inoculum, namely AHL-producing *Acidovorax radicis* (ArN53e) and its AHL synthesis mutant (ArI_{neg}), and the uninoculated control. Only genera with significantly changing bacterial effect and/or aphid main effect were selected for correlation analysis. The colour code is equivalent to a Pearson's coefficient of correlation ranging from 1 to -1. The bigger the circle, the higher the significance (i.e., the lower the p-value). Only *Bdellovibrio* showed a significant correlation with aphid load in control samples (Pearson's R = 0.800, p = 0.01). n = 3.

Table 2.2 Comparison of relative abundance change and correlation in the barley rhizosphere microbiome (AHL effect).

Displayed are those genera where rel. abundance change and direction of correlation indicate reduced aphid load upon missing AHL synthesis in inoculated *Acidovorax radicis* (ArI_{neg}). Only genera from the barley rhizosphere were selected that significantly increased or reduced in rel. abundance when AHL signalling was impaired (ArI_{neg} treatments compared to ArN35e treatments, see also Table 2.1 and Figure S10). Directions of correlation correspond to Figure 2.10. Aphid loads were considered as reduced when either reduced rel. abundance co-occured with a positive genus-aphid correlation (blue), or when an increased rel. abundance co-occurred with a negative genus-aphid correlation (red).

Changing genera	rel. abundance	correlation	aphid load
Bdellovibrio	reduced	+	reduced
Lacunisphaera	reduced	+	reduced
Spirochaeta 2	reduced	+	reduced
Sphingopyxis	increased	–	reduced
Limnobacter	increased	–	reduced
Solimonas	increased	–	reduced

In summary, a significant impact of bacterial inoculation with inhibited AHL synthesis was observed on rare microorganisms in the rhizosphere. However, these changes were only weakly correlated to aphid suppression. The rhizosphere microbiome seemed to play not the decesive, but a modulating role in microbe-plant-insect interaction in this model system and can therefore be neglected for a more functional AHL effect analysis.
These outcomes also strengthen the possibility that the inoculated AHL-producing and AHL mutant strains themselves are responsible for the respective aphid suppression effect. Also, a direct effect of AHL molecules is thinkable. In order to pursue this hypothesis, synthetic AHL molecules were introduced into a reduced axenic system where effects can be studied without additional interactions.

2.5 Direct and indirect AHL effects: AHL molecule addition

2.5.1 Persistence of synthetic OH-C10-HSL and C6-HSL in the rhizosphere

In order to substract the direct effect of AHL molecules from the totality of microbial AHL signalling effects, synthetic AHLs were applied to plants in a sterile cultivation system. For this study, the only identified AHL from *A. radicis* N35e *N*-3-hydroxy-C10-homoserine lactone (OH-C10-HSL) was investigated for its aphid suppression effect. With its hydroxy group, this homoserine lactone consists of a rather unusual chemical form and can be classified as AHL with long carbon chain length. To account for this specific characteristic, the well-studied short-chain AHL C6-homoserine lactone (C6-HSL) was selected as reference. Respectively, both synthetic AHL molecules were solved in acetonitril and added to the plant growth medium (Hoagland's solution) in an initial concentration of 10 µM. In axenic plant experiments, the impact of OH-C10-HSL and C6-HSL on aphid suppression was observed in control plants where the bare effect of AHL molecules were not masked by other factors. After 21 days, the concentration of AHL molecules was measured by a liquid biosensor assay.

Both AHLs were still detectable in the plant growth medium after 21 days (Figure 2.11 a). A liquid biosensor assay detected significantly higher AHL concentrations in all synthetic AHL treatments – approx. 0.8 µM OH-C10-HSL and up to 1.2 µM C6-HSL more – compared to

NoAHL treatments (main inoculation effect of OH-C10-HSL: $p = 0.073$., C6-HSL: $p < 0.0001$ ***, difference between OH-C10-HSL and C6-HSL $p = 0.23$ n.s., additional p-values are listed in Table S7). In the NoAHL control, the biosensor detected a baseline concentration below 0.5 µM. The biosensor was sensitive down to 0.004 µM OH-C10-HSL and 0.25 µM C6-HSL. Surprisingly, the OH-C10-HSL concentration was not higher in the ArN35e treatments, where additional AHL was presumably produced by the fully functional bacteria, compared to the other treatments. That AHL was still detectable after 21 days confirms that AHLs were persistent in the plant rhizosphere over the whole experimental course and were able to exhibit a direct effect on the plant roots.

2.5.2 Effect of synthetic OH-C10-HSL and C6-HSL on plant aphid suppression

In respect to aphid loads, the addition of both synthetic OH-C10-HSL and C6-HSL to axenically grown, not inoculated control plants revealed a constant decrease (Figure 2.11 b, see also 2.12 b). However, this bacterial effect was only small and not significant (Figure 2.12 b, OH-C10-HSL: $p = 0.96$, C6-HSL: $p = 0.31$). When the AHL concentration detected with a biosensor assay exceeded the baseline threshold of 0.5 µM, the same connection to stronger aphid suppression – ca. minus 40 % of persisting aphids, although not significant – was observed (Figure 2.11 b, OH-C10-HSL: $p = 0.30$, C6-HSL: $p = 0.39$). Only small replicate numbers were retrieved for biosensor analysis so that further correlation analysis did not reveal insightful connections. In case a stronger plant immune response was stimulated with the observed higher AHL concentration, this could lead to energy redistribution and thus to reduced plant growth. This aspect will be examined in the next paragraph.

2.5.3 Effect of synthetic OH-C10-HSL and C6-HSL on plant growth

Growth differences in plants treated with synthetic AHLs can indicate that the AHL molecules exhibit a direct effect on the plants well-being and immune state. Synthetic AHL treatment of plants revealed that OH-C10-HSL tended to increase root growth. These differences were close to significant in control plants without bacterial inoculation (compare Figure 2.11 c-f and Figure 2.13 e-h, OH-C10-HSL vs. NoAHL g: $p = 0.076$. and h: $p = 0.090$.).

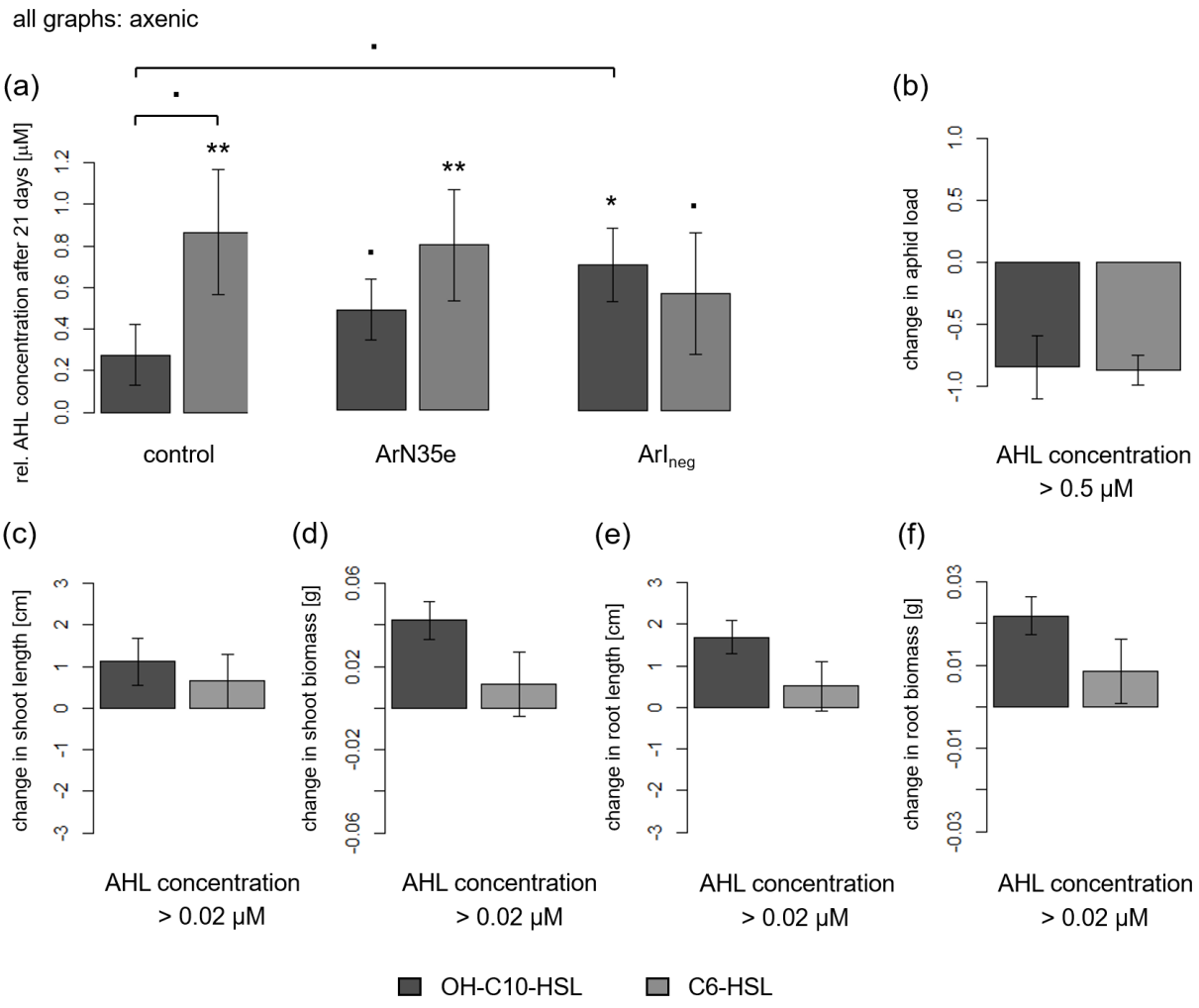

Figure 2.11 Detection of synthetic AHLs and their effect on aphid suppression and barley growth. (a) Detectable concentration of synthetic AHLs relative to the NoAHL control in plant growth medium after 21 days (data combined from AE1 and AE2 across three independent experimental runs, n = 17-24). OH-C10-HSL and C6-HSL were detectable in sign. elevated concentrations. (b) Both synthetic AHL treatments slightly, but not significantly, increased aphid suppression in control plants when detected AHL concentrations were > 0.5 µM (data normalized to the baseline threshold of 0.5 µM and combined from AE1, AE2, n = 4-5). (c-f) Plant growth was slightly increased upon synthetic OH-C10-HSL treatment when the detectable AHL concentration was > 0.02 µM (data normalized to samples with no detectable AHL and combined from AE1, AE2, n = 17-35). All Hoagland's solution samples were tested for their AHL concentration 21 days post addition with an *A. tumefaciens* liquid biosensor assay. X-Gal turnover into blue colouration was evaluated visually in comparison to an AHL standard series. Error bars ± 1 SE. Significance level p = 0.001 – 0.01 **, 0.01 – 0.05 *, 0.05 – 0.1 . visualized with asterisks or point. Brackets: pairwise comparison, no brackets: significance compared to the baseline control. Significant p-values are listed in Table S7.

When only samples were considered in which AHL was still detectable after 21 days, shoot and root length increased up to 2 cm – approx. 10 % of total root length –, but no value was significant (Figure 2.11 c-f, for example d: OH-C10-HSL vs. NoAHL p = 0.82). The reference AHL C6-HSL did not significantly change plant growth in any of the examined parameters. In the light of very small effect sizes, the minimal number of replicates needed to exceed hundreds of plants per treatment to allow robust statements about plant growth. Reasonably, this was not practicable in the frame of this thesis (see also comment on statistical power in 2.2.1 as well as 3.11 and sample size calculation example in Supplementary Table S1).

In summary, synthetic AHLs increased aphid suppression as a tendency. Synthetic OH-C10-HSL application slightly increased root growth whereas C6-HSL had no positive growth effect on barley. Reduced plant growth in combination with stronger aphid suppression could have indicated a direct immune system stimulation by the AHL molecules. This connection was not observed similarly to treatments with present or absent microbial AHLs (see 2.3.1 and 2.3.3). It is conceivable that the totality of changing AHL sensing and signalling and other signals important for interaction might lead to the observed aphid suppression differences between ArN35e and ArI_{neg}. Therefore, it is axiomatic to compare the effect of AHL alone to the combined effect with the bacterial mutant strain lacking AHL production. Also, modulated gene expression by differential AHL sensing might exhibit an important indirect AHL effect. In order to disentangle these functions more in detail, AHL sensing mutants were generated and compared for their effects in AHL complementation experiments.

2.6 Direct and indirect AHL effect: impaired AHL sensing and overlapping effects

2.6.1 Complementation effect of AHL mutants with synthetic AHL on plant aphid suppression

For a more detailed statement about direct and indirect AHL effects, AHL sensing mutants were constructed like described in the methods part (see 6.2). By gene replacement mutagenesis, the *araR* gene encoding the AHL receptor was knocked out in the ArN35e strain and in the ArI_{neg} strain, respectively, leading to the AHL sensing mutant ArR_{neg} and the double

mutant $ArR_{neg}I_{neg}$. For both newly generated strains, no AHL production was observed in a classical biosensor streak-out assay (Supplementary Figure S12). This means for the ArR_{neg} mutant that impaired AHL sensing naturally also dampened AHL production how expected. The biosensor assay was not sensitive enough to resolve the differences between ArN35e and ArR_{neg} in AHL production when complemented with synthetic AHL on plate (Supplementary Figure S13).

Comparing AHL sensing and signalling mutant effects could elucidate if bacterial self-regulation by AHL perception is playing a role in plant aphid suppression. All four AHL sensing and signalling mutants were complemented with synthetic OH-C10-HSL, and C6-HSL as reference, in axenic experiments. Only with AHL addition, differences in AHL-regulation behaviour should become apparent. In ArN35e treatments, AHL addition could lead to an additive aphid suppression effect of AHL molecules on the one hand and induced beneficial bacterial traits on the other hand – if positively regulated by AHL. Likewise, when synthetic AHL is added to the AHL-sensitive ArI_{neg} mutant, the initial effect should be reestablished. In contrast, AHL-insensitive ArR_{neg} and $ArR_{neg}I_{neg}$ should not react on supplemented AHL, so that the bare effect of AHLs on plants should become visible. Especially $ArR_{neg}I_{neg}$ can be seen as negative control where vivid cells are present but without any AHL-mediated interaction with the plant. With these additional reference points, small nuances between direct and indirect AHL effects can be distinctly compared.

Without synthetic AHL, as expected, ArR_{neg} and $ArR_{neg}I_{neg}$ treated plants revealed no significantly different aphid suppression compared to ArI_{neg} and ArN35e treatments under NoAHL conditions (Figure 2.12 a).
When synthetic OH-C10-HSL was added to the treatments, aphid loads were reduced in the control and in both AHL sensing mutant treatments (Figure 2.12 b). However, this reduction was only significant for $ArR_{neg}I_{neg}$ ($p = 0.014$ *). Surprisingly, the aphid suppression effect of ArN35e and ArI_{neg} mutant did not change in strength upon OH-C10-HSL addition. No positive effect addition could be observed. The unchanged aphid suppression effect rather pointed towards two counteracting effects of AHL molecules directly (reducing aphid loads) and the bacterial reaction towards AHL (increasing aphid loads). Only when the AHL-insensitive mutants ArR_{neg} and $ArR_{neg}I_{neg}$ were applied together with synthetic OH-C10-HSL, aphid loads

decreased significantly (Figure 2.12 b, for p-values see Supplemantary Table S8) – which was supposed to reveal the positive effect of AHL alone.

Synthetic C6-HSL alone and with ArN35e tended to decrease aphid loads. With ArI_{neg} mutant inoculation and C6-HSL, the plant showed slightly higher aphid loads.

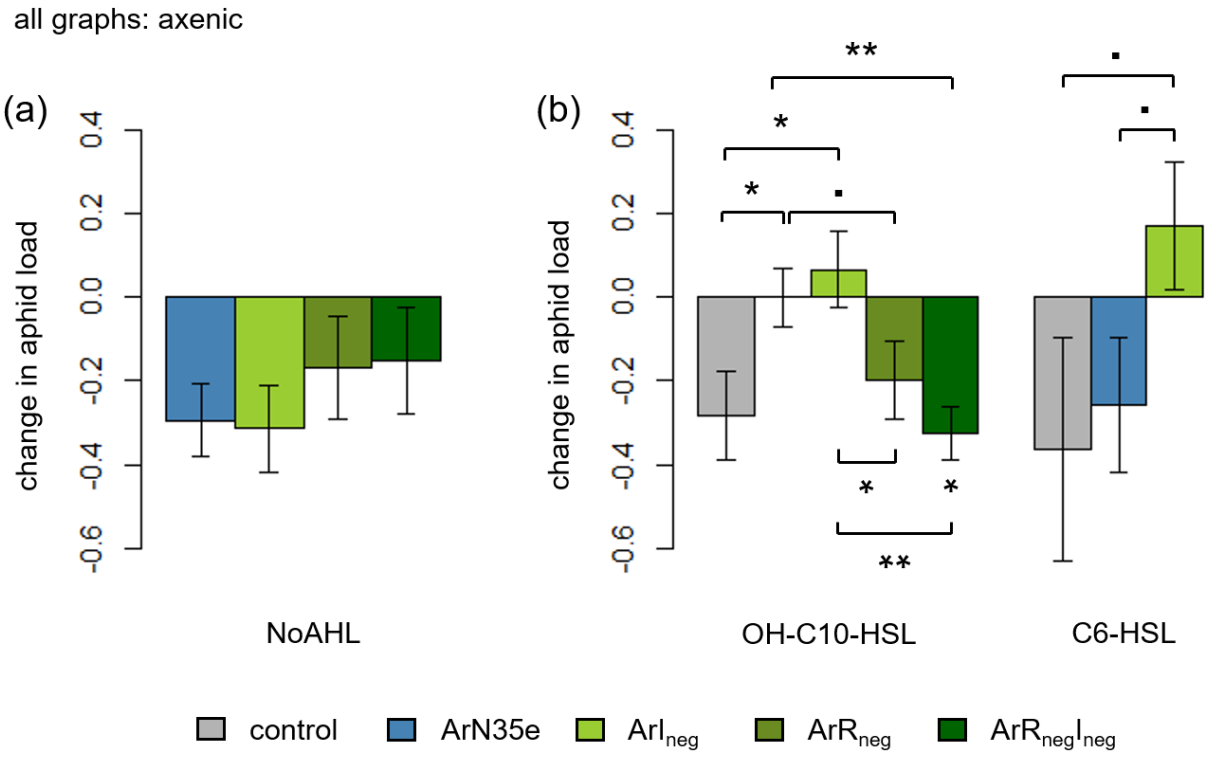

Figure 2.12 Effect of impaired AHL sensing and signalling complemented with synthetic AHL addition on aphid suppression.

(a) Inoculation with *Acidovorax radicis* AHL sensing mutants (ArR_{neg}, $ArR_{neg}I_{neg}$) resulted in a slightly lesser decrease in aphid loads than with the fully functional strain *A. radicis* (ArN35e) and *A. radicis* AHL synthesis mutant (ArI_{neg}) in axenic experiments (AE1, AE2, AE3, AE4 combined). Depicted is the change in aphid number per cm shoot length normalized by control plants within each experiment on day 21 post inoculation. (b) Addition of OH-C10-HSL led to significantly decreased aphid loads in control plants and AHL sensing mutants while aphid loads did not change in AHL synthesis mutants. Addition of C6-HSL slightly decreased aphid loads in control and ArN35e treatments but increased aphid loads in ArI_{neg} treatments (AE1, AE2 combined. a) n = 27-41, b) OH-C10-HSL: n = 21-38 and C6-HSL: n = 11-12. Error bars ± 1 SE. Significance level p = 0.001 – 0.01 **, 0.01 – 0.05 *, 0.05 – 0.1 . visualized with asterisks or point. Brackets: pairwise comparison, no brackets: significance compared to the baseline control. Significant p-values are listed in Table S8.

2.6.2 Complementation effect of AHL mutants with synthetic AHL on plant growth

Since bacterial traits under AHL control have been shown to imply beneficial growth traits like IAA production (Müller *et al.* 2009), it is conceivable that impaired AHL sensing modulates plant growth what might influence plant aphid suppression again. Therefore, also plant growth was included into the effect analysis upon the different bacterial inocula.

Without synthetic AHL addition, the new AHL sensing mutants showed no growth effect on barley, similar to the *A. radicis* AHL synthesis mutants (Figure 2.13 a-d). It has been observed before that synthetic AHL addition can slightly increase plant growth (see 2.5.3). The same tendency was visible under axenic conditions in the control treatment with synthetic OH-C10-HSL (AHL effect size, Figure 2.13 e-h, control with OH-C10-HSL vs. NoAHL g: p = 0.052 ., h: p = 0.090 ., for all p-values see Supplementary Table S9). Interestingly, $ArR_{neg}I_{neg}$ treated plants showed a similar growth increase, compared to samples without bacteria when OH-C10-HSL was applied, what is in line with the expectation that the double mutant represents a second control (Figure 2.13 e: $ArR_{neg}I_{neg}$ with OH-C10-HSL vs. NoAHL p = 0.024 *). Together with ArN35e and ArI_{neg}, reduced shoot length could be observed when OH-C10-HSL was added (Figure 2.13 e: ArI_{neg} with OH-C10-HSL vs. NoAHL p = 0.068 .). The differences between bacterial treatments appeared significant here (e: ArI_{neg} with OH-C10-HSL vs. control p = 0.0085 **). In other comparisons, plant growth did not change with combined mutant and OH-C10-HSL addition.

C6-HSL showed no plant growth promotion in uninoculated control plants and ArN35e treated plants but mainly increased plant growth together with ArI_{neg} (significant for root length in Figure 2.13 g: ArI_{neg} with C6-HSL vs. NoAHL p = 0.010 *).

Altogether, intact bacterial self-regulation and synthetic AHL molecules showed overlapping effects on plant performance. The ability of *A. radicis* N35e for AHL sensing and signalling led to significantly different outcomes especially in aphid suppression. Bacterial self-regulation induced by AHL rather had a negative effect on plant aphid suppression. The observed patterns might here be caused and/or overlapped by additional effects of the supplemented AHL on bacterial colonization (see below).

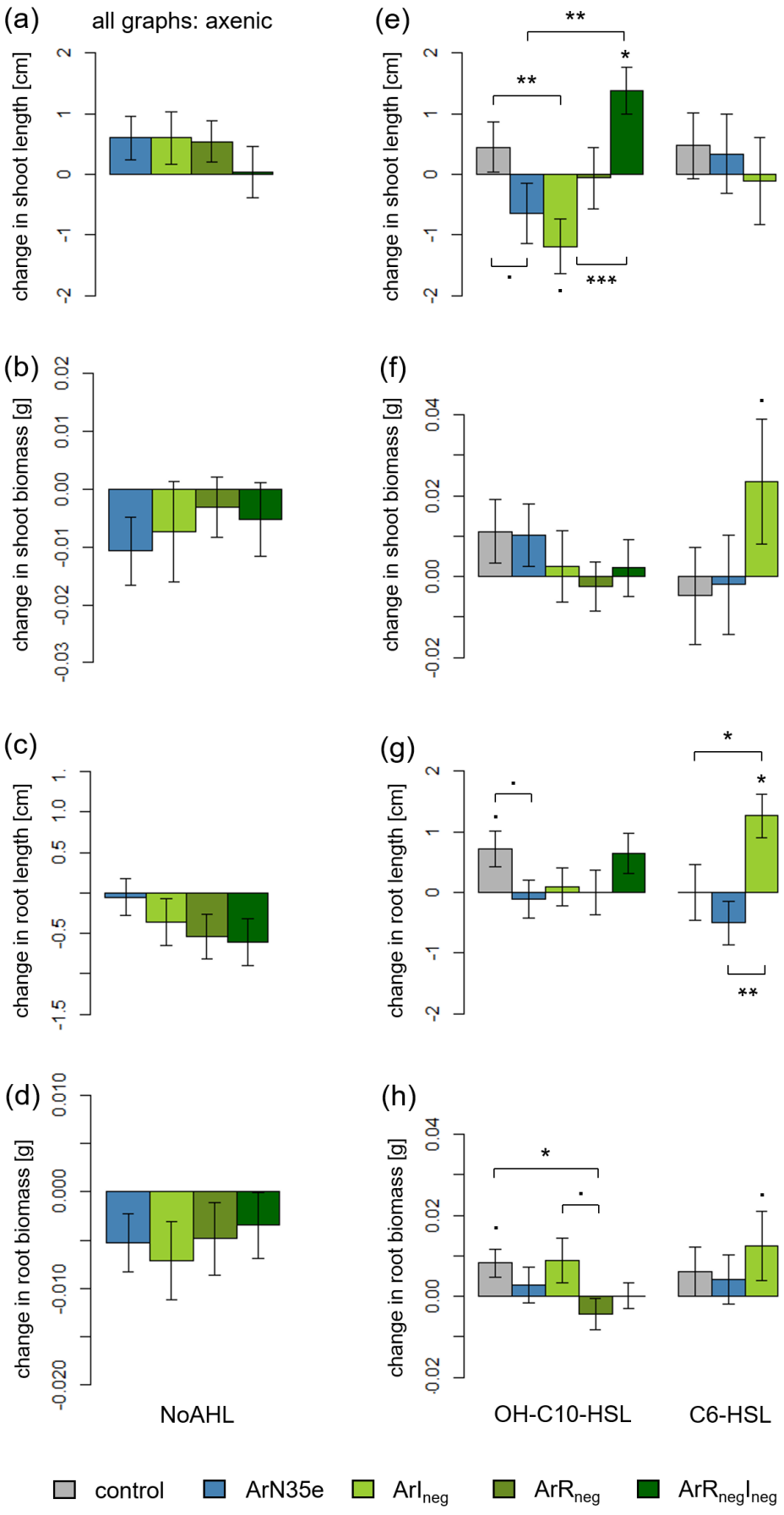
(a)
all graphs: axenic
change in shoot length [cm]
(b)
change in shoot biomass [g]
(c)
change in root length [cm]
(d)
change in root biomass [g]
(e)
(f)
(g)
(h)
NoAHL
OH-C10-HSL
C6-HSL
control
ArN35e
ArI$_{neg}$
ArR$_{neg}$
ArR$_{neg}$I$_{neg}$

Figure 2.13 Effect of impaired AHL sensing and signalling complemented with synthetic AHLs on plant growth.
Depicted is (a-d) the bacterial effect size on barley growth compared to not inoculated control plants (AE1, AE2, AE3, AE4 combined, barley cv. Scarlett, n = 59-95) and (e-h) the change in barley growth when synthetic OH-C10-HSL and C6-HSL were added to the Hoagland's solution compared to the NoAHL treatment (AE1, AE2, AE3, AE4 combined, barley cv. Scarlett, n = 36-63 and n = 23-27). Inoculation of *Acidovorax radicis* AHL sensing and signalling mutants alone did not change plant growth. Additional OH-C10-HSL increased plant growth in control and $ArR_{neg}I_{neg}$ treatments. C6-HSL increased plant growth together with ArI_{neg}. Error bars ± 1 SE. Significance level p < 0.001 ***, 0.001 – 0.01 **, 0.01 – 0.05 *, 0.05 – 0.1 . visualized with asterisks or point. Brackets: pairwise comparison, no brackets: significance compared to the baseline control. Significant p-values are listed in Table S9.

It is possible that various bacterial functions under QS control might have influenced the direct plant-microbe interaction, e.g., MAMP perception at the cell interface, nutrient acquisition, bacterial motility, biofilm formation and attachment to the roots (Chernin *et al.* 2011, Müller *et al.* 2009, Pérez-Montaño *et al.* 2014, Guan *et al.* 2021). To which extent impaired AHL sensing also influences the bacterial colonization properties on the root surface is elaborated in the following paragraph.

2.6.3 Comparison of AHL mutant colonization of plant roots

Previously, it has been observed that intact or impaired AHL sensing influenced plant aphid suppression (see 2.6.1). AHL sensing and gene regulation via the AHL feedback loop is known to be connected to bacterial colonization. In soil and under axenic conditions, *A. radicis* cells with impaired AHL signalling (ArI_{neg}) repeatedly persisted longer in the plant rhizosphere and in higher quantities than ArN35e (see 2.3.4). It is conceivable that these surprising differences are additionally influenced by impaired AHL sensing.

In order to study this in detail, colonization patterns of all *A. radicis* mutants were traced over time under axenic conditions – first of all without additional AHL – where bacteria should not be influenced by other interacting factors. Microscopic detection was performed qualitatively by taking micrographs, quantitatively by a systematic cell count of multiple microscopic fields of view and by colony forming unit (CFU) count.
For instant microscopy, fresh root material was dip-washed, embedded in Citifluor and

analyzed by CLSM the same day (AE6). In axenic experiments AE1, AE2, AE3, AE4 and AE5, root samples were fixed and stored at -20 °C so that microscopic analysis could take place days and weeks after sampling. However, fixation reduced the number of visible cells to such an extent that bacteria were hardly detectable after some weeks of storage. Therefore, the fresh sampling method was preferred for a quantitative screening of bacterial colonization. The GFP labelling was weak in all ArN35e and ArR_{neg} samples but GFP intensity was compensated in the quantitative screen with higher image brightness. In positive controls i.e., when root pieces were freshly spiked with ArN35e and ArR_{neg} cells from plate, the GFP labelling was of strong intensity so that this issue can be attributed to a reduced expression of the chromosomally integrated GFP gene in the rhizosphere.
Additionally, a diagnostic PCR method tracing the GFP gene was developed for molecular-based bacterial detection. However, these results are not included into the colonization results due to persisting random amplifications in negative control samples (for examplary gel pictures see Supplementary Figure S15).

The analysis revealed that impaired AHL sensing did not change root colonization, compared to the respective *A. radicis* strains with intact AHL sensing under axenic conditions. Similar to ArN35e (Figure 2.14 a), ArR_{neg} showed comparably low root colonization (Figure 2.14 b). ArR_{neg} cells were present in 40 – 50 % of the examined fields of view on day 4 but could not be detected anymore 10 dpi (Figure 2.15, Supplementary Figure S14).
Both AHL synthesis mutants ArI_{neg} and $ArR_{neg}I_{neg}$ showed the strongest bacterial colonization (Figure 2.14 c and d). Like in soil, ArI_{neg} persisted longer and in higher quantities also under axenic conditions compared to ArN35e (further discussed in 2.2.3). Similar to ArI_{neg}, also $ArR_{neg}I_{neg}$ appeared in biofilm-like accumulations as well as single cells spread over the whole root surface. Cells were visible in 90 – 100 % of the examined microscopic fields of view for ArI_{neg} and in 80 – 90 % of the examined fields of view for $ArR_{neg}I_{neg}$ and could both be detected up to day 10 in the rhizosphere (Figure 2.15, Supplementary Figure S14). In the microscopic cell counting, the colonization differences between both AHL synthesis mutants ArI_{neg} and $ArR_{neg}I_{neg}$ compared to other bacterial strains were highly significant at all time points (see p-values to Figure 2.15 in Supplementary Table S10). In all microscopic analyses, the cell numbers were highest at day 4 and decreased within the first ten days of observation. In the supplement, more micrographs are displayed for better over time comparison (Figure S14).

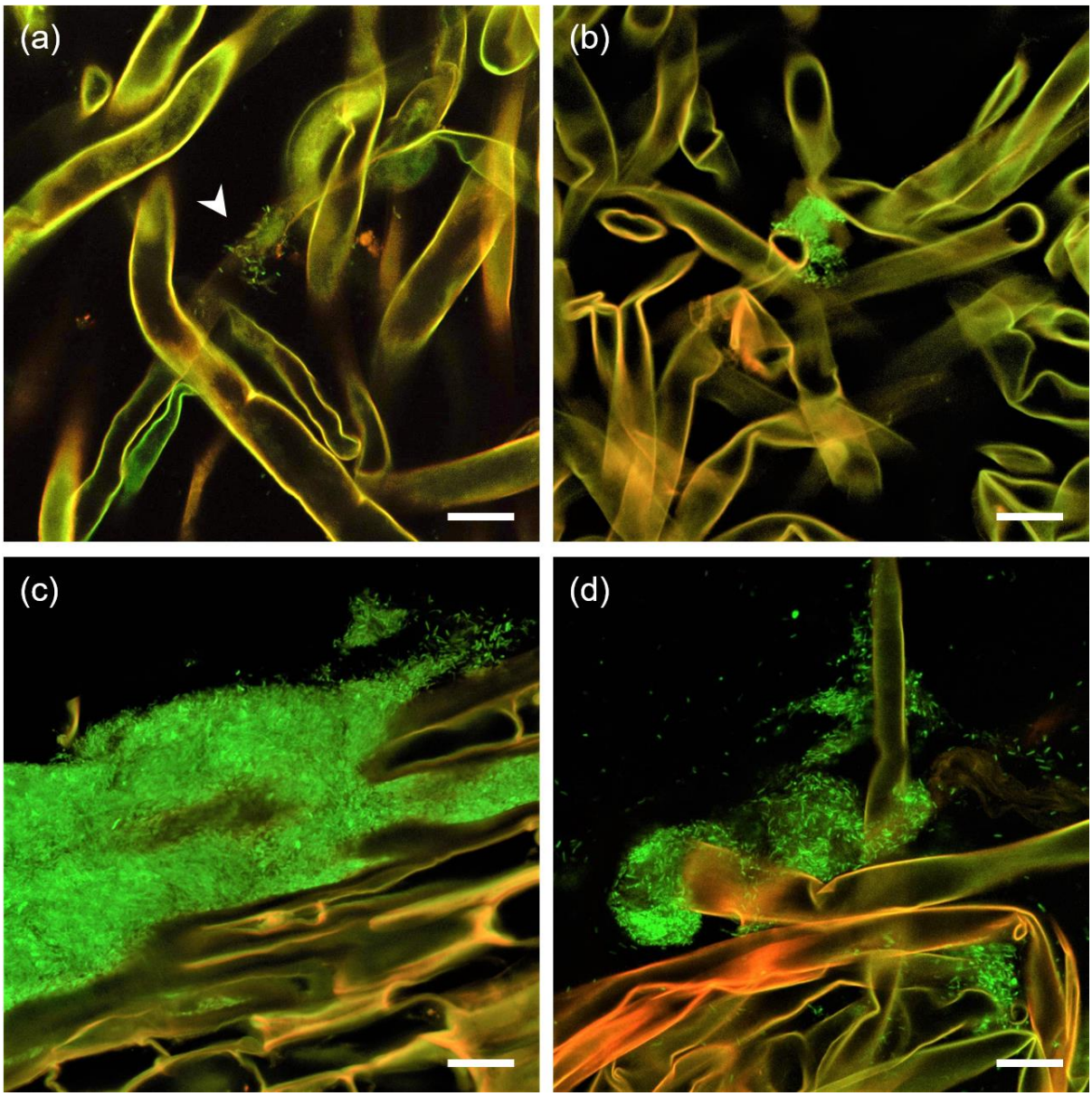

Figure 2.14 Root colonization pattern of AHL sensing and signalling mutants under axenic conditions.

Fresh root material was analyzed by Confocal Scanning Laser Microscopy on day 4, 7 and 10 days after bacterial inoculation of seedlings and cultivation under axenic conditions. Depicted are representative micrographs across these first days showing different colonization. a) The fully functional strain *Acidovorax radicis* (ArN35e) was hardly detectable (arrowhead). b) *A. radicis* AHL sensing mutant (ArR_{neg}) was detectable in low quantities. c) *A. radicis* AHL synthesis mutant (ArI_{neg}) was visible in very high quantities. d) *A. radicis* AHL double mutant ($ArR_{neg}I_{neg}$) was visible in high quantities. All bacterial strains were GFP labelled and are visible in green, root background in yellow. No additional staining performed. Micrographs were taken from AE6. Scale bar = 10 µm.

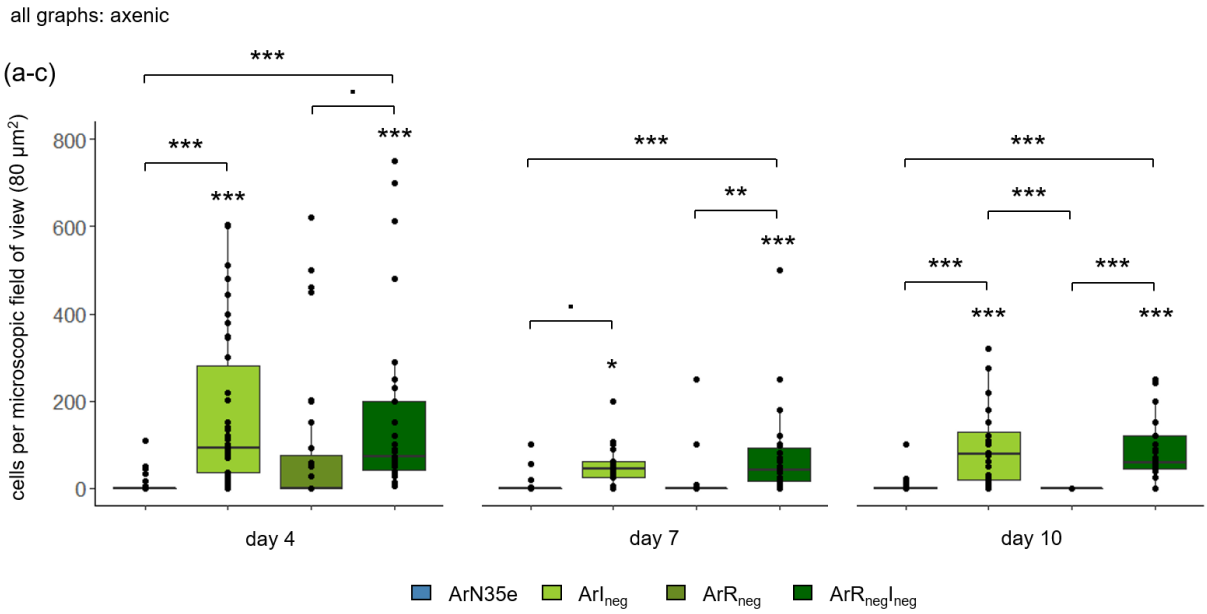

Figure 2.15 Microbial cell numbers on the root surface quantified by microscopy.
Compared are the fully functional *Acidovorax radicis* (ArN35e) strain, its AHL synthesis (ArI_{neg}) and AHL sensing (ArR_{neg} and $ArR_{neg}I_{neg}$) mutants. Fresh root samples were taken for a quantitative screen of bacterial colonization in axenic experiment AE6 and analyzed immediately without fixation. With Confocal Laser Scanning Microscopy, representative fields of view were selected randomly and adjusted so that a maximal number of cells was captured on micrographs (n = 24-31). Cells numbers were estimated per field of view (80 µm x 80 µm). On day 4, four data points of more than 50,000 cells in ArI_{neg} and $ArR_{neg}I_{neg}$ treatments were left outside the graph boundaries for the sake of y axis resolution and treated as outliers i.e., NA, for statistics. Control fields were counted but zero and are therefore not displayed for better vizualisation. When GFP fluorescence was low, picture brightness was manually increased for counting. Significance level $p < 0.001$ ***, 0.001 – 0.01 **, 0.01 – 0.05 *, 0.05 – 0.1 . visualized with asterisks or point. Brackets: pairwise comparison, no brackets: significance compared to the uninoculated control. Significant p-values are listed in Table S10.

Additional results from CFU counting corroborate these observations (Figure 2.16). In three independent axenic experiments, it could be observed that ArI_{neg} cells were most abundant at the first sampling days. Repeatedly, the double AHL mutant $ArR_{neg}I_{neg}$ was half as abundant as ArI_{neg} but approximately twice as abundant as ArN35e. Again, ArN35e was five times more abundant than the ArR_{neg} mutant which had the lowest cell numbers in most cases. In the course of all experiments, the cell numbers decreased over time but it was variable at which time point this reduction happened. In the end of the experiments, mainly the AHL signalling mutants ArI_{neg} and $ArR_{neg}I_{neg}$ were still detectable by CFU count while ArN35e and ArR_{neg} disappeared nearly completely.

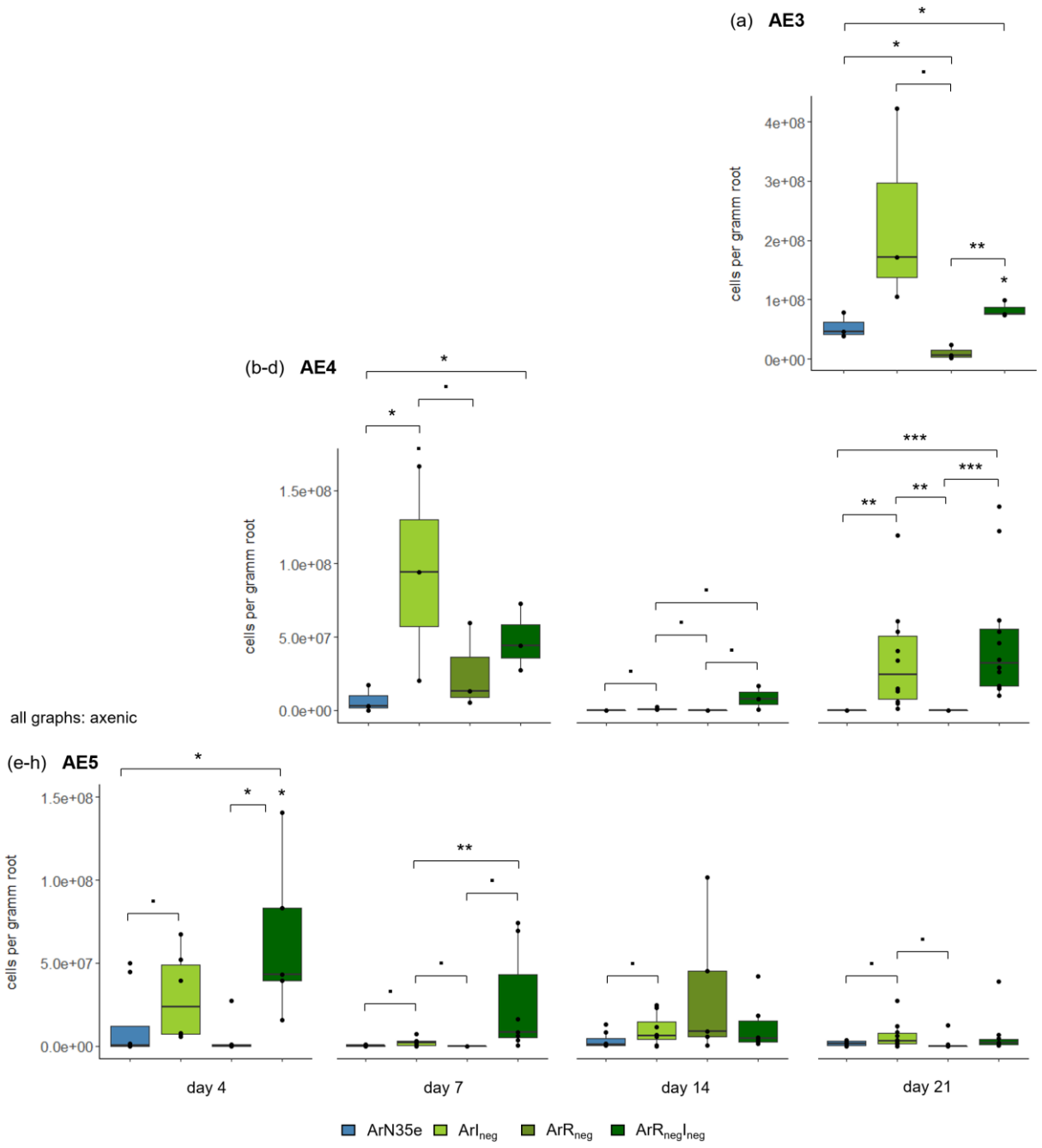

Figure 2.16 Microbial cell numbers in the barley rhizosphere quantified by CFU analysis. Compared are the fully functional *Acidovorax radicis* (ArN35e) strain, its AHL synthesis (ArI$_{neg}$) and AHL sensing (ArR$_{neg}$ and ArR$_{neg}$I$_{neg}$) mutants. Root samples from independent axenic experiments AE3, AE4 and AE5 were dip-washed, weighed and grinded in 1 mL 1x PBS. A 1:10 dilution series was established and at least three technical replicates of at least two dilutions were plated out on NB plates with the respective antibiotic combination. From colony forming units (CFU) cell numbers per g root fresh weight were counted back. Control counted but not displayed for better vizualisation. (a-c) n = 3, (d) n = 12, (e-g) n = 8, (h) n = 12. Significance level p < 0.001 ***, 0.001 – 0.01 **, 0.01 – 0.05 *, 0.05 – 0.1 . visualized with asterisks or point. Brackets: pairwise comparison, no brackets: significance compared to the uninoculated control. Significant p-values are listed in Table S11.

These results support the microscopic observations and show that mutants with impaired AHL synthesis colonized the rhizosphere in higher numbers and more persistently compared to the respective strain capable of AHL production. Impaired AHL sensing decreased the bacterial colonization capacity only in two cases significantly compared to the respective strains with intact AHL sensing (Figure 2.16 a: ArN35e vs. ArR_{neg} p = 0.037 * and f: ArI_{neg} vs. $ArR_{neg}I_{neg}$ p = 0.007 **, for all p-values see Supplementary Table S11). In total, impaired AHL sensing did not change the colonization pattern. Taken all results together, both mutants with impaired AHL synthesis (ArI_{neg} and $ArR_{neg}I_{neg}$) had similar colonization numbers two or even four times higher than ArN35e and ArR_{neg}.

2.6.4 Effect of synthetic AHL on bacterial root colonization

If impaired AHL signalling makes a difference for bacterial colonization, it is likely that synthetic AHL addition can modulate the bacterial root colonization. In fact, fewer colony forming units could be observed when synthetic OH-C10-HSL was added to the roots compared to NoAHL treated plants (Figure 2.17). The differences for ArI_{neg} (p = 0.056 .) and $ArR_{neg}I_{neg}$ (p = 0.067 .) were close to significant, while the difference between $ArR_{neg}I_{neg}$ without OH-C10-HSL and ArI_{neg} with OH-C10-HSL addition turned out significant (p = 0.020 *). Interestingly, the addition of synthetic OH-C10-HSL led to slightly fewer ArI_{neg} colonization compared to the cell numbers of the AHL-insensitive double mutant $ArR_{neg}I_{neg}$ (p = 0.095 .). Both strains with intact AHL synthesis (ArN35e and ArR_{neg}) already disappeared from the rhizosphere 21 dpi so that differences could not be observed for these bacteria.

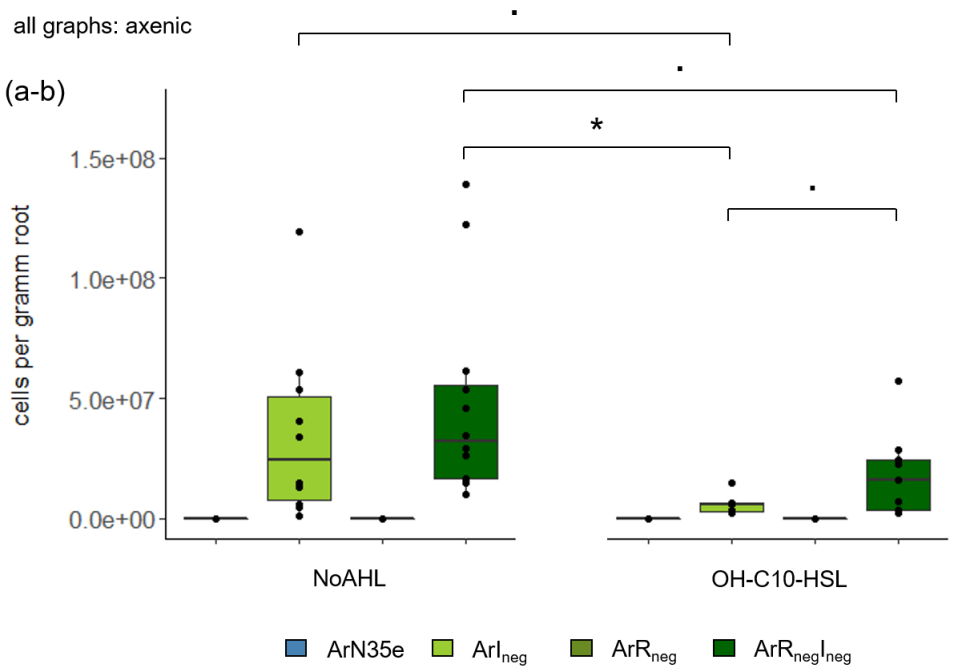

Figure 2.17 Microbial cell numbers in the barley rhizosphere in the presence and absence of synthetic AHL.
Compared are the fully functional *Acidovorax radicis* (ArN35e) strain, AHL synthesis (ArI$_{neg}$) and AHL sensing (ArR$_{neg}$ and ArR$_{neg}$I$_{neg}$) mutants. Colony forming units (CFU) were determined in axenic experient AE4 at 21 dpi when a) no AHL or b) synthetic OH-C10-HSL was applied. With synthetic AHL addition the cell numbers were almost significantly reduced (ArI$_{neg}$: NoAHL vs. OH-C10-HSL p = 0.056 . and ArR$_{neg}$I$_{neg}$: NoAHL vs. OH-C10-HSL p = 0.067 .). ArN35e and ArR$_{neg}$ were not detectable 21 dpi (all values = 0). Root samples were dip-washed, weighed and grinded in 1 mL 1x PBS. A 1:10 dilution series was established and at least three technical replicates of at least two dilutions were plated out on NB plates with the respective antibiotic combination. From colony forming units (CFU) cell numbers per g root fresh weight were counted back. Control counted not displayed for better vizualisation. n = 12. Significance level p = 0.01 – 0.05 *, 0.05 – 0.1 . visualized with asterisks or point. Brackets: pairwise comparison.

Taken together, missing AHL had a positive effect on bacterial colonization, synthetic AHL displayed a negative effect while bacterial self-regulation induced by synthetic AHL slightly reduced colonization as well. It would have been conceivable that the counted differences in cell numbers originate from a more dispersed colonization mode when AHL signalling is impaired. However, in this study, both colonization modi were observed in all four mutants.

In order to investigate the role of AHL on the colonization mode further, a more in-depth analysis of biofilm formation and cell attachment could have been insightful. Although biofilm formation has been described previously for *A. radicis* N35e (Li 2011), no biofilm formation could be confirmed for any of the *A. radicis* strains in a classical staining assay with crystal

violet (Supplementary Figure S17). However, on the root surface, ArI_{neg} and $ArR_{neg}I_{neg}$ were shown to build biofilm-like structures that stretched over several 100 µm. Moreover, enhanced swarming capacity could have led to a quicker and more spread-out root attachment of bacteria during seedling incubation. But also swarming assays on semisolid agar plates turned out negative for all *A. radicis* strains (Figure S16). Nevertheless, differences in cell aggregation and proliferation cannot be excluded and likely could have contributed to the observed differences in microbe-plant-insect interaction.

Discussion

Insect feeding causes enourmous yield loss by physical plant damage and the transfer of plant diseases (Dhaliwal *et al.* 2015). This way, pest insects threaten global agriculture that is already under pressure in times of climate change and a growing world population (Sharma *et al.* 2017). In order to secure food production also for future generations, sustainable pest management is urgently required (Douglas 2018). As ecologically friendly biocontrol agents, beneficial rhizobacteria harbour a largely unexploited potential to improve plant health, nutrition and resilience (Bender *et al.* 2016, Parray *et al.* 2016). When inoculated to plant roots, they can confer resistance against various biotic stressors including insects (Glick 2012). Although, for the successful application of these plant growth promoting bacteria (PGPRs), it would be crucial to understand in detail how this beneficial interaction is established and which modulating factors are involved (Disi *et al.* 2019). Among other microbial compounds, AHL signalling molecules have been postulated to play a key role in beneficial microbe-plant interaction (Hartmann *et al.* 2014). AHLs are essential for microbial communication by quorum sensing and regulate diverse bacterial functions related to colonization and pathogenicity (Fuqua *et al.* 1994, Eberl 1999). In lab trials, synthetic and microbially-derived AHLs repeatedly increased plant growth and resistance to pathogens (Mathesius *et al.* 2003, Schuhegger *et al.* 2006, Schikora *et al.* 2011, Shrestha *et al.* 2020). Therefore, it is very likely that AHLs also stimulate the plant immunity to better deal with pest insects. Nevertheless, the role of microbial AHL signalling in microbe-plant-insect interaction has been scarcely studied.

In order to address this knowledge gap, the present thesis investigated the role of AHL signalling on aphid suppression in barley using the rhizobacteria *Acidovorax radicis* N35e (producing OH-C10-HSL) and *Rhizobium radiobacter* F4 (producing an AHL mix) across soil and axenic cultivation. The aim of this thesis was (1) to test if the selected rhizobacteria successfully increase aphid suppression in the selected model system, (2) to explore if presence and absence of microbial AHL signalling has an impact on plant aphid suppression and (3) to disentangle the direct effect of synthetic AHL molecules from eventual indirect AHL effects.

This study revealed that *A. radicis* (ArN35e) represents a promising candidate for investigating the role of microbial AHL signalling because of its recurrent aphid suppression effect in barley (discussed in chapter 3.1). In comparison, *R. radiobacter* (RrF4) showed no consistent aphid suppression effect (chapter 3.2).

Surprisingly, *A. radicis* inoculation with impaired AHL synthesis (ArI_{neg}) increased aphid suppression even stronger than the AHL-producing strain (chapter 3.3). Most likely, this effect is not attributed to growth differences (chapter 3.4), but to an increased root colonization of the AHL mutant (chapter 3.5), which might have provoqued a stronger plant immune response. The induced immune pathways, however, seemed to differ between barley cultivars (chapter 3.6). Moreover, the flavonoid biosynthesis pathway likely played a mechanistic role in plant aphid suppression (chapter 3.7).

Direct AHL effects causing an immediate reaction in plant tissues and indirect effects via altered AHL-regulated bacterial traits both contributed to the observed changes (chapter 3.8). Synthetic AHL addition as well as *A. radicis* mutants impaired in AHL sensing (ArR_{neg} and double mutant $ArR_{neg}I_{neg}$) showed slightly modulated effects on plant aphid suppression, suggesting a role of AHL molecules themselves and the bacterial self-regulation coupled to AHL alike. The rhizosphere microbiome did not play the decisive role for aphid suppression. In general, barley genotype and the cultivation conditions strongly influenced microbe-plant-insect interaction (chapter 3.9 and 3.10).

3.1 *A. radicis* N35e as suitable candidate for investigating AHL effects

In order to establish a basis for investigating AHL effects, the rhizobacterium *A. radicis* N35e (ArN35e) was tested for its aphid suppression capacity in the selected model system. Preliminary studies have demonstrated that ArN35e can positively influence growth and resistance of barley (Li *et al.* 2012, Han *et al.* 2016). In this thesis, ArN35e inoculation indeed increased aphid suppression across all experiments – significantly in Barke (cultivated in soil) and with a strong tendency in Scarlett (axenic and soil). This result is in accordance with observations from greenhouse experiments where ArN35e reduced aphid density up to 10 % overall (Zytynska *et al.* 2020). However, the aphid biocontrol effect was most prominent in the barley cultivars Grace and Chevallier and, in contrast to the findings herein, less prevalent in

Scarlett and Barke (Zytynska *et al.* 2020). Similarly, Xi & Zytynska (2022) found that the survival of aphid genotypes Fescue and Sickte was visibly reduced upon ArN35e inoculation in Chevallier and Irina but less affected in Barbarella. Together, these studies corroborate that ArN35e robustly enhance aphid suppression in barley, but with variable effect between cultivars.

Despite the often described trade-off between plant defense and growth (He *et al.* 2022), aphid resistance seemed not to be negatively correlated to plant growth in this study. In total, ArN35e showed no clear plant growth promoting effect. Only in Barke, a slight increase in root biomass ($p = 0.022$ *) and shoot length ($p = 0.092$) could be observed while, for all other cultivars, the plant growth changes remained minimal (see Figure 2.3). This result was in contrast to Li *et al.* (2012) who reported a significant increase in barley shoot biomass and dry weight upon ArN35e inoculation. Han *et al.* (2016) observed that ArN35e increased Barke fresh weight after two months in soil (Han *et al.* 2016). Zytynska *et al.* (2020) suggested that ArN35e allocated energy from shoot to the belowground tissues resulting especially in root growth promotion. However, these studies underline that PGP effects induced by ArN35e might require prolonged cultivation times (Han *et al.* 2016, Zytynska *et al.* 2020). The possibility can therefore not be excluded that the incubation time of 21 days chosen for this work did just not suffice to reveal plant growth promotion by ArN35e.

Apart from these beneficial effects, ArN35e was proven to successfully colonize the root surface, which is an important prerequisite for plant-microbe interaction (Kloepper & Schroth 1980). However, ArN35e disappeared from the rhizosphere within 7 to 10 dpi. Nevertheless, a long-lasting positive effect on plant aphid suppression could be observed at later time points. The native rhizosphere microbiome was not altered significantly by ArN35e inoculation (Figure 2.9). This finding is in accordance with an earlier study where ArN35e did not dominate the rhizosphere microbiome but was present in low abundance with still strong ecological effects (Zytynska *et al.* 2020). This property might be advantageous for application of *A. radicis* in the field while preserving the existing microbiota (Zytynska *et al.* 2020).

Altogether, because of its subtle but consistent biocontrol effect, ArN35e was considered as a promising candidate to investigate the role of microbial AHL signalling on microbe-plant-insect interaction in the following thesis.

3.2 *R. radiobacter* F4 as not reliable candidate for investigating AHL effects

As second candidate for analysing the role of AHL signalling in microbe-plant-insect interaction, the well-known rhizobacterium *Rhizobium radiobacter* F4 (RrF4) was tested. In contrast to ArN35e, RrF4 treatment showed no consistent aphid suppression effect in barley. In two soil experiments, either an increase or an unwanted decrease of aphid suppression was observed (Figure 2.2 b,c).

This result was unexpected since previous studies showed that beneficial RrF4 convincingly induced resistance against powdery mildew in barley (Sharma *et al.* 2008), *Xtt* in wheat (Glaeser *et al.* 2016, Alabid *et al.* 2020) and *Pst* in *Arabidopsis* (Glaeser *et al.* 2016). In respect to insect resistance, several *Rhizobium* species were able to decrease aphid abundance (Martinuz *et al.* 2012, Basu *et al.* 2021b). Other studies only report rather weak (Heath & Lau 2011, Dean *et al.* 2014) or inconsistent aphid suppression effects for rhizobia (Kempel *et al.* 2009). In some cases, however, *Rhizobium ssp.* inoculation even improved aphid performance or reduced their parasitoids which in turn benefits aphids (Dabré *et al.* 2022). For example, beans treated with a commercial *Rhizobium* fertilizer showed higher *Aphis fabae* numbers than non-inoculated plants (Naluyange *et al.* 2014, 2016). Similarly, aphids feeding on nodulating soybean, comprising *R. etli* as symbiont, reached higher abundances than non-nodulating plants (Whitaker *et al.* 2014).

These different outcomes might result from the complex feedback loops determining microbe-plant-insect interaction (Pineda *et al.* 2010, 2013, Mahdavi-Arab *et al.* 2014). On the one hand, additional nitrogen provided by N_2-fixing *R. radiobacter* might have increased resistance by the production of nitrogen-based defense compounds (Mattson 1980, Kempel *et al.* 2009). On the other hand, the rhizobial nitrogen supply might positively influence plant nutritional quality for foraging aphids (Dean *et al.* 2014). This second mechanism might have outweighted the beneficial resistance effect in this work, leading to no or even increased aphid performance (Kempel *et al.* 2009).

Observations on plant growth match with this assumption. When aphid performance increased in RrF4-inoculated Barke, shoot growth and root length increased as well (see soil experiment SE4, Figure 2.3 c). Probably, promoted plant growth positively affected aphid fitness here. In two other experiments, RrF4 inoculation did not influence aphid suppression while especially

shoot and root biomass strongly decreased in Barke (SE2 and SE3 combined, Figure 2.3 b). Aphid performance might here again directly result from the negative plant growth changes. The detrimental effect of RrF4 on plant growth, however, cannot be readily explained. Usually, *R. radiobacter* strains efficiently promoted plant biomass and yield in important crops including barley (Humphry *et al.* 2007, Sharma *et al.* 2008, Glaeser *et al.* 2016, Guo *et al.* 2017, Hadide *et al.* 2019, Singh *et al.* 2020a, Kumar *et al.* 2021). *Rhizobium* is an essential part of many available biofertilizers and successfully applied to the field in many countries (Basu *et al.* 2021a).

For investigating AHL effects on microbe-plant-insect interaction, no detrimental growth and an ecologically relevant aphid suppression effect would have been advantageous. Because of the just described inconsistencies, RrF4 was considered to be not a reliable candidate for a more functional AHL analysis but an informative reference point for basal comparison with *A. radicis*.

3.3 Increased plant aphid suppression upon ArI_{neg} inoculation compared to ArN35e

In a first approach to investigate the role of AHL molecules in microbe-plant-insect interaction, the ArN35e effect on plant aphid suppression was compared to the effect of a QS mutant in which the *araI* gene was disrupted. Previous characterization already revealed that this ArI_{neg} mutant strain was unable to produce OH-C10-HSL molecules (Han *et al.* 2016).

Based on earlier observations, it was hypothesized that inoculation of ArI_{neg} would reduce plant aphid suppression compared to the AHL-producing strain ArN35e. Microbial AHLs are usually considered as positive signal for the plant which induces plant resistance and facilitates a beneficial interaction (Hartmann *et al.* 2014). However, in this study, ArI_{neg} inoculation resulted in even stronger plant aphid resistance than ArN35e inoculation, significantly in soil and as a tendency under axenic conditions.

This result was surprising since earlier studies mostly stated that AHL synthesis mutants were less effective in inducing plant resistance (Schuhegger *et al.* 2006, Müller *et al.* 2009, Pang *et al.* 2009, Shrestha *et al.* 2019, Alabid *et al.* 2020). For instance, AHL synthesis mutants of *P. putida* and *S. liquefaciens* showed reduced ISR against the fungus *A. alternata* in tomato (Schuhegger *et al.* 2006). Similarly, *S. plymuthica* AHL synthesis mutants were repeatedly

unable to induce resistance against *B. cinerea* and other diseases in tomato, bean and cucumber (Müller *et al.* 2009, Pang *et al.* 2009). The *aiiA* expressing transconjugant *Rhizobium radiobacter* F4NM13 reduced resistance against *X. translucens* in barley and wheat (Alabid *et al.* 2020) while the AHL mutant *E. meliloti* attM showed a higher *Bgh* disease incidence in many barley cultivars than the AHL-producing wildtype (Shrestha *et al.* 2019). However, Ryu *et al.* (2013) suggest that the impaired AHL signalling effect on microbe-plant interaction might be pathogen-dependent. The researchers verified that the missing AHL stimulus in lactonase-expressing tobacco reduced *S. marcescens*-mediated ISR against *Pectobacterium carotovorum* but enhanced plant resistance against the *Cucumber mosaic virus* (Ryu *et al.* 2013).

The mentioned literature examples illustrate that an increase in plant resistance upon AHL mutant treatment was only rarely observed. However, one recent study – specifically dealing with AHL effects on plant-insect interactions – also reported a negative effect of AHLs on insect suppression. Heidel *et al.* (2010) showed that belowground addition of C6-HSL 4-fold increased the larval mass of the tobacco hawk moth *Manduca sexta*. AHL are here proposed to weaken the plant immune system by inhibiting JA-dependent defense responses. Important defense factors against herbivory, like the trypsin proteinase inhibitor TPI, were downregulated upon AHL treatment (Heidel *et al.* 2010). However, direct and indirect AHL effects – by AHL uptake into plant tissues or mediated by other AHL-sensitive rhizobacteria which might in turn affect plant responses – could not be further disentangled (Heidel *et al.* 2010).

In contrast, Wehner *et al.* (2021) showed that oxo-C14-HSL-producing *E. meliloti* strain expR^{+}ch helped to reduce feeding and reproduction of the aphid *Rhopalosiphum padi* in barley cultivar Morex, while the AHL transconjugant attM did not induce plant resistance against herbivory. However, the barley cultivar BCC1415 did not respond by showing differences in aphid resistance when treated with either AHL-producing or AHL-defective *E. meliloti*, suggesting a different AHL-sensitivity of both barley cultivars (Wehner *et al.* 2021).

While the results of Heidel *et al.* (2010) fit to the present findings, Wehner *et al.* (2021) is in striking contrast to the herein observed aphid suppression increase upon impaired AHL production. Likely explanations, how this surprising increase could have been produced, will be elaborated in the following sections.

3.4 Link between plant growth and increased plant aphid suppression

Several explanations for the aforementioned aphid resistance increase upon ArI_{neg} inoculation are thinkable. One explanation involves the well-known trade-off between plant growth and defense (He *et al.* 2022). Many studies reported that AHL mutants were less effective in promoting plant growth compared to the respective wildtype (Cai *et al*. 2020, Alabid *et al.* 2020). Therefore, it is possible that AHL mutant inoculation reduced plant growth, so that more energy was avaiblable for defense.

However, in this study, plant growth seemed not causally linked to the increase in plant aphid resistance with ArI_{neg} inoculation. In Scarlett (soil), shoot and root length slightly increased with ArI_{neg} inoculation (Figure 2.7). At the same time, the significant increase in shoot length and root biomass with ArN35e treatments was lost in ArI_{neg} treatments in Barke. A previous study reported that ArI_{neg} had the same plant growth promotion effect in Barke as the wildtype after two months in soil, but no effect under axenic conditions (Han *et al.* 2016). This fits to the overall observation in this work, that plant growth differences were more pronounced in soil than in axenic environments.

In the present study, the strongly reduced shoot and root biomass in *R. radiobacter* F4-inoculated Barke was reverted in the AHL signalling mutant (RrI_{neg}) treated plants. An effect attenuation could also be observed in other studies, however, of opposite direction. For instance, a positive effect of RrF4 disappeared in AHL mutant treated wheat in experiments conducted by Alabid *et al.* (2020). Also the plant growth stimulation of closely related *Bradyrhizobium* on *Arabidopsis* (Zúñiga *et al.* 2013) and rice (Cai *et al.* 2020) was abolished with deficient AHL accumulation. These literature examples suggest that AHL might play a positive role in improving plant growth, but only under certain conditions, which could not be reproduced in this work.

The present study overall revealed variable and very small plant growth changes and therefore assumes that energy allocation from plant growth to defense was rather not decisive for the herein observed AHL-dependent aphid suppression.

3.5 Increased root colonization of ArI_{neg} explaining plant aphid suppression

Reflecting on the totality of available data, it appears most likely that the observed increase in aphid suppression upon ArI_{neg} inoculation has been caused by the increased colonization of ArI_{neg} mutant cells.

Unexpectedly, ArI_{neg} and $ArR_{neg}I_{neg}$ colonized the rhizosphere in significantly higher numbers – up to four times more cells per g biomass – than the respective strain with intact AHL synthesis and persisted longer in the rhizosphere. This result was astonishing since AHL signalling is known to play an essential role for colonization (Miller & Bassler 2001). In most reports, AHL synthesis mutants were largely unable to colonize the rhizosphere efficiently or were less competetive in co-inoculation assays (Pang *et al.* 2009, Steindler *et al.* 2009, Pérez-Montaño *et al.* 2014, Alabid *et al.* 2020, Cai *et al.* 2020, Xiong *et al.* 2020). AHL synthesis mutants of *Pseudomonas* and *Burkholderia* were compromised in their colonization capacity (Wie & Zhang 2006, Zúñiga *et al.* 2013). Similarly, Han *et al.* (2016) observed lower and more dispersed cell numbers of ArI_{neg} on the root surface of barley. This previous finding, however, was only based on rough microscopic estimations – instead of CFU counting and systematic quantification – which might explain the discrepancy between Han *et al.* (2016) and the results of this work. A few studies, nevertheless, report a similar unexpected increase in bacterial root colonization with impaired AHL signalling (von Bodmann *et al.* 1998, Stoodley *et al.* 1999, Koutsoudis *et al.* 2008). In *P. aeruginosa*, inheriting two LuxI/LuxR systems, colonization only increased when the production of oxo-C12-HSL was impaired, while C4-HSL production was still intact (Stoodley *et al.* 1999). Colonization of *Pantoea stewartii* increased in an *esaI* AHL synthesis mutant, while it remained unaltered in the respective *esaR* mutant (von Bodmann *et al.* 1998). In most cases, the exact mechanisms behind this altered colonization are unclear. However, many of these examples suggest that rhizosphere colonization was linked to a) biofilm formation, b) motility and c) other bacterial traits under QS control.

a) Biofilm formation differences altering root colonization

Typically, AHL positively regulates biofilm formation which is an important features for cell attachment (Davies *et al.* 1998). Biofilm formation might likely be suppressed or activated when the AHL signalling circuit is interrupted (Hammer & Bassler 2003).

In this study, microscopic observations point towards an increasing biofilm formation capacity of the AHL synthesis mutants compared to the respective strains with intact AHL synthesis. ArI_{neg} and $ArR_{neg}I_{neg}$ cells prevailed in widespread biofilm-like accumulations on the root surface as well as in a dispersed single cell mode. ArN35e and ArR_{neg} cells only appeared in smaller aggregates. This finding is in strong contrast to previous observations by Li (2011) who described biofilm-like structures for ArN35e. Other scientists report that *Acidovorax* strains were indeed able to produce biofilms (Bahar *et al.* 2009, Shrestha *et al.* 2013, Yang *et al.* 2023). However, in the classical staining essay with crystal violet, *A. citrulli* formed no or only a nearly invisible ring under very specific cultivation conditions (Wang *et al.* 2016, Ji *et al.* 2022). Similarly, no biofilm formation could be verified with crystal violet staining in this work – neither for *A. radicis* N35e, nor for the AHL mutants. Still, the microscopic observations suggest that the missing AHL stimulus in ArI_{neg} positively influenced biofilm formation.

Existing literature on the genus *Acidovorax* speculates that biofilm formation can be positively as well as negatively regulated by QS. Kusada *et al.* (2014) could promote biofilm formation in *Acidovorax sp.* strain MR-S7 by exogenous AHL addition. Accordingly, reduced biofilm formation was repeatedly observed for AHL mutants (von Bodmann *et al.* 2003, Pérez-Montaño *et al.* 2014, Wie & Zhang 2006, Zúñiga *et al.* 2013, Xiong *et al.* 2020). In some bacteria, including *A. citrulli* (Fan *et al.* 2011), biofilm formation remained unaffected when AHL production was interrupted (Schuhegger *et al.* 2006, Müller *et al.* 2009, Johnson & Walcott 2013, Cai *et al.* 2020). At the same time, several other studies support the impression that colonization can increase with interrupted AHL synthesis. For example, for *A. citrulli*, Wang *et al.* (2016) found strongly increased biofilm formation in *aacI* and *aacR* mutants, and Guan *et al.* (2021) reported a similar increase for an *aclR* mutant. An AHL synthesis mutant of *P. aeruginosa* accumulated to thick biofilms, which continued growing, while the wildtype repeatedly detached from the surface (Stoodley *et al.* 1999). Cyclic colonization and dispersal of cells in periods of a few days have been reported already for several bacteria including *Vibrio* and *Pseudomonas* (Dalton *et al.* 1996). In *P. aeruginosa*, AHLs were proposed to control the expression of *algL*, encoding for alginate lyase, which is involved in the detachment of biofilm cells (Boyd & Chakrabarty 1994, Stoodley *et al.* 1999). In a similar way, reduced detachment might alternatively explain the increased bacterial biomass of the AHL synthesis mutants observed in this study.

b) Motility differences altering root colonization

Bacterial swarming on semi-solid agar plates could not be identified for any *A. radicis* strain in this work. Using the same assay, however, Li (2011) previously confirmed a similar swarming ability for ArN35e and ArI_{neg}. Still, in other bacterial species, motility has been shown to be affected by impaired AHL synthesis (Morohoshi *et al.* 2009). For example, AHL-deficient mutants of the pathogenic *A. citrulli* showed reduced swimming (Fan *et al.* 2011), twitching (Wang *et al.* 2016) and compromised flagella formation (Guan *et al.* 2021). In contrast, motility appeared enhanced in AHL mutants of *E. chrysanthemi* and *P. syringae* (Quiñones *et al.* 2005, Hussain *et al.* 2008). In the lactonase expressing AHL mutant of *S. plymuthica*, the swimming ability increased while colonization and biofilm formation remained unaffected (Müller *et al.* 2009). Even though a different swarming behaviour was not observed in this work, the possibility cannot be ruled out that motility differed between *A. radicis* mutant strains contributing to increased colonization and modulated microbe-plant-insect interaction.

c) Other bacterial traits altering root colonization

The increased root colonization of AHL mutants might also be determined by multiple other bacterial traits that might be upregulated when AHL is missing (Morohoshi *et al.* 2009). These traits include the production of exopolysaccharides, cell-wall degrading enzymes, antibiotic compounds, etc. (Pérez-Montaño *et al.* 2014, Maddula *et al.* 2006). Exopolysaccharide production is connected to biofilm formation and can be altered in AHL mutants as it has been shown by von Bodmann *et al.* (1998) and Krysciak *et al.* (2014). Antibiotic compounds, like pyrronitrin and chitinases, play an essential role in interspecies competition in the rhizosphere and have been demonstrated as important AHL-dependent compounds for successful colonization (Persello-Cartieaux *et al.* 2003, Liu *et al.* 2007, Müller *et al.* 2009). The upregulation of cell-wall degrading enzymes could facilitate bacterial adhesion and intracellular colonization (Zhang & Zhang 2013, Dutilloy *et al.* 2022). Moreover, secretion systems might be regulated differently by AHLs and could change how the bacterial cell is perceived by the plant (Henke & Bassler 2004). At least, in non-pathogenic *A. citrulli*, a T3SS mutant proliferated significantly less compared to the wildtype (Johnson *et al.* 2011).

Finally, it cannot be excluded that AHL-deficient mutants might have an initial growth and fitness advantage that facilitated cell proliferation in the rhizosphere. While AHL mutants can still profit from social dynamics in the bacterial population, they instantly save energy for other metabolic processes than AHL production (Ruparell *et al.* 2016). This energy advantage over AHL-producing wildtype bacteria, as recently shown for *P. aeroguinosa* QS cheaters (Mund *et al.* 2017), might have reinforced colonization on a short term scale. However, over time, communication and cooperation between closely related cells in microcolonies should have an indirect kin selection advantage (Hamilton 1964, Keller & Surette 2006), embracing QS as an evolutionary stable strategy (Hense *et al.* 2007).

Which of the mentioned bacterial traits have altered the colonization capacity of the AHL mutants in this study, could be analyzed by a comparative study on *A. radicis* gene expression in the future (further outlined in 3.9.3). Transcriptomic analysis by RNA-Seq and/or a more specific RT-qPCR targeting the mentioned traits could give valuable insights in the origin of the detected colonization differences.

3.6 Scenarios of bacterial recognition in different barley cultivars

As discussed above, the increased root colonization of ArI_{neg} implies a more intense physical interaction of colonizing cells and the host plant. This could signify that plant-bacteria recognition changed upon the missing AHL stimulus. ArI_{neg} mutant might be perceived differently by the plant compared to ArN35e, either because of the absent AHL itself or because of AHL-mediated changes in the bacterial phenotype. If this assumption is veracious, a different immune gene expression could be visible in ArI_{neg} and ArN35e treated plants, which might mechanistically explain the unexpected increase in plant aphid suppression upon ArI_{neg} inoculation.

Indeed, the pattern of analyzed defense gene expression revealed a stronger systemic immune response upon ArI_{neg} than ArN35e inoculation in both barley cultivars. However, ArI_{neg} inoculation activated a *PR* gene related immune response, comparable to SAR, in Barke (see 3.6.1), while *PR* genes were suppressed in a more ISR-like defense reaction in Scarlett (3.6.2). This result gave a hint that AHL-induced plant resistance is determined very specifically on plant genotype level, as discussed more in detail in the following paragraphs.

3.6.1 Differently induced plant immune response in Barke

The analysis of five selected immune response genes revealed substantial differences in the plant immune response between bacterial inoculation, barley cultivar and cultivation system. In Barke, the ArN35e treatment did not provoque significant expression changes in the selected plant defense genes. However, when AHL signalling was impaired in ArI_{neg} treatments, the pathogen-related genes *PR1* and *PR17b* were upregulated up to two times. *PR* genes are commonly considered as marker genes for Systemic Aquired Resistance (SAR) which is induced by the encounter with a pathogen (Ward *et al.* 1991, van Loon *et al.* 2006). Upon local pathogen perception in root tissues, the plant reacts with a pathogen triggered immunity (PTI) and an eventual effector triggered immunity (ETI), in which *PR* proteins counteract bacterial effectors (Jones & Dangl 2006, Tsuda & Katagiri 2010). In distal shoot tissues, the same SA-dependent mechanisms can lead to a systemic SAR response (Pieterse *et al.* 2014).

An activation of SAR-like immunity in Barke appears additionally plausible since *WRKY22* was significantly upregulated in ArI_{neg} treated Barke leaves. In line with this result, Dey *et al.* (2014) reported that Barke plants showed a 2- to 3-fold upregulation of *WRKY22* when induced with the soilborne pathogens *P. syringae* and *Xanthomonas translucense*. *WRKY* transcription factors are regarded as master regulators of pathogen-induced immunity since they activate *PR* gene expression encoding for antimicrobial proteins (Wang *et al.* 2006, Muthamilarasan & Prasad 2013). These PR proteins have been shown to fend-off biotrophic pathogens but also, to a smaller extent, herbivorous insects (Morkunas *et al.* 2011, Dey *et al.* 2014).

The observed upregulation of *PR* genes might indicate that Barke perceived the ArI_{neg} mutant strain rather as a pathogen than a beneficial interaction partner. However, the exclusive pathogen-stimulated activation of *PR* genes has been questioned in several studies recently (Niu *et al.* 2011, Jiang *et al.* 2016, Nie *et al.* 2017, Beris *et al.* 2018). For example, Kim *et al.* (2015) reported that *PR* genes were induced in response to a plant growth promoting *Bacillus sp.* strain in tobbaco. Similarly, beneficial *Bacillus subtilis* MBI600 stimulated a significant *PR1a* gene expression in tomato (Samaras *et al.* 2021). Therefore, the pathogenic or beneficial nature of this interaction cannot be reliably deduced from the defense gene expression pattern. Nevertheless, the significant change in *PR* gene expression in ArI_{neg} treated plants points towards a change in bacterial perception by the plant.

In respect to AHL signalling, the upregulation of *PR* genes in ArI_{neg} treated Barke plants was rather surprising. It would have been hypothesized that the absent positive AHL stimulus would lead to less immune system stimulation. In previous studies, *PR1b* and *PR17b* were indeed less upregulated when treated with the AHL-deficient mutant *E. meliloti* attM compared to the AHL-producing wildtype (Bziuk *et al.* 2022). Similarly, *PR1* and *PR17b* gene expression was induced by oxo-C14-HSL addition in the AHL-sensitive barley cultivar Golden Promise but not in the AHL-insensitive cultivar Gaulois (Hernández-Reyes *et al.* 2014, Shrestha *et al.* 2019). These literature examples assume AHL as a positive stimulus that facilitates bacterial recognition and beneficial interaction. Also in this work, pure OH-C10-HSL addition slightly increased root growth in Scarlett, suggesting the beneficial character of AHL (see 3.8).

If this is the case, AHL might have played a mitigating role in bacterial plant perception and defense induction (illustrated in Figure 3.1). As a positive stimulus, AHLs might have lowered the plant defense response towards the AHL-producing bacterium that would otherwise be perceived as a more harmful invader. Thanks to the mitigating AHL, ArN35e would thus be rather perceived as harmless so that the plant tolerated its presence for a beneficial interaction. When the AHL signal was now missing in ArI_{neg}, this strain was probably perceived as more dangerous which alerted the plant immunity, leading to a stronger plant defense against this microbe and indirectly also against invading aphids.

As only drawback, this scenario does not explain the increased colonization of ArI_{neg} and $ArR_{neg}I_{neg}$ cells (see 3.5). In contrary, the plant immune system should allow a dense colonization of harmless wildtype bacteria while the pathogen-like ArI_{neg} mutant should be fended off rigorously by the plant. Therefore, this scenario additionally suggests that the AHL-deficient mutant became more colonization competent, to overcome increased plant defenses. The indirectly AHL-mediated colonization increase here outweighted the missing direct AHL effect, with positive outcome for aphid suppression. This scenario is also discussed in Sanchez-Mahecha *et al.* (2022).

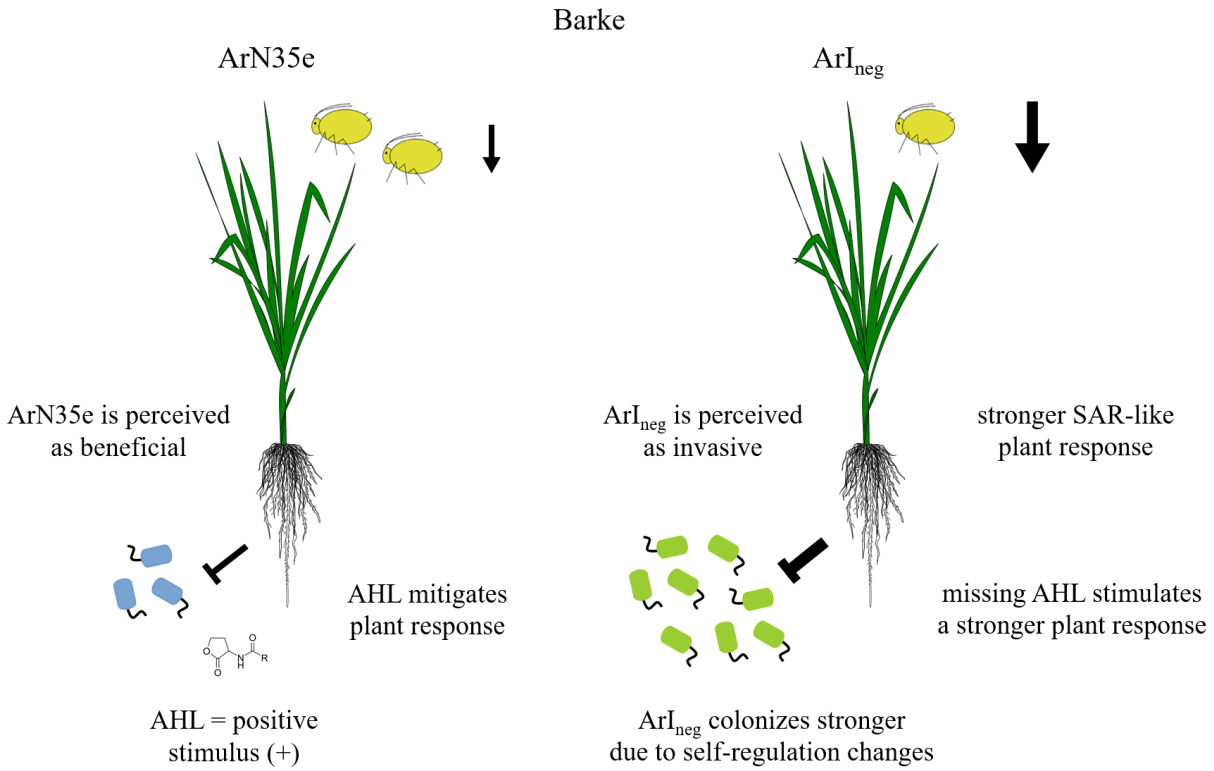

Figure 3.1 Most probable scenario for AHL-mediated plant aphid suppression in Barke. Upregulated *PR* genes suggest a SAR-like immune response of barley cultivar Barke towards ArI$_{neg}$ (green bacteria, right side) which was likely perceived as more invasive than ArN35e (blue bacteria, left side, representative AHL molecule depicted). In ArN35e, the positive AHL stimulus mitigated the plant response to the bacterium which was perceived as more beneficial. When AHL was absent in ArI$_{neg}$, accordingly, the immune response was stronger towards the bacterium and systemically also towards aphids (depicted as larger arrows and less aphids on the right). However, a stronger immune response against ArI$_{neg}$ does not explain the stronger colonization of ArI$_{neg}$ (depicted as more green cells on the right). Therefore, additional self-regulation changes might have turned the ArI$_{neg}$ mutant more competent in colonization, so that the bacterium could overcome enhanced plant defenses.

3.6.2 Differently induced plant immune response in Scarlett

In barley cultivar Scarlett, a different scenario might be the case. Reflecting on the SAR-like defense induction in Barke, it was striking to find the opposite defense gene expression pattern in Scarlett leaves. In Scarlett, the inoculation of ArI$_{neg}$ did not elicit an upregulation of both *PR* genes but a 2-fold downregulation in soil and to the same extent also under axenic conditions, although not significant (Figure 2.6). The gene expression of *WRKY22* remained unchanged. This observation suggests that the ArI$_{neg}$ mutant inoculation did rather not stimulate the SAR defense line. In contrary, this downregulation might signify that *PR* gene expression was

actively suppressed while a different immune pathway was favoured.
In the complex network of phytohormones, it is widely recognized that the SA-induced SAR pathway is antagonistically linked to induced systemic resistance (ISR, Pieterse *et al.* 2014). ISR is usually triggered by beneficial rhizobacteria and depends on ET/JA signalling (Pieterse *et al.* 2014). Ethylene-responsive transcription factors have been reported to directly suppress *PR* gene expression (Lorenzo *et al.* 2003). Indeed, in this study, a simultaneous upregulation could be observed in the ethylene-responsive factor *ERF-like* when ArI_{neg} was inoculated compared to ArN35e. A similar pattern could be observed in Barke, but the upregulation was only significant in Scarlett. This gene expression pattern might indicate that the ArI_{neg} mutant was rather not perceived as a pathogen but as more beneficial by the barley cultivar Scarlett, specifically. At least, almost all studies report that ISR induced by beneficial PGPR did not induce *PR* gene expression (Pieterse *et al.* 1996, van Loon *et al.* 2007, van Wees *et al.* 2008). When encountering a pathogenic *Fusarium* fungus, Scarlett reacted with a high upregulation of *PR1* and *PR1b* genes (Yang *et al.* 2010), confirming that the *PR* pathway of Scarlett was very sensitive to pathogens. Wehner *et al.* (2019) advocates that highly pathogen susceptible barley cultivars, like Scarlett, might be more efficient in developing ISR. Therefore, the induction of a more ISR-like immune response by ArI_{neg} seems most likely. This immune pathway can convey protection against pest insects, since ISR uses with JA and ET the same phytohormones that are also stimulated by insect feeding (Pineda *et al.* 2010, Balmer *et al.* 2013, Walters *et al.* 2013). Alternatively, other effective mechanisms independent of *PR* genes could have been triggered by *A. radicis* in Scarlett that have yet to be explored.

Comparing soil and axenic cultivation conditions, the defense gene expression was similar in ArI_{neg} treated Scarlett leaves, although not significant under axenic conditions. At the same time, it was surprising to find a substantial change in gene expression for ArN35e comparing cultivations. Under axenic conditions, *PR* genes appeared 5-fold downregulated in comparison to a not significant 1-fold downregulation in soil. This result indicates that the rhizosphere microbiome, shaped by present or absent AHL, and physical soil properties additionally altered bacterially-induced plant defenses (discussed more in detail in 3.10).

The downregulation of *PR* genes upon ArI_{neg} inoculation suggests that the AHL synthesis mutant was perceived rather not as pathogenic stimulus, but as more beneficial than ArN35e.

This is in contrast to the hypothesis that AHL facilitates a beneficial interaction, as a positive stimulus (see synthetic AHL effect), while the missing AHL stimulus in ArI_{neg} would constrain a beneficial interaction. In a possible scenario (depicted in Figure 3.2), this missing direct AHL effect might have been outweighted by a positive AHL-mediated self-regulation that might have altered bacteria-plant recognition and/or enhanced the production of beneficial QS-dependent bacterial traits (see indirect AHL effects). Consequently, bacterial colonization of ArI_{neg} might thus have been favoured since the plant allowed closer contact of the more beneficial interaction partner. But also causally, a positive self-regulation effect on bacterial colonization might have strengthened the physical interaction in the rhizosphere which might have been perceived as beneficial by the plant. In both cases, this scenario might have led to the enhanced aphid suppression effect.

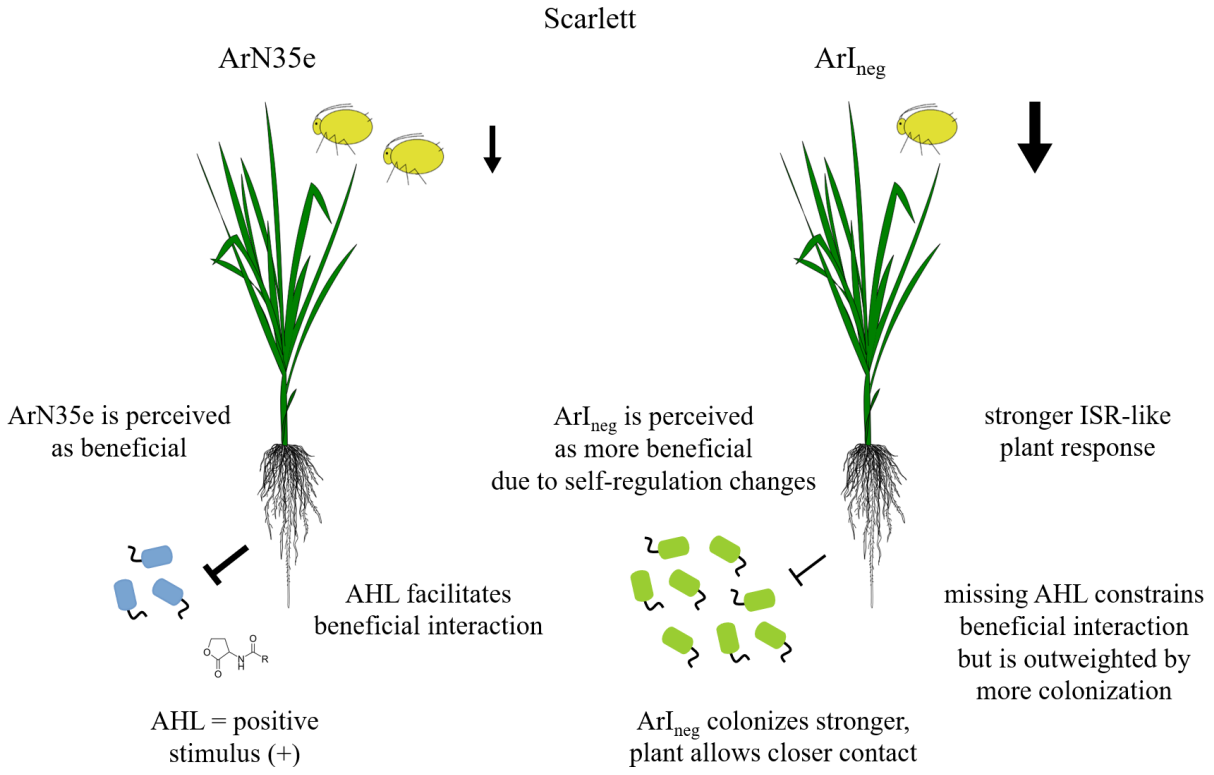

Figure 3.2 Most probable scenario for AHL-mediated plant aphid suppression in Scarlett. Downregulated *PR* genes suggest an ISR-like immune response of barley cultivar Scarlett towards the *A. radicis* AHL synthesis mutant ArI_{neg} (green bacteria, right side) which were most likely perceived as more beneficial than the AHL-producing strain ArN35e (blue bacteria, left side, representative AHL molecule depicted). This is in accordance with the colonization increase observed for ArI_{neg}, as the plant might allow more contact (depicted as more green cells and a smaller blocking arrow on the right). At

the same time, synthetic AHL was determined as rather positive stimulus for the plant so that present AHL should directly facilitate a beneficial interaction. Therefore, missing AHL should theoretically constrain beneficial interaction in ArI_{neg} treatments. However, self-regulation changes due to missing AHL might alter bacteria-plant recognition and/or bacterial traits (see indirect AHL effect in 3.8). The indirect AHL effect might thus outweight the missing direct AHL effect, leading to enhanced aphid suppression (depicted as larger down-pointing arrow and less aphids on the right).

It has to be mentioned that both presented scenarios are based on various assumptions – like the beneficial nature of *A. radicis* N35e (Li *et al.* 2012, Han *et al.* 2016, Siani *et al.* 2021) or the positive AHL stimulus (Schikora *et al.* 2016, Han *et al.* 2016, Wehner *et al.* 2021) – that cannot be deduced with certainity from the present data. Also other scenarios are conceivable, but only the most adequate explanations are discussed here for the sake of stringency. More insights into plant response as well as bacterial transcriptional regulation would be needed to better judge the beneficial or detrimental character of microbe-plant-insect interactions. Nevertheless, it can be specified that the barely genetic background strongly determines AHL-related interaction outcome. Apart from this, also the cultivation system visibly modulated plant responses and thus need to be considered when interpreting plant gene expression results. Both described scenarios strengthen the notion that indirect AHL effects via self-regulation changes played a substantial role in microbe-plant insect. The strength and extent of direct and indirect AHL effects is explored more in detail in 3.8.

3.7 ArI_{neg}-induced flavonoid production explaining increased plant aphid suppression

Apart from the just described scenarios of altered bacterial recognition, another mechanism related to plant defenses might have influenced aphid suppression. Although ISR and SAR pathways have been shown to affect insects, the differential expression of *PR*, *WRKY* and *ERF-like* genes might not ultimately explain the increased aphid resistance induced by ArI_{neg}. At least in Scarlett, it is likely that the enhanced resistance results from the increasing production of plant secondary metabolites, namely flavonoids.

In axenically-grown Scarlett, the expression of the UDP-glucuronosyl transferase (UGT) gene was significantly increased upon ArI_{neg} inoculation while also in soil and the cultivar Barke a similar tendency was observed. The UGT transcript is important for early flavonoid

biosynthesis in which it adds sugar to flavonoid precursors (Bowles *et al.* 2006). The final phenolic compounds exhibit insecticidal activity and thus efficiently counteract insect feeding on plant leaves (Simmonds 2003, Pangesti *et al.* 2013, Rashid *et al.* 2018, Ghitti *et al.* 2022). In accordance with this thesis, Han *et al.* (2016) reported already that flavonoid-related genes were upregulated in ArI_{neg} treated barley cultivar Barke. Also the respective compounds accumulated in plant leaves (Han *et al.* 2016). Interestingly, oxo-C12-HSL and oxo-C14-HSL previously induced UGT expression among other flavonoid synthesis genes (Mathesius *et al.* 2003, Schenk *et al.* 2014).

Han *et al.* (2016) points out that the unusual AHL of *A. radicis* N35e with its hydroxy-substitution (3-OH-C10-HSL) might have operated differently than common AHLs and might have inhibited flavonoid biosynthesis. This could explain the less suppressed expression of flavonoid-related genes in ArI_{neg} treated plants when AHL was absent (Han *et al.* 2016). This study, however, rather suggests that the increasing root colonization of ArI_{neg} (discussed in 3.4), coupled to AHL and bacterial recognition, might have stimulated the flavonoid-related pathway. This way, flavonoid biosynthesis might mechanistically explain the observed bacterially-induced aphid suppression on plant level.

In addition, flavonoid metabolites are generally thought to shape the root microbiome (Bag *et al.* 2022). Flavonoids responded to AHLs and induced root nodules after oxo-C14-HSL treatment in *M. truncatula* (Veliz-Vallejos *et al.* 2014). This way, activated flavonoid gene expression could have modified the altered microbiome composition upon ArI_{neg} inoculation (see 3.8.1).

3.8 Direct and indirect AHL effects on plant aphid suppression

The presented mechanisms outlined that complex AHL effects might have played a role in AHL-mediated plant resistance. AHL molecules can here not only stimulate plant tissues directly, but might also act indirectly by influencing bacterial gene expression and other rhizosphere microorganisms with aphid suppression activity. To which extent these direct and indirect AHL effects contributed to the oberved interaction outcomes, are disentangled more functionally in the following discussion.

The results of this thesis suggest that the rhizopshere microbiome did not play the major role

for AHL-mediated aphid suppression (3.8.1). Instead, the overall effect of synthetic OH-C10-HSL and C6-HSL molecules proposes a positive role on plant growth and resistance. However, these direct AHL effects seemed to be outweighted by bacterial colonization effects (3.8.2). Synthetic AHL also slightly abolished colonization differences, but indirect AHL effects probably determined colonization more importantly. AHL sensing and self-regulation modulated microbe-plant-insect interaction additionally, in which a negative regulation of beneficial bacterial traits might have played a special role (3.8.3). Complementation of AHL mutants with AHL revealed that multiple counteracting effects might have attenuated each other while other effects added up, but only to a certain limit.

3.8.1 The role of the rhizosphere microbiome

The rhizosphere microbiome was analyzed to find out if other soilborne microbes contributed to the increased aphid suppression effect in ArI_{neg} treatments. Amplicon sequencing of the 16S rRNA gene revealed that ArI_{neg} inoculation significantly influenced the belowground microbial community (Figure 2.9 c). Particularly, the relative abundance of rare microbiome members was affected by ArI_{neg} inoculation. This result was expected since the presence or absence of AHL might alter abundance and interspecies interaction, above all of QS-sensitive taxa, which occur enriched in the rhizosphere (Elasri *et al.* 2001). In contrast to the AHL mutant, ArN35e inoculation had no effect on the microbiome profile (Figure 2.9 a). This observation is in accordance with a comparable study by Zytynska *et al.* (2020) in which inoculated ArN35e did not significantly affect the Barke rhizosphere microbiome as well.

In respect to species richness and evenness, no significant differences could be observed. Interestingly, Ibal *et al.* (2021) found a lower microbial alpha-diversity in C10-HSL treated gingseng while bacterial abundance remained unchanged. Also, Liang *et al.* (2020) reported that AHL treatment reduced the bacterial diversity in agricultural soil while interacting with bacteriophages. A high bacterial diversity is commonly associated with enhanced ecosystem functioning (Wagg *et al.* 2019). The diverse genetic potential of a rich microbial community might provide resilience to environmental stress while microbiome dysbiosis often goes along with a loss of diversity and function (Ibal *et al.* 2021, Arnault *et al.* 2022). In the present study, contrarily, the stable richness pointed towards no disturbance of a healthy microbiome.

In the present work, sixteen genera responded differently to ArN35e and ArI_{neg} inoculation (see Table 2.1, AHL effect), while only three genera changed upon aphid infestation alone, namely *Dyadobacter, Hirschia* and *Stenotrophomonas*. Six of the mentioned genera showed interesting tendencies in which a relative abundance change co-occurred with reduced aphid loads when comparing ArN35e and ArI_{neg} treated plants: *Bdellovibrio*, *Lacunisphaera*, *Limnobacter*, *Spirochaeta 2*, *Sphingopyxis* and *Solimonas* (see Table 2.2). However, the correlation between aphid load and the relative abundance of all these OTUs was not significant in any treatment, except *Bdellovibrio*. These weak correlations suggest that the rhizosphere microbiome was not the driving force behind the observed aphid suppression effects of ArI_{neg}.

Nevertheless, it is still possible that certain taxa contributed to the enhanced plant resistance, indirectly mediated by AHL. This might particularly apply to the nine mentioned genera which were linked to reduced aphid loads (Table 2.1 and 2.2) and might therefore bear potential for AHL-mediated aphid suppression.

For some interesting taxa – *Hirschia, Lacunisphaera, Limnobacter* and *Sphirochaeta* – no information on plant interaction or biocontrol activity are available. Other genera have been already associated with enhanced plant resistance, like *Dyadobacter* (Fu *et al.* 2017, Siegel-Hertz *et al.* 2018, Taparia *et al.* 2021). This genus has been proposed as key stone taxon in healthy suppressive microbiomes (Wei *et al.* 2019). However, in this study, the abundance of *Dyadobacter* descended significantly with both *A. radicis* treatments which does not suggest an active recruitment by the inoculated plants.

Similarly, *Solimonas* members have been already associated with microbially induced resistance in *Arabidopsis* and plant protection in wild banana (Sommer *et al.* 2021, Singh *et al.* 2023). In this study, *Solimonas* was significantly enriched in the ArI_{neg} treated rhizosphere while aphid loads were reduced in a negative correlation (red). This might hint towards a potential response of this genus to the presence or absence of AHL produced by *A. radicis,* which in turn influences aphid suppression.

The same pattern applies to *Sphingopyxis*. In contrast, this genus is so far only known for promoting plant growth in strawberry (Dias *et al.* 2009) and causing the corky root disease in lettuce (van Bruggen *et al.* 2014). Although biocontrol activity of *Sphingopyxis* remains to be examined, these genera might hence be worth further investigation.

Apart from these taxa, *Bdellovibrio* represents a common microbiome member which might have indirectly influenced plant aphid suppression. *Bdellovibrio* displayed the only significant positive correlation between bacterial abundance and aphid loads in the control. This might be a hint that the presence of *Bdellovibrio* facilitated aphid growth, counteracting the beneficial effect of *A. radicis*. The relative abundance of *Bdellovibrio* decreased in ArI_{neg} compared to ArN35e treatments, so that its positive effect on aphid loads might also be reduced. It is possible that the growth of *Bdellovibrio* was compromised by the higher colonization density of ArI_{neg}. Moreover, *Bdellovibrio* comprises pedatory bacteria that have shown to consume Gram-negative bacteria like *A. citrulli* (Dwidar *et al.* 2012, Aharon *et al.* 2021). However, no hints could be found that predation influenced the abundance and AHL signalling of *A. radicis*. In contrast to all aforementioned genera, *Stenotrophomonas* has been related to insect biocontrol before (Berg *et al.* 2010). The genus was prominently enriched in the microbiota of whitefly-infested pepper (Kong *et al.* 2016). *Stenotrophomonas* isolates killed aphids and termites with high mortality (Baazeem *et al.* 2022, Jabeen *et al.* 2018) and controlled fungal pathogens like *Fusarium* in wheat and *Ralstonia* in potato (Dal Bello *et al.* 2002, Messiha *et al.* 2007). *Stenotrophomonas* abundance was also significantly correlated to aphid presence in this work. However, its abundance remained constant between bacterial treatments and is therefore unlikely influenced by *A. radicis* and its AHL signalling.

In summary, collected information on the most prominently changing genera did not suggest a significant role of other soilborne microorganisms in AHL-mediated aphid suppression. None of the exemplarily discussed genera revealed a reasonable relation between abundance change, correlation profile and biocontrol history. Hence, it appears unlikely that on of these microbiome members played a decisive role in increasing aphid suppression in ArI_{neg} treatments. Nevertheless, the identified genera might represent interesting candidates in the search for new biocontrol agents.

Although the rhizosphere harbours a high proportion of bacteria with AHL signalling capacity (Elasri *et al.* 2001), the changing genera could not be identified as specifically AHL-sensitive. Apart from a direct AHL effect on the microbiome, two other possible reasons for the differences between ArN35e and ArI_{neg} treatments need to be mentioned at this point. On the one hand, the stronger and more persistent ArI_{neg} colonization likely influenced niche

occupation in the rhizosphere (see 3.5). The biofilm-like ArI_{neg} accumulations might have outcompeted certain taxa or at least complicated the establishment of other plant interaction partners. On the other hand, the differences in aphid density between the bacterial inocula could have modulated the microbiome composition top-down as well. High or low aphid loads might have altered root exudation or nutrient availability in the soil (Liu *et al.* 2020, Potthast *et al.* 2022). Several of the discussed taxa also prevail in the aphid gut microbiome, including *Dyadobacter* (He *et al.* 2021), *Stenotrophomonas* (Xu *et al.* 2023) or *Sphingopyxis* (McLean *et al.* 2019). Aphid-associated cells could have been transferred to the soil via the plant sap, honeydew excretion or dislocated animals, as confirmed before by Seeger & Filser (2008) and Wolfgang *et al.* (2023).

Despite of these reciprocal feedback ways, an indirect AHL effect via the rhizosphere microbiome was regarded as not decisive for microbe-plant-insect interaction in this thesis. The observed increase in aphid suppression upon ArI_{neg} inoculation might more likely be attributed to a direct effect of AHL molecules or the QS-dependent bacterial gene expression, as elaborated in the following.

3.8.2 The role of direct AHL effects

In order to assess the direct effect of AHL molecules on barley, synthetic OH-C10-HSL (the same AHL type as produced by *A. radicis* N35e) and C6-HSL (as comparison) were applied to axenically-grown plants. Biosensor assays confirmed that the concentration of both synthetic OH-C10-HSL and C6-HSL was still significantly elevated in the growth medium after 21 days compared to the NoAHL control (Figure 2.11). The OH-C10-HSL concentration was not higher in the ArN35e treatments, where additional AHL was presumably produced by the bacterial cells, compared to the other treatments. However, this finding can be well explained by the short colonization time of ArN35e (see 3.1). As AHL concentrations can still trigger plant responses in the nanomolar range (Mathesius *et al.* 2003, Bai *et al.* 2012, Palmer *et al.* 2014), the remaining AHL concentrations between 0.3 to 0.8 µM after 21 days was considered to be high enough to trigger plant responses throughout the whole experiment.

a) Synthetic AHL effect on plant aphid suppression

In respect to plant resistance, both synthetic AHLs tended to increase aphid suppression across all analyses, although not significantly (see Figure 2.12 b and 2.5.2). For OH-C10-HSL, this small increase was expected since long chain AHLs are commonly thought to induce resistance against a range of phytopathogens by activating plant immunity (Mathesius *et al.* 2003, Schuhegger *et al.* 2006, Schikora *et al.* 2011, Schenk *et al.* 2012, Pazarlar *et al.* 2020). Previous data from Han *et al.* (2016) indicated a small stimulation effect of *A. radicis*-derived OH-C10-HSL on early immune response genes in barley. Similarly, Nawaz *et al.* (2020) proposed a positive effect of OH-C10-HSL and other purified AHLs from *Aeromonas sp.* on wheat defenses. However, for synthetic OH-C10-HSL specifically, no literature on plant resistance is currently available. Only unsubstituted C10-HSL application has already been shown to upregulate important detox genes in barley and yam bean (Götz-Rösch *et al.* 2015), induced defense genes in *Arabidopsis* (Cao *et al.* 2022) and stimulated resistance to *B. cinerea* in tomato (Hu *et al.* 2018).
AHLs with C3 hydroxy substitutions are rather unconventional in bacterial QS. Therefore, it is conceivable that barley was less susceptible to OH-C10-HSL. Earlier studies already indicated that the side chain substitution makes a substantial difference for AHL priming (Schikora *et al.* 2011, Bai *et al.* 2013). For instance, OH-C14-HSL induced resistance in *Arabidopsis* to a lesser extent than oxo-C14-HSL (Schikora *et al.* 2011). Similarly, only oxo-C10-HSL induced adventitious root formation in mung bean while the plant did not respond to the unsubstituted long chain AHL (Bai *et al.* 2013). Future examinations of OH-C10-HSL would be needed to resolve the functionality of this specific AHL.

For C6-HSL, the tendency for induced plant resistance is in accordance with literature as well. Synthetic C6-HSL activated the expression of auxin- and cytokinin-dependent defense genes in *Arabidopsis* (van Rad *et al.* 2008) and upregulated detox genes in barley and yam bean, similar to synthetic C10-HSL (Götz-Rösch *et al.* 2015). However, at the same time, the addition of synthetic C6-HSL led to a 4-fold increase in larval mass of the tobacco hawk moth *Manduca sexta*, probably subjected to an interaction of AHL with the JA-dependent defense pathway (Heidel *et al.* 2010). Hence, these studies caution that also direct AHL effects are not straightforward (von Rad *et al.* 2008, Heidel *et al.* 2010). The herein observed small increase

in aphid suppression suggests nevertheless, that the OH-C10-HSL as well as C6-HSL stimulus alone rather positively influenced plant immunity.

This positive role of AHL molecules contradicts the observed increase in aphid suppression upon missing AHL in ArI_{neg} treatments. In case AHL represents a positive signal for the plant, the lack of AHL production would have plausibly led to less immune system stimulation. This result strengthens the assumption that the stronger colonization of ArI_{neg} outweighted direct AHL effects in the present model system. Most likely, direct AHL effects only modulated microbe-plant-insect interaction to a smaller extent in the present work.

b) Synthetic AHL effect on bacterial colonization

Importantly, the described effects of synthetic AHLs cannot be judged without taking into account that AHL molecules naturally also influence bacterial colonization. According to common literature, AHLs are supposed to induce surface attachment and biofilm formation in QS-responsive bacteria (Davies *et al.* 1998, Hammer & Bassler 2003, Subramani & Jayaprakashvel 2019). However, in this study, *A. radicis* mutants impaired in AHL synthesis colonized the roots in significantly higher amounts than the respective strain with intact AHL synthesis (discussed in chapter 3.5). In case the absent AHL molecules are directly responsible for the colonization changes, synthetic AHL addition should be able to completely abolish the colonization differences.

Indeed, the complementation of ArI_{neg} and $ArR_{neg}I_{neg}$ with synthetic OH-C10-HSL showed a small but nearly significant decrease in cell numbers per g root compared to NoAHL trials (see Figure 2.17). The AHL addition thus partly alleviated the differences in *A. radicis* mutant colonization. This indicates that the presence or absence of AHL molecules made a difference for colonization. Since the added AHL reduced colonization also in $ArR_{neg}I_{neg}$, in which AHL-induced gene expression should be unfunctional, a direct negative effect of OH-C10-HSL on colonization can be assumed (see 3.5).

Though, it has to be noted that the respective ArN35e colonization pattern could not be restored by AHL addition. This suggests that the colonization differences not only resulted from the AHL stimulus itself but might have been caused by a complex combination of altered plant perception, bacterial self-regulation and other factors influencing both interaction partners.

In summary, these results illustrate that AHL probably represents a positive stimulus for the plant, slightly inducing aphid resistance. However, discriminating the effect origin remains difficult. Especially the differences in bacterial colonization – again influenced by AHL itself – overlapped with the direct AHL effect. Thus, the missing positive AHL effect in ArI_{neg} treatments is most likely outweighted by enhanced plant-microbe interaction due to increased root colonization. Direct AHL effects appeared not to be the driving force for aphid suppression. Instead, they only seem to modulate plant resistance together with indirect AHL effects acting on QS-dependent bacterial traits, as discussed in the following.

3.8.3 The role of AHL-dependent gene expression

In addition to the described direct AHL effects, microbial AHL molecules can also indirectly affect interkingdom interactions by changing bacterial gene expression. Many bacterial traits have been proven to be AHL-regulated, including nitrogen acquisition (Daniels *et al.* 2002), iron and sulfur uptake (Chapalain *et al.* 2013, Iwanicka-Nowicka *et al.* 2007), ACC deaminase (Jung *et al.* 2020) and phytohormone production (Müller *et al.* 2009, Zúñiga *et al.* 2013), virulence factor secretion (Rutherford & Bassler 2012) and other metabolic functions (Altaf *et al.* 2017). Also the QS-dependent production of antimicrobial compounds like phenazine, pyrollnitrin and pyoluteorin have been shown to affect bacterial interaction with its host plant (Schmidt *et al.* 2009, Yan *et al.* 2007, Chernin *et al.* 2011). These traits can plausibly be altered when AHL synthesis is artificially inhibited in signalling mutants. Most prominently, the interrupted AHL feedback loop can affect bacterial colonization ability (Wei & Zhang 2006), which appeared to be the major cause for interaction differences in the present thesis. These AHL-mediated colonization effects were already extensively discussed further above (see chapter 3.5). But also other QS-dependent traits can modulate microbe-plant-insect interaction. To disentangle direct from indirect AHL effects more in detail, the aphid suppression effect of *A. radicis* strains with intact AHL perception (ArN35e and ArI_{neg}) can be compared to AHL sensing mutants (ArR_{neg} and $ArR_{neg}I_{neg}$) which should not respond to AHL.

a) Bacterial self-regulation effect on aphid suppression

Without AHL addition, it can be expected that both AHL receptor mutants ArR_{neg} and $ArR_{neg}I_{neg}$ show a similar aphid suppression effect like the synthesis mutant ArI_{neg}. In all three strains, the AHL feedback loop would then be interrupted because of missing AHL or receptor molecules, respectively. Like expected, under NoAHL conditions, no significant differences could be found in plant aphid suppression between these three mutant inocula (see Figure 2.12 a). Though, visible differences should appear when synthetic OH-C10-HSL was added.

In complementation assays with OH-C10-HSL, the differences between AHL sensing and signalling mutant effects became highly significant (Figure 2.12 b). However, multiple effects of the same or counteracting directions overlapped and therefore remain difficult to discern. In AHL-sensitive ArN35e and ArI_{neg}, the supplemented OH-C10-HSL was expected to induce beneficial bacterial traits under QS control. Complemention of ArI_{neg} treatments should here reestablish the ArN35e effect, while in ArN35e treatments the synthetic AHL might additionally boost the AHL feedback loop – if not yet exhausted. Together with a direct effect of AHL molecules, this should result in an additive aphid suppression effect of the same positive direction. However, no positive effect addition could be observed in both cases. On the one hand, beneficial aphid suppression might have already reached its maximum in the ArN35e treatment combination. On the other hand, this outcome might be explained by two overlapping effects: while AHL molecules could have enhanced aphid suppression directly, other beneficial traits might have been downregulated by negative AHL regulation, counteracting the direct AHL effect. This is also supported by the aphid suppression pattern observed for the AHL receptor mutants. In comparison to the respective AHL-sensitive mutants, plants inoculated with ArR_{neg} and $ArR_{neg}I_{neg}$ strains showed significantly increased aphid suppression, when complemented with OH-C10-HSL. Here, the missing AHL receptor could have prevented counteracting QS-regulation effects upon AHL application.

Negative regulation of beneficial traits has been suggested already analyzing root colonization and gene expression data (see 3.5 and 3.6) and has also been reported previously e.g., by Müller *et al.* (2009). For example, production of the plant growth hormone IAA was upregulated in a *S. plymuthica* mutant in which AHL accumulation was inhibited, leading to enhanced growth

of oilseed rape (Müller *et al.* 2009). Similarly, iron-complexing siderophores have been shown to be overproduced when AHL production was impaired in *cepI* mutants of *Burkholderia* strains (Lewenza *et al.* 1999, Chapalain *et al.* 2013). Antibiotic production against phytopathogens was no longer repressed in AHL-deficient mutants of beneficial *Pseudomonas sp.* M18 (Yan *et al.* 2007) and *Lysobacter* (Ling *et al.* 2009). A similar negative regulation of other QS-related genes – including biofilm formation and root colonization – could likely also benefit bacteria-plant interaction and plant aphid resistance. This mechanism could thus partly explain increased aphid resistance upon missing AHL, as already proposed in Sanchez-Mahecha *et al.* (2022).

Likewise, it is conceivable that bacterial traits with detrimental plant effect, which are positively QS-regulated, were no longer induced upon missing AHL but activated with supplemented AHL again. Examples for such QS-regulated bacterial traits with detrimental plant effect mainly comprise the production of virulence factors (Rutherford & Bassler 2012), MAMPs like flagella proteins (Felix *et al.* 1999) and secretion systems (Henke & Bassler 2004). Secretion systems inject effectors directly into plant cells and play a global role in bacterial transport and communication (Pena *et al.* 2019). Again, these detrimental traits have been shown to be either QS-induced or QS-repressed depending on the respective response regulator (Chapon-Hervé *et al.* 1997, Winzer & Williams 2001, Quiñones *et al.* 2005, Lesic *et al.* 2009). In the light of this complexity, the possibility is given that numerous beneficial and detrimental bacterial traits were activated or repressed differently in ArI_{neg} with or without supplemented AHL.

It has to be stressed that the observed aphid suppression differences between AHL sensing and synthesis mutant inoculation might predominantly derive from colonization changes. As discussed previously (see 3.5), root colonization can likely be negatively QS-regulated. Indeed, ArI_{neg} showed reduced colonization when its self-regulation was activated by OH-C10-HSL compared to the AHL-insensitive $ArR_{neg}I_{neg}$ (see Figure 2.17). This result underlines that bacterial AHL-mediated gene expression – as one of many mechanisms – contributed to overall effects. Yet, the exact effect origin is hard to pinpoint due to the mentioned complexity. A more extensive RT-qPCR analysis of bacterial gene expression under QS control could reveal this further.

b) Further investigation of QS-dependent traits

The discussed results show that bacterial self-regulation mediated by AHL contributed to aphid suppression effects, although effect direction and strength cannot be undoubtedly determined. Which genes were differently regulated in the four applied *A. radicis* strains in this work, could be revealed by an extensive transcriptomic analysis combined with targeted RT-qPCR in the future. A first *in vitro* comparison of *A. radicis* gene expression profiles across exponential and stationary growth phase could provide general information on the bacterial phenotype and the role of AHL, followed by subsequent insights into the bacterial behaviour on plant roots with and without synthetic AHL. As a result, diverse genes involved in microbe-plant interaction are expected to differ in their expression which can also serve as interesting targets for RT-qPCR confirmation.

For this envisaged investigation, the doctoral researcher Roberto Siani (HMGU) already identified gene candidates which stand under QS control in *A. radicis* N35e. Among them were several genes involved in flagellar assembly (like *flgF* and *flagJ*), peptidoglycan synthesis (*murJ*), potassium transport (*kdpC*), glycerol metabolism (*glpK*). In addition, QS-controlled secretion systems would be interesting targets for investigation in *A. radicis*, in which at least a type II secretion system was identified (motif identification by Roberto Siani). An extensive pan-genome analysis by Siani *et al.* (2021) pointed out that *Acidovorax* strains with pathogenic lifestyle differed from commensals by increased secretion system functioning. Especially the activity of T3SS could thus provide insights in the beneficial or detrimental nature of microbe-plant interaction (Siani *et al.* 2021). Moreover, the secretion systems play a key role in flagellar morphogenesis and motility since they secret flagellar components and are thus involved in bacterial colonization (Noh *et al.* 2015). If differentially regulated secretion systems thus influenced bacterial communication, can be elucidated with a more detailed analysis of whole transcription profiles generated by RNA-Seq. However, this analysis is time-expensive, requires special know-how and therefore remains to be realized in future research cooperations.

In total, indirect AHL effects via bacterial gene expression seemed to modulate plant aphid resistance in this work. However, due to multiple overlapping effects and the small effect size, a statement about direct and indirect effect strength and direction remains challenging. As mentioned in the previous sections, different possibilities exist how mixed direct and indirect

AHL effects might have modulated the visible plant aphid resistance in barley. To disentangle these effects, additional replications with higher sample sizes would be definitely needed. Additionally, AHL mutant effects should be urgently compared to the respective rescue mutants with vectorially introduced intact *araI* or *araR* genes. Due to limited time and experimental size, the generation and application of rescue mutants was postponed in this thesis. However, rescue mutants would be crucial to unambiguously attribute the observed effects to the interrupted AHL signalling, while unwanted off-target effects could be excluded. In such larger-scale experiments comprising all possible controls – together with the aforementioned transcriptomic analysis – direct and indirect AHL effects could be more deeply understood.

c) Off-target effect contribution to indirect AHL effects

Regarding the complexity of the AHL signalling effects on microbe-plant-insect interaction, it cannot be ruled out that the gene replacement mutagenesis led to unwanted off-target insertions or gene knockouts essential for bacterial AHL signalling, colonization and microbe-plant-insect interaction. It is possible that the open reading frame harbouring the *araI* gene encodes also for other bacterial traits which could have been unintentionally interrupted by the tetracycline marker gene. In addition, the tetracycline resistance cassette, which was introduced in ArI_{neg} mutants and encoded a membrane-integrated TetA efflux transporter (Hoang *et al.* 1998), might have influenced also other cellular functions since transporter activation alters membrane potential and ion homeostasis (Berens & Hillen 2003).

Finally, it cannot be excluded that yet undiscovered signalling elements balanced the observed effects. In *A. radicis*, Fekete *et al.* (2007) only detected OH-C10-HSL as the single homoserine lactone. But many bacteria harbour more than one LuxR/LuxI-type two component systems or LuxR solo homologue (Tobias *et al.* 2020). In *A. citrulli*, still new LuxR-type transcriptional regulators were recently identified (Guan *et al.* 2020). Also in *A. radicis* N35e, other regulatory elements might have compensated the sensing and signalling functions that were inhibited in AHL mutants. Thanks to ongoing research, new groups of molecular signals like photopyrones (Brachmann *et al.* 2013) or dialkylresorcinols (Brameyer *et al.* 2015) are discovered that enlarge our understanding about bacterial crosstalk (Tobias *et al.* 2020).

3.9 Barley cultivar influencing microbe-plant-insect interaction

The results of this thesis highlight that plant sensitivity and breeding history strongly determine multitrophic interaction outcome. Barley cultivars are widely known to vary in their native response towards aphid infestation (Tétard-Jones *et al.* 2007, Zytynska & Weisser 2016). Among the selected barley genotypes, Scarlett represents the most susceptible cultivar against foliar diseases while Chevallier inherited the highest resistance against herbivory. Barke and Grace harboured intermediate aphid resistance in previous tests. In this work, Barke appeared as most resistant while Scarlett showed indeed the most pronounced aphid infestation. Previous reports argued already that Scarlett lacks substantial defenses and might therefore exhibit lower expression of major defense genes (Hickey *et al.* 2017, Al Daoude *et al.* 2020). Modern breedings, like Scarlett, have frequently lost original resistance traits against herbivorous insects during domestication (Moreira *et al.* 2018). Also, when exposed to abiotic stress, the relatively modern cultivar Scarlett followed a different response strategy than older cultivars (Cantalapiedra *et al.* 2017).

The differences between barley cultivars was most striking in the plant gene expression analysis. The observed pattern suggests that the inoculated bacterial strains have been perceived differently in Barke and Scarlett. Shrestha *et al.* (2019) already reported that barley genotype determines the outcome of beneficial bacteria-induced resistance against the fungus *Blumeria graminis*. Also in Leybourne *et al.* (2019) and Mehrabi *et al.* (2014), defense gene expression differed between susceptible and more resistant wild barley genotypes infested with aphids. Schwarczinger *et al.* (2021) underlined that susceptible and resistant barley cultivars can differ in their *PR* gene expression. Other studies suggest that aphid feeding can induce SA-regulated immune pathway more strongly in susceptible crop plants, like in Scarlett, so that *PR* gene expression might be initially more elevated in control plants (Moran & Thompson 2001, Leybourne *et al.* 2019). Hence, different initial transcription levels might underly the direction change that was visible between the barley cultivars.

Similarly, the sensitivity to AHL has been shown to differ substantially between plant cultivars. Shrestha *et al.* (2019) demonstrated that a range of barley genotypes responded with altered immunity to oxo-C14-HSL addition. Wehner *et al.* (2019) underpinned that barley cultivars showed different efficiency in defense induction against *Bgh* governed by AHL-producing *E. meliloti* exp^{+}ch. The results of this thesis emphasize that the genetic background of the chosen

model organisms has to be taken into account when evaluating microbial AHL effects on plant resistance. The variability between plant cultivars complicates the prediction of microbe-plant-insect interaction outcome (Zytynka *et al.* 2020). Hence, embracing contrasting cultivars in future studies could help to better understand and overcome these inconsistencies for successful PGPR application in the field (Zytynka *et al.* 2020).

3.10 Cultivation conditions influencing microbe-plant-insect interaction

Apart from the genetic background of the model plant, also reciprocal effects with the abiotic and biotic environment have to be considered for successful biocontrol. This thesis highlights that the cultivation context strongly influenced microbe-plant-insect interaction. Interestingly, aphid suppression and plant growth effects of *A. radicis* N35e appeared more pronounced in soil, which is in accordance with Han *et al.* (2016). Also other studies report more pronounced PGPR effects under non-sterile conditions, in which the inherent microflora catalyzed beneficial trait production (Kloepper & Schroth 1980, Khalid *et al.* 2004, Gholami *et al.* 2009). Only in respect to plant defenses, ArN35e inoculation to Scarlett plants induced *PR* gene expression stronger under axenic conditions. Under these conditions, the plant might be more directly exposed to the bacterial stimuli without any buffering soil effects. As demonstrated for *Azospirillum brasilense* (Schloter & Hartmann 1998) and *Pseudomonas* (Buddrus-Schiemann *et al.* 2010), bacteria colonize better in axenic environments without any interspecies competition. The natural properties of soil, which is rich in physical niches, diffusion obstacles, parasites and other microorganisms, might hinder the widespread distribution of *A. radicis* cells (Lugtenberg *et al.* 2001, Arora *et al.* 2010). This study revealed a successful but short colonization of all *A. radicis* mutants in soil as well as under axenic conditions. A comparative analysis of root colonization in soil and under axenic cultivation conditions remains to be performed.

Moreover, it is conceivable that AHL molecules produced by ArN35e distributed more equally in the liquid cultivation medium, so that AHL effects could be more pronounced under axenic conditions (Hense *et al.* 2007). Simultaneously, AHL degradation occurs with a high frequency in soil, due to lactonase production of indigenous microbiome members (Wang & Leadbetter 2005). Additionally, other microorganisms might modulate their own abundance and behaviour

when encountering the AHL signalling of ArN35e and might thus differentially stimulate plant immunity.

In general, it is thought that PGPR exhibit more pronounced effects under stressful conditions, like low nutrient availability, salinity or drought in soil (Nadeem *et al.* 2013, Vocciante *et al.* 2022). The tremendous influence of nutrient availability, macroscopic soil engineers like earthworms and climate change on *A. radicis* inoculation effects were examined more in detail by our cooperation partners (Zytynska *et al.* 2020, Sanchez-Mahecha *et al.* 2022). Altogether, these observations emphasize that bacterially-induced herbivore control is strongly context-dependent (Pacheco de Silva *et al.* 2022). For interaction studies, it is therefore highly recommended to compare effects from reduced experimental setups with more complex designs, which take into account the versatile biotic and abiotic parameters from natural environments (Zytynska 2021). Still, *in vitro* studies can only be translated with difficulties to the field; many allegedly robust PGP effects disappear when transferred from controlled greenhouse experiments to agricultural soils (Berg 2009, Timmusk *et al.* 2017, Basu *et al.* 2021a). Therefore, multifactorial experiments can be of great benefit to deepen our knowledge about rhizobacterial biocontrol and its fruitful application in sustainable food production.

3.11 Comment on statistical power

As already mentioned, all conclusions from herein performed experiments have to be regarded in the light of the small statistical power. Several hundreds of true biological replicates would have been needed to secure reliable statements on plant aphid suppression (see 6.11.3 for prior power analysis). Small statistical power is a frequent phenomenon in ecological studies (Fairweather 1991, Waite & Campbell 2006, Gent *et al.* 2018). Plants often show high intraspecific heterogeneity and effect variability (Albert *et al.* 2011, Herrera 2017) while, in this study, the estimated effects size was comparably low. Unsufficient sample size together with small plant effects lead to large type II errors which means that relevant effects may not be identified as such (Gent *et al.* 2018).

In this work, following advices for power improvement by Madden & Paul (2011), experiments were independently repeated and data sets combined in a meta-analysis-like approach. An average sample sizes of n = 25 was reached with this while other plant studies recommended

sample sizes of 8 (Raudonius *et al.* 2017) to 21 (de Souza *et al.* 2023). As proposed by Gent *et al.* (2018), alpha levels – determining the false discovery rate – were loosened by considering p-values between 0.05 and 0.1 as meaningful tendencies. Moreover, the multifactorial design allowed to increase power by reducing residual variance when covariates were integrated into the analysis (Cohen 2013, Zytynska *et al.* 2021). Still, follow-up experiments should be specifically tailored for refining the postulated mechanisms in microbe-plant-insect interaction.

Conclusion

The present thesis showed that microbial AHL signalling played a complex role in microbially-mediated plant aphid resistance. Inoculation of the OH-C10-HSL-producing rhizobacterium *Acidovorax radicis* N35e revealed a small but consistent aphid suppression effect in barley, whereas *Rhizobium radiobacter* F4 was not further used as model strain due to inconsistent biocontrol effects. Surprisingly, when AHL was missing in *A. radicis* synthesis mutant ArI_{neg}, aphids were even more suppressed compared to the AHL-producing ArN35e in soil and as a tendency also under axenic conditions. This increase could be traced back to a significantly higher root colonization by the AHL synthesis mutant compared to the AHL-producing strain. Plant immune response suggested that the AHL mutant was perceived differently by the plant than the fully functional ArN35e. However, different immune pathways were activated in the two barley cultivars Barke and Scarlett, suggesting that the plant genotype strongly determines the reaction to the bacterial mutants. Since synthetic OH-C10-HSL tended to have a positive effect on plant aphid resistance, it can be assumed that AHL directly mitigates the plant defense against *A. radicis* N35e and facilitates a more beneficial interaction. The enhanced colonization and increased aphid suppression most likely results from an additional AHL-mediated change in bacterial gene expression, which might have boosted colonization capacity and/or other beneficial traits. Thus, in addition to the direct perception by the plant, AHL is also indirectly influencing the plant via its role in bacterial self-regulation. Both direct and indirect AHL effects thus appeared to have important modulating effects on bacterially-induced plant resistance. The rhizosphere microbiome was significantly altered by ArI_{neg} inoculation, likely due to its enhanced colonization, but no other microbial genus could be identified as cause for the aphid suppression changes. An upregulation of flavonoid biosynthesis in barley leaves was found upon AHL synthesis mutant inoculation, which might mechanistically underly the altered aphid suppression. Complementation assays with AHL sensing mutants confirmed that overlapping direct and indirect AHL effects of different directions most probably contribute to microbe-plant-insect interaction. All AHL effects here strongly depend on the sensitivity of the barley cultivar and the cultivation system. Thus, this thesis highlights the complexity of AHL effects in interkingdom communication and encourages more multifactorial studies which can be powerful to better understand the role of microbial signalling in insect biocontrol.

Outlook

This thesis adds new perspectives on the role of AHL in bacterially-induced plant resistance to pest insects. Comprehensively, additional experimental replications – including rescue mutants with vectorially introduced intact *araI* or *araR* genes – would be crucially needed to verify the small and overlapping AHL effects revealed in the interaction between *A. radicis* N35e, barley and aphids. The specificity of direct AHL effects could be further clarified by including C10-HSL, as unsubstituted comparison to OH-C10-HSL, and well described plant stimulators as oxo-C14-HSL (Schikora *et al.* 2011) into inoculation experiments. Moreover, plant defense gene expression upon synthetic AHL addition could give insights into direct AHL effects on barley resistance, including additional key genes for induced resistance like MYB72 (van der Ent *et al.* 2008). Bacterial functions modulated by the AHL feedback loop could be pinned down by global transcriptome profiling of *A. radicis* mutants *in vitro* and *in planta,* followed by a targeted RT-qPCR on selected QS-regulated genes. Comparative analysis of *A. radicis* gene expression could moreover elucidate how the increased colonization upon missing AHL synthesis was produced. All analyses should here consider that bacterial and AHL effects eventually become visible only at certain time points. Time series comparisons, with a longer experimental span up to several months (Han *et al.* 2016), could hence be appropriate. In addition, this thesis opens up further questions on the context-dependency of AHL effects. Extensive multifactorial study designs, from phytochambers to the field, could elucidate the reliability of AHL effects across various model plants, rhizobacteria, insect herbivores and environmental conditions. Since microbial AHL is unlikely the only important signal in interkingdom communication (Rodriguez *et al.* 2019), it would be of great benefit to investigate the totality of factors involved in bacterially-mediated biocontrol. This understanding could be an important step forward to successfully combat pest insects in more sustainable agriculture.

Material and methods

6.1 Bacterial strains and cultivation

All strains used for experiments in this study are listed in Table 6.1. Unless further specified, they were cultivated in nutrient broth medium (NB) consisting of 5 g Meat peptone, 3 g Meat extract, 1 L MilliQ, pH 7.0 (No. 4, Fluka, Steinheim, Germany) with respective antibiotics. All microorganisms were re-cultivated on NB agar plates every 3-4 weeks and kept at 4 ºC.

Table 6.1 Bacterial strains for plant inoculation experiments used in this study.

Bacterial strain	**Abbre-viation**	**GFP labelling**	**AHL signalling**	**Resistances**	**Reference**
Acidovorax radicis N35e	ArN35e	-	wildtype fully functional	-	Klein 2003
Acidovorax radicis N35e GFP	ArN35e	chromosomal	fully functional	Kan^R 50 µg/mL Amp^R 100 µg/mL	Li, 2011
Acidovorax radicis N35e *araI::tet*	ArI_{neg}	-	no AHL production	Tc^R 20 µg/mL	Li, 2011
Acidovorax radicis N35e *araI::tet* GFP	ArI_{neg}	vectorial	no AHL production	Kan^R 50 µg/mL Tc^R 20 µg/mL	Han, 2017
Acidovorax radicis N35e *araR::gen* GFP	ArR_{neg}	chromosomal	no AHL sensing	Kan^R 50 µg/mL Amp^R 100 µg/mL Gm^R 20 µg/mL	This study
Acidovorax radicis N35e *araI::tet, araR::gen* GFP	$ArR_{neg}I_{neg}$	vectorial	no AHL production, no AHL sensing	Kan^R 50 µg/mL Tc^R 20 µg/mL Gm^R 20 µg/mL	This study
Rhizobium radiobacter F4	RrF4	-	wildtype fully functional	-	Sharma *et al.* 2008
Rhizobium radiobacter F4 GFP	RrF4	vectorial	fully functional	$Spec^R$ 100 µg/mL	Glaeser *et al.* 2016
Rhizobium radiobacter F4 NM13	RrI_{neg}	-	AHL degraded by lactonase	$Tell^R$ 100 µg/mL	Li, 2011
Rhizobium radiobacter F4 NM13 GFP	RrI_{neg}	vectorial	AHL degraded by lactonase	$Tell^R$ 100 µg/mL $Spec^R$ 100 µg/mL	Glaeser *et al.* 2016
Agrobacterium tumefaciens A136	-	-	biosensor strain via *lacZ* fusion	Tc^R 5 µg/mL	Stickler *et al.* 1998

Before plant inoculation experiments, all strains were cultivated from the original glycerol stock. For preparation of glycerol stocks, bacteria were grown in 5 mL liquid NB medium over

night. Flocculations were resuspended, concentrated from several liquid cultures if needed and washed twice in 1x PBS. Finally, the bacteria were resuspended in 1 mL NB medium mixed with 1 mL glycerol. Cryostocks were stored at -80 ºC.

6.1.1 Cultivation and specifications of *Acidovorax radicis* strains

The endophytic rhizobacterium *Acidovorax radicis* N35e was isolated from surface sterilized wheat (Klein 2003). It forms rough colonies on plate surface and dense flocculations in liquid medium. *Acidovorax radicis* N35e has the ability to irreversibly switch from a rough colony form to a smooth swarming form (*A. radicis* N35v, described by Li 2011). To avoid this switching, all *A. radicis* strains were cultivated on NB agar plates containing the respective antibiotics at 30 °C for 2-4 days. Liquid cultures, in which the switching occurs to a higher proportion, were used only for strain confirmation and cryostock generation. Herefore, the strains were loop inoculated in 5 mL liquid NB medium containing the respective antibiotics and shaken by 180 rpm at 30 °C over night. In this study, only the rough form was used for experiments because of its better colonization properties (Li *et al.* 2011). For microscopic detection, ArN35e was chromosomally GFP-labelled by Li (2011) using pJBA28 as carrier plasmid for mini-Tn5-Km-PA1/04/03-RBSII-gfpmut3*-T^0-T^1 (Anderson *et al.* 1998).
The *A. radicis araI* knock-out mutant ArI_{neg} was generated previously by a tetracycline resistance gene into the *araI* gene responsible for AHL production by a homologuous recombination technique (Han 2017). The ArI_{neg} mutant was no longer able to produce OH-C10-HSL but still had its AHL sensing capacity. Afterwards, the strain ArI_{neg} was GFP-labelled via the plasmid pBBR1MCS-2-GFPmut3* by Han (2017).
Both GFP-labeled strains were used as basis for the generation of *araR* knock-out mutants so that the new strain ArR_{neg} carried the same chromosomal GFP-label as ArN35e while $ArR_{neg}I_{neg}$ was labelled via the aforementioned plasmid like ArI_{neg}.

6.1.2 Cultivation and specifications of *Rhizobium radiobacter* strains

Rhizobium radiobacter F4 was isolated from its host fungus *Piriformospora indica* DSM 11827 (Sharma *et al.* 2008). Prior to this work, the *R. radiobacter* AHL-negative mutant was

constructed by vectorially integrating an *aiiA* lactonase gene into the cell which degrades the entire produced AHLs (Li 2011). This was achieved by conjugational transfer with an *E. coli* donor strain carrying the plasmids pMLBAD-aiiA and pRK600, as described in detail in Li (2011). Lactonase activity is induced by 0.2 % arabinose.
Rhizobium radiobacter F4 and the AHL-degrading mutant NM13 was GFP-labelled using plasmid pLH6000 by Glaeser *et al.* (2016).

Agrobacterium tumefaciens was cultivated in 5 mL NB liquid medium with the respective antibiotics at 28 °C at 180 rpm over night.

6.2 Gene replacement of the *araR* gene for AHL sensing

The bacterial mutant impaired in its AHL production (ArI_{neg}) was already generated and characterized previously to this work by knock-out mutagenesis of the *araI* gene in *A. radicis* (Li 2011). However, this strain is still able to sense AHLs via the *araR* endcoded AHL receptor and regulate its gene expression accordingly. To disentangle the effect of bacterial self-regulation from the AHL effect itself, *araR* gene knock-out mutagenesis was performed by homologuous gene transfer as described below.

6.2.1 Plasmid construction

The plasmid construction procedure contained several steps. The idea was 1) to integrate the *araR* gene into an amplification vector, 2) to disrupt *araR* by inserting the gentamicin resistance gene so that *araR* becomes unfunctional, 3) to excize *araR::gen* and clone it into the final gene replacement vector, 4) to transform *A. radicis* cells with this vector where homologuous recombination is taking place and 5) to excise unnecessary vector residues by counterselection. Another cloning procedure (amplifying only the gentamicin gene with *araR*-overhangs on the primers) was tried but not further followed since ligation was not successful. The sentitivity of *A. radicis* to gentamicin was verified on plates prior to plasmid contruction since this is a crucial prerequisite for the later selection procedure. All cloning steps were carefully planned

in silico beforehand with the software Benchling (2021, https://benchling.com). Plasmids and primers used for mutant construction are listed in Table 6.2. The cloning procedure is schematically depicted in Figure 6.1 and described in the following.

Table 6.2 Plasmids and primers used for *araR* gene replacement.

Plasmid / Primer	**Sequence**	**Specification**	**Reference**
pEX18-Gm	backbone	Gene replacement vector with Gm^R	Hoang *et al.* 1998
pSCA-Amp/Kan	backbone	Cloning vector with Amp^R and Kan^R	Stratagene
pSCA-Amp/Kan-*araR*	backbone + *araR* gene	Cloning vector with Amp^R and Kan^R, AHL sensing gene integrated	This study
pSCA-Amp/Kan-*araR::gen*	backbone + *araR* gene disrupted by gentamicin resistance gene	Cloning vector with Amp^R and Kan^R, AHL sensing gene interrupted	This study
pEX18-Gm-*araR::gen*	backbone + *araR* gene disrupted by gentamicin resistance gene	Gene replacement vector with Gm^R, AHL sensing gene interrupted	This study
araR_EcoRI_F	5'-GC**GAATTC**ATGGCG CGTAATTCGCG-3'	amplification of *araR*, overhang adding **EcoRI**	This study
araR_EcoRI_R	5'-GC**GAATTC**TCAGCCCAG CAACCCC-3'	amplification of *araR*, overhang adding **EcoRI**	This study
gen_BbsI_F	5'-GCGAAGACGC**ACGA**TT GACATAAGCCTGTTCGG TTCG-3'	amplification of gentamicin resistance gene (Gm^R) including promotor sequence, overhang adding **BbsI** cutting and binding site	This study
gen_BbsI_R	5'-GC**TCGT**GCGTCTTCTTAG GTGGCGGTACTTGGGTC-3'	amplification of gentamicin resistance gene (Gm^R) including promotor sequence, overhang adding **BbsI** cutting and binding site	This study
sacB_F	5'-TCAGCAGGAAGCTAGG CG-3'	amplification of *sacB* for mutant verification	This study
sacB_R	5'-CTGACGGCACTGTCGC-3'	amplification of *sacB* for mutant verification	This study

1) Integration of *araR* into an amplification vector

The complete coding sequence of the *araR* gene in *A. radicis* has been identified previously by Li (2011) and is provided in the supplement (Figure S18). Sequence identity has been re-checked by BLAST search against common *luxR* homologues. The *araR* gene was amplified from pure genomic DNA extracted from *A. radicis* N35e with the primers araR_EcoRI_F and

araR_EcoRI_R containing an EcoRI overhang (Table 6.2). A KOD PCR system with high proofreading quality was used for gene amplification (see 6.10). Amplified fragments were visualized on a 1 % agarose gel and bands from the right size (777 bp) were excized and purified by a NucleoSpin Gel and PCR Clean-up Kit (Macherey-Nagel, Düren, Germany). To introduce the *araR* amplicon into the pSC-A-Amp/Kan expression vector, a poly-A tail needed to be added to the *araR* fragments what was achieved by an additonal DreamTaq PCR reaction (see 6.10) that naturally leads to sticky single-strand overhangs. Afterwards, the *araR* fragment was ligated to pSCA-Amp/Kan which is provided as an open vector with sticky overhangs by the StrataClone PCR Cloning Kit (Agilent Technologies, Frankfurt, Germany) what simplifies fragment integration by topoisomerase I and Cre recombinase and does not require enzymatic treatment. For plasmid multiplication, *E. coli* cells from the StrataClone SoloPack Competent Cells (Agilent Technologies, Frankfurt, Germany) were transformed with pSCA-Amp/Kan-*araR* (for further information on transformation see 6.2.8).

2) Disruption of *araR* by gentamicin insertion

The gentamicin resistance gene (*gen*) including its promotor sequence were amplified from the vector plasmid pEX18-Gm, extracted with the NucleoSpin Plasmid MiniPrep Kit (Macherey-Nagel, Düren, Germany). The primers (Table 6.2) comprised BbSI overhangs. For optimal quality, the Gm^R gene fragment was amplified in a KOD PCR reaction as specified in 6.10. The Gm^R gene fragment (*gen*, 769 bp) was visualized and extracted from a 1 % agarose gel like described above. The sequence of the gentamicin resistance gene including promotors is provided in the supplement (Figure S19).
In order to disrupt the *araR* gene on the pSCA-Amp/Kan-*araR* vector, the vector as well as the Gm^R gene (*gen*) were cut with the restriction enzyme BbsI since *araR* naturally contains a unique BbSI restriction site in the middle of its sequence. For digestion and clean-up procedure see 6.2.3 and 6.2.4. Fragments and linearized vector were ligated and introduced into *E. coli* StrataClone competent cells as described in 6.2.5. Transformants containing the new plasmid pSCA-Amp/Kan-*araR::gen* were selected on gentamicin (20 µg/mL) containing LB plates.

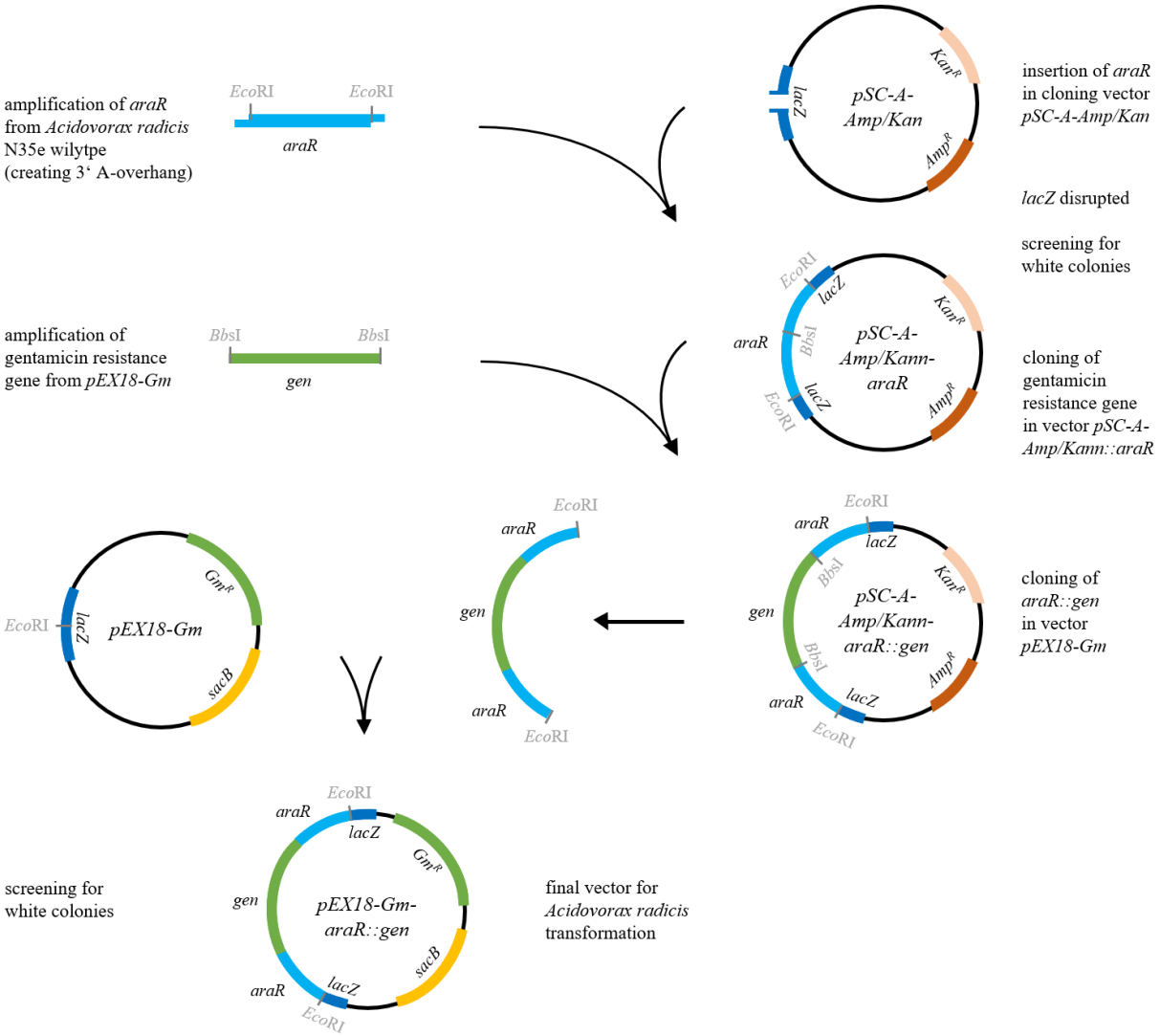

Figure 6.1 Schematic overview of *araR* gene replacement vector construction.
From *Acidovorad radicis* N35e the *araR* gene was amplified by introducing EcoRI cutting sites. It was ligated with the backbone of the pSC-A-Amp/Kan plasmid. The vector pSC-A-Amp/Kan-*araR* was cut by the naturally occuring BbsI cutting site. The gentamicin resistance gene (*gen*) including promoter sequences was amplified by introding a BbsI cutting site and combined with the digested plasmid for interrupting the *araR* gene. The insert *araR::gen* was digested again with EcoRI and ligated to the backbone of pEX18-Gm. In all cloning steps, plasmid amplification was achieved in chemically competent *E. coli* StrataClone cells while the final gene replacement vector was introduced into electro-competent *A. radicis* cells from ArN35e and ArI_{neg}. Counterselection (see below) led to ArR_{neg} and $ArR_{neg}I_{neg}$ mutants.

3) Integration of *araR::gen* into the gene replacement vector

In order to construct the final gene replacement vector, the newly constructed plasmid pSCA-Amp/Kan-*araR::gen* was extracted as mentioned previously and digested with EcoRI (see 6.2.4). This restriction site was introduced in the first step as primer overhangs during *araR* amplification and excises the entire *araR::gen* sequence. Similarly, the gene replacement vector pEX18-Gm was digested with EcoRI (see 6.2.3). The linearized vector and the digested fragment were ligated as described in 6.2.5. Finally, *E. coli* StrataClone competent cells were transformed with the final gene replacement vector for amplification. The vector pEX18-Gm-*araR::gen* contains two Gm^R genes so that selection by plating on gentamicin containing medium is not sufficient since also transformants carrying empty vectors show gentamicin resistance. Therefore, mutants with the correct plasmid could only be identified by blue-white screening.

4) Homologuous recombination in *Acidovorax radicis*

The constructed gene replacement vector pEX18-Gm-*araR::gen* was extracted as described above. Electrocompetent *A. radicis* cells were transformed with this plasmid as described below (6.2.6). Within *A. radicis* cells, one part of the *araR* sequence is supposed to naturally align to the chromosomal *araR* version. With a certain frequency, this alignment is followed by a homologuous recombination process which integrates the whole plasmid into the bacterial chromosome as depicted in Figure 6.2. The *araR* gene is here disrupted by *araR::gen* and other vectorial elements. Mutants carrying the recombinant *araR* version were selected on NB agar plates containing gentamicin (20 µg/mL).

5) Counterselection

In order to eliminate the remaining parts of the vector from the bacterial chromosome, mutants carrying the recombinant *araR* version were plated on NB plates containing 5 % sucrose. The *sacB* gene, that was also integrated into the bacterial genome (compare to Figure 6.2), encodes for the precursor gene of levansucrase that turns over sucrose into levans which is deadly for most Gram-negative bacteria (Gay *et al.* 1985). In the presence of sucrose, these mutants

frequently excise the unwanted *sacB* and additional genetic elements in its proximity from their genome what leads to the desired *araR* knock-out genotype.

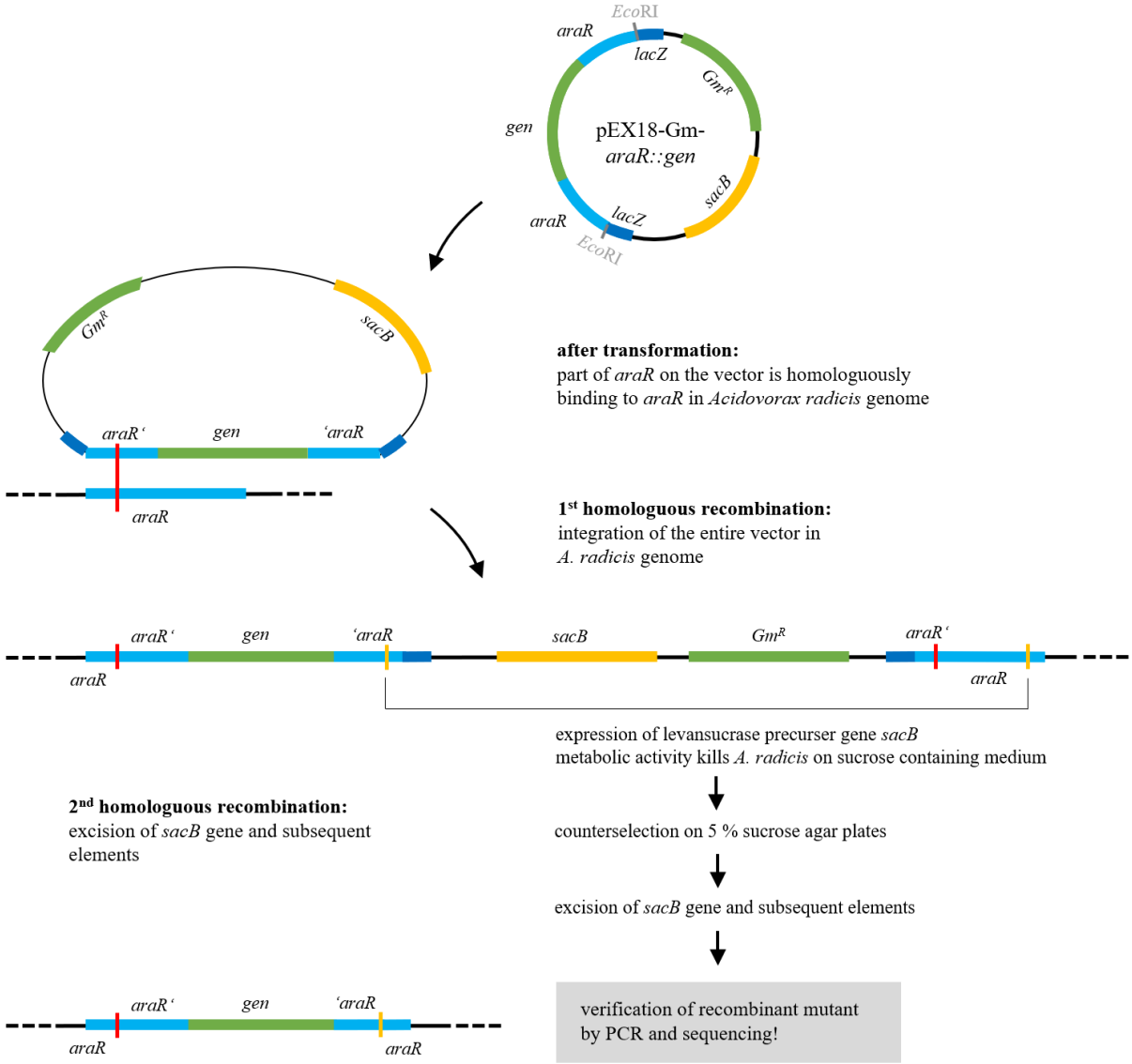

Figure 6.2 Schematic overview of homologuous recombination for *araR* knock-out in *Acidovorax radicis* genome.
Cells of the AHL-producing strain *Acidovorax radicis* N35e (ArN35e) and its AHL signalling mutant (ArI_{neg}) were transformed with the final replacement vector containing the *araR* gene interrupted by a gentamicin resistance cassette (*gen*). Homologuous recombination of *araR::gen* with the chromosomal *araR* gene from *A. radicis* leads to complete plasmid integration. Subsequently, unwanted vector parts were eliminated by counterselecting for *sacB*. Recombinant mutants with successfully integrated knock-out were verified. For more details, see description in the text and sequences in the supplement.

6.2.2 Competent cells

Chemically competent *E. coli* cells were purchased from the StrataClone SoloPack Competent Cell Kit (Agilent Technologies, Frankfurt, Germany) and directly used (see transformation protocol in 6.2.6). For the final gene replacement vector integration into *A. radicis* cells, electrocompetent cells were generated following a modified protocol from Hanahan (1983). Liquid cultures were grown over night in several 50 mL Erlenmeyer flasks at 30 °C, moderately shaking at 100 rpm, to ensure optimal growing of the rough cell type. Cultures were cooled on ice for 15 min. Flocculations from all flasks were concentrated by centrifugation at 5000 rpm at 4 °C for 5 min. The pellet was washed 1x in ice-cooled sterile MilliQ and 1x in ice-cooled and sterile-filtered 10 % Glycerol. Finally, the pellet was resuspended in 1 mL 10 % Glycerol. Aliquots of 100 µL were immediately shock-frozen in liquid nitrogen and stored at -80 °C.

6.2.3 Digestion of vectors

For vector amplification, transformed *E. coli* cells were cultured over night in liquid LB medium at 37 °C shaking at 180 rpm. Vectors were extracted using the NucleoSpin Plasmid MiniPrep Kit (Macherey-Nagel, Düren, Germany). Plasmid DNA was eluted in NFW for minimal ion concentration improving further cloning steps.

For digesting vectors, 5 µL 10x Cut-Smart buffer, 1.5 µL plasmid DNA (1 µg/µL) and 2 µL enzyme were mixed and filled up with NFW to a final volume of 50 µL. The reaction was incubated at 37 °C for 1 h and inactivated at 65 °C for 20 min in a thermocycler or heat block. The digested vectors were loaded on a 0.8 % agarose gel with big combs. Linearized vectors were cut out and extracted with a NucleoSpin Gel and PCR Clean-up Kit (Macherey-Nagel, Düren, Germany) including two washing steps and elution in 20 µL NFW. To prevent the linearized vector from closing, a phosphatase treatment step was performed afterwards. Per 17 µL of vector (30 ng/µL), 1 µL FastAP phosphatase enzyme and 2 µL 1x FastAP buffer were added while keeping the reaction on a heat block. The mixture was incubated at 37 °C for 20 min, mixed every 2 min by inverting twice and inactivated at 75 °C for 7 min. The linearized fragment was stored at -20 °C.

6.2.4 Digestion of PCR fragments

For digesting PCR fragments, 2.85 µL 10x Cut-Smart buffer, 0.25 µL enzyme and 23 µL cleaned PCR product (concentration as high as possible) were mixed. The reaction was incubated at 37 °C for 1 h and inactivated at 65 °C for 20 min in a thermocycler or heat block. Digested PCR fragments were subsequently cleaned-up with the MagSi-NGSPrep Plus Kit (Magtivio, The Netherlands). PCR reactions and magnetic beads were mixed in a ratio of 1:0.7 and washed once with 70 % EtOH. Digested and cleaned fragments were eluted in 30 µL NFW and stored at -20 °C.

6.2.5 Ligation

For ligation, 15.8 µL clean digested PCR fragment (concentration as high as possible) was added to 2 µL linearized vector (25 ng/µL) together with 0.2 µL T4 Ligase and 2 µL 10x Ligase buffer. The ligation mix was incubated for 2 h at 22 °C and 8 h at 12 °C in a thermocycler. Subsequently, the enzyme was deactivated for 10 min at 65 °C. The ligation mix was used immediately or stored at -20 °C for several trials.

6.2.6 Transformation of chemocompetent *Escherichia coli* cells

For one transformation process, two aliquots of *E. coli* StrataClone cells were thawed on ice. To each tube, 2 µL of ligation mix was added and incubated on ice for 20 min. Afterwards, the transformation mix was heat-shocked at 42 °C for 45 sec, incubated on ice for 2 min and recovered with 250 µL pre-warmed LB medium. Transformed cells were recovered for 1-2 h at 37 °C horizontally shaking at 180 rpm. Respectively, 100 µL mix were plated out on LB plates containing necessary antibiotics and 40 µL X-Gal (from 20 mg/mL stock solution) for subsequent blue-white screening. Plates were incubation over night at 37 °C. Occasionally, additional reactions were carried along for no-plasmid-control and positive control with the enclosed pUC18 control vector from the StrataClone PCR Kit.

6.2.7 Blue-white screening

Only *E. coli* cells bearing the vector with antibiotic resistances were expected to grow on the respective agar plates. Additionally, when inserts were successfully integrated into the cloning vectors, the *lacZ* reporter gene was disrupted leading to a white colony phenotype instead of a blue colouration indicating an empty vector. The *lacZ* gene encodes for a beta-galactosidase that turns over 5-Bromo-4-chloro-3-indoxyl (X-Gal) so that indoxyl is oxidized to blue indigo as a final product (Burn 2012). After 24 h incubation, 5-10 white colonies were picked. Inoculated liquid cultures were cultivated at 37 °C over night. From promising mutant candidates, plasmids were extracted and sequenced for correct plasmid integration (see 6.2.12). Cryostocks were generated from all mutant candidates as backup.

6.2.8 Transformation of electrocompetent *Acidovorax radicis* cells

Acidovorax radicis cells were transformed by an electroporation protocol after Dower *et al.* (1988). In this method, an external electrical field locally preforates the bacterial cell wall so that plasmid DNA can be transferred into the cell.
Electrocompetent cells were thawed on ice and approx. 500 ng plasmid was added. The reaction was gently mixed and chilled on ice for 30 min. Shortly before electroporation, 1 mL NB medium was prepared in a pipet tip. The transformation mix was carefully added between the metal plates of a cooled but dry 2 mm cuvette (Peqlab, Erlangen, Germany). The electrical shock was accomplished with a Gene Pulser (Bio-Rad, Munich, Germany) at a voltage of 2.5 kV pulsing for 4.5 – 5.5 msec and a resistance of Ω = 100. Afterwards, the cells were recovered in 1 mL NB medium as quick as possible and genty shaken at 30 °C for 1 h. At last, the cells were plated on NB agar plates with the usual antibiotics and 20 ng/µL gentamicin.

6.2.9 *Acidovorax radicis* mutant screening

Since *A. radicis* was tested sensitive to gentamicin, only cells with integrated plasmid (either with insert or empty vector because of endogenous gentamicin resistance of pEX18-Gm) should grow on NB agar plates with gentamicin. Additionally, the suicide vector pEX18-Gm

contains an origin of replication that is only recognized by *E. coli* and thus should not replicate in *A. radicis* outside the genome. Therefore, all growing mutant colonies should bear the correct plasmid integrated into the bacterial genome by homologuous recombination of *araR::gen* with the orginial *araR* gene from *A. radicis*. Naturally, only few growing colonies were expected due to low efficiency of the described transformation approach.
Mutant candidates were picked and streaked out on new NB plates. From subsequent liquid cultures, plasmids were extracted and sequenced for correct plasmid integration into the genome (see 6.2.12).

6.2.10 Counterselection

In the prevailing mutant candidates, the gene replacement vector should have integrated entirely into the *A. radicis* genome by homologuous recombination (compare Figure 6.2). To eliminate unwanted vectorial elements in a final step, counterselection was performed by streaking out the respective mutant cells (resuspended culture with several dilution steps) on NB agar plates containing 5 % sucrose. The *sacB* gene encodes for a levansucrase precurser gene that degrades sucrose into levans which is deadly for most Gram-negative bacteria (Gay *et al.* 1985). The excision of *sacB* and genetic elements in the proximity should result in survival of the cell and the desired clean *araR::gen* knock-out sequence. A high proportion of cells was expected to accomplish the gene excision. Counterselected mutant candidates were picked and streaked out on new NB plates containing sucrose. From liquid cultures, plasmids were extracted as usual, used as PCR templates for mutant verification (6.2.11) and sequenced for the correctly knocked-out *araR* gene (see 6.2.12).

6.2.11 Mutant verification by PCR

Whether the desired gene knock-out was achieved, can be quickly evaluated by comparing the gene size of the original *araR* (777 bp) with the interrupted version *araR::gen* (1546 bp). In correct mutants, no *sacB* gene (1071 bp) should be present after successful counterselection. As positive control, genomic DNA was extracted from ArN35e and ArI_{neg} with the original *araR* gene and carried along. The pEX18-Gm-*araR::gen* gene replacement vector was used as

additional control where the longer gene version should be prevelant while the *sacB* gene is still detectable.

Plasmid DNA from promising mutant candidates (10 ng) was added to DreamTaq PCR reactions in triplicates targeting 1) the *araR* gene and b) the *sacB* gene with the primers listed in Table 6.2. The standard DreamTaq protocol (see 6.10) was used with an annealing temperature Tm = 56 °C, 35 cycles with elongation of 1 min 20 sec. Bands sizes were visualized on a 1 % agarose gel like described previously. The verification PCR was repeated at least two times.

6.2.12 Mutant verification by sequencing

Mutants candidates with correct PCR verification result were analyzed by Sanger sequencing to confirm the introduced knock-out additionally. From agarose gels, the respective *araR::gen* bands were cut and purified with the NucleoSpin Gel and PCR Clean-up Kit (Macherey-Nagel, Düren, Germany). Amplificate samples (20 ng/µL) were pre-mixed with the forward primer araR_EcoRI_F and further processed by the Eurofins Genomics Sequencing Service (Ebersberg, Germany). Sequencing results were aligned with the original and designed *araR* sequences in the software Benchling.

Verified strains were studied in detail about their growth behaviour, AHL production, swarming and switching ability and biofilm formation (see below) before they were finally used in plant experiments. For final evalution, the respective mutant strains should have been additionally verified by a phenotypic rescue with the original *araR* gene introduced on an expression vector. Due to time limitation, this essential complemetation step was skipped and still needs to be performed for secure validation.

6.3 Characterization of *Acidovorax radicis* mutant strains

6.3.1 AHL production

AHL production of *A. radicis* N35e *araI* and *araR* mutants was tested with a plate cross-streak assay against the AHL biosensor strain *Agrobacterium tumefaciens* A136 bearing *traR*- and *traI-lacZ* fusion genes (Stickler *et al.* 1998). The biosensor strain was streaked veritically on NB agar plates containing 40 µg/mL X-gal following a protocol of Ravn *et al.* (2001). The bacterial strains under investiagtion were cross-streaked horizontally close to the biosensor line. The plates were incubated at 30 °C for 24-48 hours. AHL production was considered as positive as soon as a blue colouration emerged at the point of closest contact between the *A. tumefaciens* biosensor and the *A. radicis* strains.

For testing the AHL production of *A. radicis* mutant strains when complemented with synthetic AHLs, a similar drop assay format was developed. Bacterial liquid cultures from the strains under investigation and the AHL biosensor strain *A. tumefaciens* A136 were established over night (see 6.1.1 and 6.1.2). Also, fresh material from plates were tested leading to similar results. Bacterial pellets were resuspended in fresh NB medium and adjusted approx. to an OD_{600} = 0.2. Additional NB medium was prepared containing X-Gal for a final concentration of 40 µg/mL. Synthetic AHL was stepwise diluted in NFW from stock solutions (e.g., 3685 g/mol OH-C10-HSL) for 100 µM and 10 µM. In separate tubes, test bacteria, biosensor strain, NB medium with X-Gal and synthetic AHLs (or blank NB medium) were mixed in the ratio 1:1:1:1. This way, the content should be comparable in all reactions with and without the single components. A final AHL concentrations of 5 µM was chosen. Droplets of 10 µL were pipetted on NB plates in triplicates, air-dryed carefully and transferred to 30 °C for 24-48 h. AHL production indicated by the intensity of blue colouration can be compared between droplets containing only the bacterial mutant strains, only synthetic AHLs or both combined. Negative controls containing *A. tumefaciens* A136 only should give white droplets.

6.3.2 Swarming

Bacterial swarming was examined according to Caiazza *et al.* (2005) with modifications. Overnight liquid cultures of *A. radicis* strains were washed in 1x PBS, resuspended in fresh

NB medium and diluted to approx. OD_{600} = 0.1. Each bacterial culture was pipetted in 10 µL droplets in the center of semi-solid agar plates (0.5 % (w/v) agar) containing the respective antibiotics (see Table 6.1). The plates were air-dryed, incubated to 30 °C and the halo formation examined by vizual documentation after 24 h and 48 h. As positive control, *Pseudomonas sp.* SCA7 (Kuhl-Nagel *et al.* 2022) was cultivated accordingly on NB semisolid agar without antibiotics. The swarming assay was performed in duplicates and repeated three times independently. A streak-out format was tried in addition leading to the same motility pattern.

6.3.3 Biofilm formation

Biofilm formation of *A. radicis* strains was tested in a crystal violet microtiter format following a protocol of O'Toole (2011). Bacterial cultures were grown over night (conditions see Table 6.1). Flocks were 1x washed, resuspended in fresh NB medium and the OD_{600} adjusted to approx. 0.1. In eight technical replicates, 100 µL of the bacterial cultures were transfered to the outer wells of a translucent flat-bottom 96-well plate (Greiner) and pregrown without shaking at 30 °C. Although minimal medium is recommended at this point, only NB medium could assure normal growth of *A. radicis* strains. After 24 h, cell growth was controlled by measuring the OD_{600} with a plate reader (SpectraMax iD3, Molecular Devices). Medium and unattached cells were dumped out of the plate on a paper towel. The plate was carefully washed by submerging it twice in a MilliQ bath. Subsequently, 125 µL of 0.1 % crystal violet (Roth, Germany) solution was added to each well. After 15 min, the plate was rinsed three times with MilliQ water and dried over night before visual documentation. Biofilms should become visible as violet ring-shaped matrix encircling the wells. For better vizualisation, biofilms were solubilized with 125 µL of 30 % acetic acid (Roth, Germany) subsequently. Biofilm-forming *Pseudomonas simiae* WCS417 served as positive control while *Escherichia coli* DH5α should not produce biofilms under these conditions and served as negative control. The experiment was repeated four times.

6.4 Plant experiments

All experiments were conducted in the Munich Model EcoSystem Analyser research facility TUMmesa (Weihenstephan, Germany) or in phytochambers (Weiss Technik, Modell SGC120PG2, Germany) at the HMGU (Neuherberg, Germany). All soil experiments are listed in Table 6.3. All axenic experiments are listed in Table 6.4.

Table 6.3 Overview of soil experiments.

	Main focus	**Location**	**Bacteria**	**GFP**	**Barley cultivar**	**Aphids**	**Sampling**	**Analysis**
SE1	test strain effect on aphids	TUM-mesa	ArN35e RrF4	no	Barke Chevallier Scarlett	Sickte Fescue	day 21	microbiome
SE2	AHL effect on aphids	TUM-mesa	ArN35e ArI_{neg}	yes	Barke Grace Scarlett	Fescue	day 21	microbiome plant gene expression
SE3	AHL effect on aphids	phyto-chamber	ArN35e ArI_{neg} RrF4 RrI_{neg}	both	Barke	Sickte	day 21	
SE4	inoculation doses	phyto-chamber	RrF4	yes	Barke	Sickte	day 21	
SE5	colonization over time	phyto-chamber	ArN35e ArI_{neg}	yes	Scarlett	no	day 4, 7, 10, 14, 18, 21	diagn. PCR
SE6	colonization over time	phyto-chamber	ArN35e ArI_{neg}	yes	Scarlett	no	day 4, 7, 10, 14, 21	

Tabel 6.4 Overview of axenic experiments.

	Main focus	**Bacteria**	**AHLs (10 µM)**	**Aphids**	**Sampling**	**Analysis**
AE1	AHL effect on aphids	ArN35e ArI_{neg}	OH-C10-HSL C6-HSL	Sickte (offspring removed)	day 21	biosensor
AE2	AHL effect on aphids	ArN35e ArI_{neg}	OH-C10-HSL C6-HSL	Sickte (offspring removed)	day 21	biosensor ELISA plant gene expression
AE3	AHL effect on aphids	ArN35e ArI_{neg} ArR_{neg} $ArR_{neg}I_{neg}$	OH-C10-HSL	Sickte (offspring removed)	day 21	CFU count diagn. PCR

AE4	AHL effect on aphids	ArN35e ArI_{neg} ArR_{neg} $ArR_{neg}I_{neg}$	OH-C10-HSL	Sickte (offspring removed)	day 7, 14, 21	CFU count diagn. PCR
AE5	colonization over time	ArN35e ArI_{neg} ArR_{neg} $ArR_{neg}I_{neg}$	no AHLs	no aphids	day 4, 7, 14, 21	CFU count
AE6	colonization over time	ArN35e ArI_{neg} ArR_{neg} $ArR_{neg}I_{neg}$	no AHLs	no aphids	day 4, 7, 10	CLSM screen

6.4.1 Sterilization of barley seeds

Seeds of barley (*Hordeum vulgare* L.) cultivars Barke, Chevallier and Scarlett were purchased from Saatzucht Breun GmbH and barley cultivar Grace from Ackermann Saatzucht GmbH and stored at 4 °C in paper bags or falcon tubes until use.

For axenic experiments, where sterility is crucial, max. 250 seeds were handled at the same time (in 50 mL falcon tubes, filled up to 15 mL) and washing steps were facilitated using a conventional metallic sieve. In SE1, SE2 and SE7, seeds were surface sterilized in 12 % NaOCl (Roth, Germany) for 20 min and germinated on wet paper towels for five days in the dark. In all other experiments, seeds were shortly washed in MilliQ, shaked in 1 % Tween80 (Sigma, USA) for 2 min and incubated in 70 % EtOH for 5 min. Afterwards, they were incubated in 4 % NaOCl for 20 min while slowly shaking at 120 rpm. The bleach was removed entirely by washing the seeds at least five times in MilliQ. Subsequently, they were left in 0.6 mg/mL penicillin and 0.25 mg/mL streptomycin for 30 min. The antibiotics were not removed. Up to 12 seeds were placed on one NB agar plate with the germination furrow facing down and covered each with two droplets of liquid NB medium to increase humidity. Seeds were germinated for three days at room temperature in the dark.

6.4.2 Preparation of bacterial inocula

For preparing inoculation solutions, *A. radicis* strains were pregrown on NB agar plates as described in chapter 6.1. The bacterial lane was washed off and resuspended in 10 mM $MgCl_2$

solution until an optical density of approx. OD_{600} = 1.0 (Beckman Coulter DU 720 Spectophotometer), corresponding to $1x10^8$ cells, was reached. For better mixing, bacteria were homogenized by pipetting up and down during the resuspending process and 1 % Tween20 was added.

For *R. radiobacter* inoculation, liquid NB overnight cultures (see 6.1) were washed twice in 10 mM $MgCl_2$ by centrifugation at 5.000 g for 2 min. The optical density was adjusted to OD_{600} = 1. Only in soil experiment SE4, where several inoculation doses were tested, the inoculate was diluted to OD_{600} = 1.5 ($1x10^7$ cells/mL), OD_{600} = 0.6 ($5x10^6$ cells/mL) and OD_{600} = 0.15 ($1x10^6$ cells/mL).

6.4.3 Inoculation procedure

After three days of germination, uncontaminated seedlings were selected, dip-washed twice in sterile MilliQ to wash of remaining antibiotics and transferred to petri dishes. Subsequently, they were covered with 40 mL of the respective bacterial solution for 1 h without moving. $MgCl_2$ background solution served as control. To keep the inoculation times comparable, several inoculation steps were performed handling only 10-20 seedlings at the same time.

6.4.4 Aphid propagation

English grain aphids (*Sitobion avenae*, genotype Fescue and Sickte) were reared under phytochamber conditions on barley plants cv. Kym or Chanson in soil. Population density was kept low by infecting new plants every three weeks. For experiments, five (in soil experiments) or two (in axenic experiments) 4^{th} instar adults were selected and transferred to the plant manually with a paintbrush. Between treatments, paintbrush was dip-sterilized first in 70 % EtOH and second in sterile MilliQ water to facilitate aphid attachment and to prevent bacterial transfer between plants.

6.4.5 Synthetic AHL addition

Under sterile conditions, pure AHL molecules should persist longer compared to soil environments. Therefore, their effect can be studied best in axenic experiments when added to the plant watering solution. Synthetic *N*-3-hydroxy-C10-homoserine lactone (OH-C10-HSL) and C6-homoserine lactone (C6-HSL) were purchased from Sigma-Aldrich (Darmstadt, Germany) in powder form and resolved in acetonitril for a concentration of 10 mg/mL. For plant treatments, working solutions of 100 µM OH-C10-HSL and C6-HSL in MilliQ were established, sterile filtered through a 2 nm membrane and added 1:10 to Hoagland's solution for plant watering in a final concentration of 10 µM.

6.4.6 Design and conditions of soil experiments

Soil experiments were conducted with low nutrient pot soil (Floradur multiplication substrate, Floragard). Inoculated barley seedlings were individually planted in squared plastic pots, kept in trays and watered only from below every three days with tap water. Trays always contained the same bacterial treament and were randomly redistributed between chambers/shelves 2-3 times in the course of the experiment. After five days, five aphids (see 6.4.4) and earthworms of a total biomass of 1.0-1.25 g (in soil experiments SE1 and SE2) were introduced. On top, pots were covered with air- and moisture-permeable cellophane bags, fixed with rubber bands, to prevent aphid escape. On the bottom, pots were covered with mesh, fixed with rubber bands, to prevent earthworm escape.

Soil experiments SE1 and SE2 were conducted in two temporal blocks in four climate chambers under the following conditions: 20 °C, 65 % relative humidity, with 10 h of full light (850 PAR), 8 h of total darkness and a 3 h sunrise/sunset gradient between during which the light was gradually increasing/decreasing. All other soil experiments were performed in one phytochamber at full light intensity, 23 °C, 55 % humidity, day-night-cycle of 12 h:12 h. All experiments were entirely randomized and blinded.

6.4.7 Design and conditions of axenic experiments

All axenic experiments were conducted in phytochambers and with barley cultivar Scarlett. Seedlings were sterilized (see 6.4.1), inoculated with bacteria (6.4.2 and 6.4.3) and planted into sterile glass tubes filled up to a height of 10 cm with sterile glass beads (approx. 75 g of beads) and 10 mL Hoagland's solution (Hoagland's No. 2 Basal Salt, Sigma-Aldrich, Germany) with or without synthetic AHL addition (see 6.4.5). Prior to use, glass beads were washed twice with VE water, rinsed twice with 70 % EtOH and air-dried over night. The use of glass beads as plant growth matrix should keep the adherence of AHLs minimal and still allows comparably good root development. The glass tube was clogged with a sterile cotton wool pad to prevent any contamination from aphids or handling. Additionally, a circle of sterile backing paper was placed on top of the cotton wool to keep aphids from entangling in the cotton wool. The whole glass tube was covered with an air- and moisture-permeable cellophane bag to prevent aphid escape. The bag was fixed with a rubber band and toothpick for convenient handling. Tubes were sorted after randomized numbers and placed in racks in a controlled phytochamber for 21 days (full light intensity, 23 °C, 55 % humidity, day-night-cycle of 12 h:12 h).
After 10 and 18 days, 5-10 mL plants were watered with Hoagland's solution (inkluding 10 µM of AHLs on day 10 as booster) up to a filling line of 9 cm. For this, the cotton wool was passed by with a long syringe needle adding the solution directly on top of the glass beads. The setup of the axenic system is depicted in Figure 6.3.

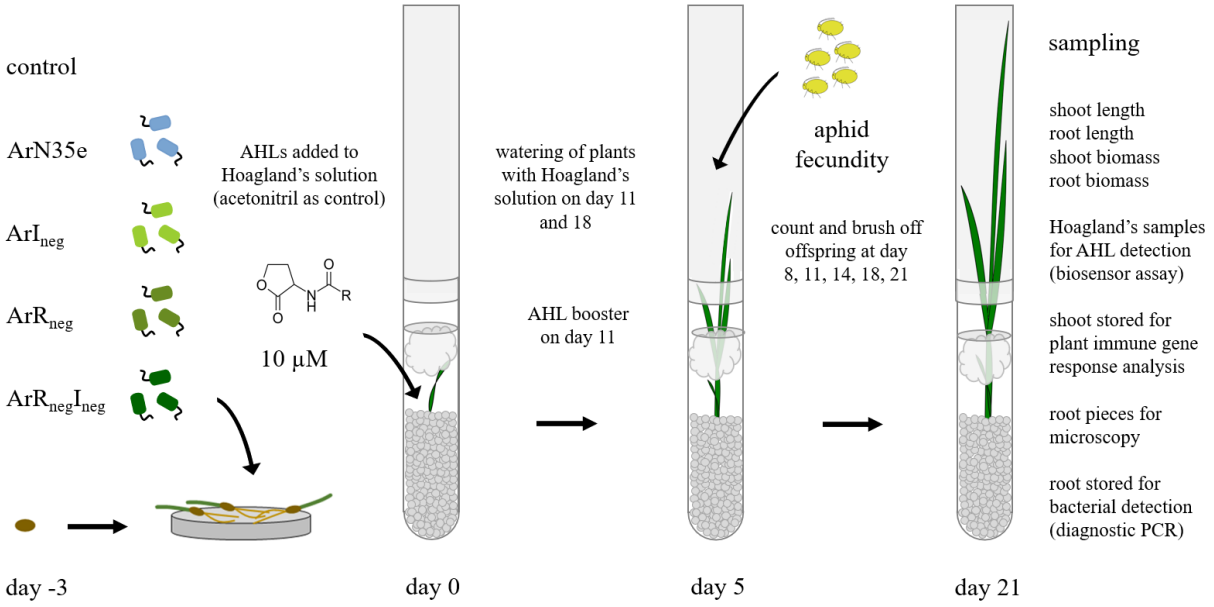

Figure 6.3 Setup of axenic experiments.
All axenic experiments were conducted in sterile glass tubes filled with glass beads (schematically depicted). Seeds of barley cv. Scarlett were sterilized and pregerminated for three days in the dark. Seedlings were inoculated for 1 h in the respective bacterial suspension in petri dishes. For the inoculation, the AHL-producing rhizobacterium *A. radicis* (ArN35e, depicted as blue cells), its AHL synthesis mutant (ArI_{neg}, light green), AHL sensing mutant (ArR_{neg}, middle green) and double mutant ($ArR_{neg}I_{neg}$, dark green) were resuspended in $MgCl_2$, control = $MgCl_2$ only. Synthetic OH-C10-HSL, C6-HSL (representative AHL molecule depicted) or acetonitril was added to the Hoagland's solution prior to watering. Cotton wool and backing paper circles were added to the tube to prevent contaminations. Glass tubes were covered with a moist- and air-permeable cellophane bag. For more information see decription in the text (6.4.7).

6.4.8 Shoot and root harvesting

Shoot length was measured from the base of the seed up the the tip of the longest leave. Shoot biomass was measured excluding the remaining seed. Chlorophyll content was recorded with a chlorophyll measurement device (Konica Minolta SPAD-502). Samples were immediately packed into aluminium foil, frozen in liquid nitrogen and stored at -80 °C. In axenic experiments, 50 mg of green shoot material was weighed in tubes prior to analysis and shock frozen as decribed.

The roots were recovered by reversing the container and shaking out the soil or beads carefully. Roots were washed in 1x PBS so that all beads and loosly attached soil was removed. The remaining soil on the roots should be equivalent to the "rhizosphere". Roots were placed on paper towels and air-dried for max. 10 min so that PBS content is not influencing the root biomass measurement. Root length was measured from the base of the root excluding the seed to the tip of the longest root. All samples were immediately packed into aluminium foil, frozen in liquid nitrogen and stored at -80 °C.

6.4.9 Root harvesting for bacterial detection by microscopy

For microscopic analysis, one 1 cm root pieces was cut from the proximal end of the root, ca. 1 cm below the base, and one distal piece, ca. 1 cm from the root tip. In SE1 and SE2, root pieces were fixed in 4 % PFA solution (preparation see below) over night at 4 °C and endfixed, after three washing steps in 1x PBS, in a 1:1 PBS:EtOH (v/v) solution and stored at -20 °C. In

all other experiments, where the bacteria are GFP-labelled and do not require further staining steps, root pieces were directly fixed in a 1:1 PBS:EtOH (v/v) solution and stored at -20 °C.

The PFA fixative was prepared manually by heating 45 mL MilliQ water to 65 °C and adding 2 g PFA powder. A few droplets of 10 M NaOH was added until the solution became clear. After adding 5 mL of 10x PBS, the solution was cooled down to room temperature and the pH was adjusted to pH = 4.2 – 4.6. The 4 % PFA solution was sterile filtered through a 0.2 µm Millipore filter and stored at -20 °C for maximum one week.

6.4.10 Aphid measurements

In soil experiments, the aphid population was counted after 21 days leave by leave with a click counter and removed from by wiping them off manually. In axenic experiments, offspring was counted and removed every third day carefully with a paintbrush. After 21 days, all aphids were counted and removed from the shoot.

6.5 Microbiome analysis by 16S Amplicon sequencing

For microbial profiling of soil experiments, biological triplicates were randomly chosen across bacterial, barley, aphid and earthworm treatments (n = 108 in SE1, n = 110 in SE2). Due to the high number of treatments, replicate numbers had to be kept at a minimum.

6.5.1 Library preparation

From frozen root samples with closely attached soil, 200-500 mg were set aside on dry ice. Genomic DNA was extracted with the FastDNA SPIN Kit for soil (MP Biomedicals GmbH, Germany). Precipitated proteins were removed in 2 mL microcentrifuge tubes instead of 15 mL falcons and DNA was eluted in 50 µL NE buffer. Final DNA concentrations were quantified with a NanoDrop One spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). Library preperation was performed according to the protocol of Thompson *et al.* (2017). The

V4-V5 region of the 16S rRNA gene was amplified with the NEBNext High Fidelity PCR Kit (New England Biolabs, Frankfurt, Germany) with the primers 515-F (5'-GTGYCAGCMGCC-GCGGTAA-3') and 806-R (5'-GGACTACNVGGGTW-TCTAAT-3') (Caporaso *et al.* 2011) tagged with the flowcell adaptor sequence (Metabion International AG, Germany). For each sample, the tagmentation PCR was performed in triplicates and pooled afterwards to eliminate amplification bias. PCR reactions included denaturation at 98 °C for 5 min, followed by 20 cycles at 98 °C for 10 sec, 55 °C for 30 sec, 72 °C for 30 sec and one final elongation step at 72 °C for 5 min. The amplified gene products (approx. 250 bp) were visualized on 1 % agarose gels.

Small gene fragments and primer dimers were removed from PCR reactions using the MagSi-NGSPrep Plus Kit (Magtivio, The Netherlands). PCR reactions and magnetic beads were mixed in a ratio of 1:0.8 for left side size selection. DNA was eluted in 20 µL NE buffer. Subsequently, the purity of DNA was analyzed by Cornelia Galonska at the Institute of Comparative Microbiome Analysis, HMGU, with the bioanalyzer DNA 7500 Chip (Agilent Technologies, USA) using Kit 374. Amplicon concentration was quantified using the Quant-iT PicoGreen dsDNA Assay Kit (Thermo Scientific, Germany) and a plate reader (SpectraMax iD3, Molecular Devices) with excitation at 480 nm and emission at 520 nm.

For each sample, 10 ng DNA was barcoded in an Index-PCR with Dual Index Primer Set1 (#E7600S, NEBNext Multiplex Oligos for Illumina, New England Biolabs, Frankfurt, Germany) and were amplified with the NEBNext High Fidelity PCR Kit (New England Biolabs, Frankfurt, Germany). PCR conditions comprised denaturation at 98 °C for 30 sec, followed by 8 cycles at 98 °C for 10 sec, 55 °C for 30 sec, 72 °C for 30 sec and one final elongation step at 72 °C for 5 min. Similar to the description above, PCR reactions were cleaned using the MagSi-NGSPrep Plus Kit and amplicon concentration was quantified with the Quant-iT PicoGreen dsDNA Assay Kit. Finally, barcoded PCR products were diluted to a concentration of 4 nM DNA. For each sample, 5 µL were transferred to the library pool.

6.5.2 Illumina sequencing

Libraries were sequenced at the Institute of Comparative Microbiome Analysis (COMI) at HMGU by Susanne Kublik using MiSeq Reagent Kit v3 for Illumina sequencing (Illumina,

San Diego, CA, USA). Final demultiplexed data were provided by the sequencing service as .fastq files for further processing and analysis.

6.5.3 Microbial community analysis

Illumina amplicon sequencing data were processed with a Perl-based remultiplexor resulting in separated forward and reverse reads. Subsequently, these data were analyzed using the pipeline provided on the IMNGS platform (https://www.imngs.org/, Lagkouvardos *et al.* 2016) using the UPARSE algorithm (Edgar 2013) with an implemented USEARCH11 package (Edgar 2010). Read lengths were adjusted to min. 200 bp and max. 400 bp. Primers were trimmed by 19 bp at the 5'-end and 20 bp at 3'-end. The cut-off threshold was set to 0.0001 % in SE1 and 0.001 % in SE2. Downstream analysis such as normalization, diversity analysis, taxonomic binning, statistical comparison and calculation of correlations were done with the Rhea pipeline for R (Lagkouvardos *et al.* 2017). Mitochondrial and chloroplast reads were removed from the OTU table.

6.6 Quantitative expression of plant immune response genes

Plant immune gene expression was analyzed in soil experiment SE2 by Oriana Sanchez-Mahecha (TUM) and in axenic experiment AE2 following the same protocol with the same reagents and instruments. In SE2, samples were chosen across bacteria, aphid and earthworm treatments for barley cv. Barke and Scarlett. In AE2, samples were chosen across bacteria and aphid treatments from barley cv. Scarlett without synthetic AHL addition.
Oriana Sanchez-Mahecha selected six target genes by literature research that were associated with microbe-induced plant defenses and were also relevant for plant-aphid interactions in previous studies (Delp *et al.* 2009). These genes included two pathogenesis-related genes *PR1* and *PR17b*, ethylene-responsive factor *(ERF)*, the transcription factor *WRKY22* and an *UGT*-related flavonoid biosynthesis gene (MLOC10956). The gene *EF1α*, encoding for the essential elongation factor 1α, was used as housekeeping gene and has been validated before by Dr. Soumitra Paul Chowdhury (INET). Primer sequences are listed in Table 6.5.

Table 6.5 Primer list for plant immune gene expression analysis

Nº	Primer name	Sequence	Gene name annotation	Source	Primer type
1	Hv*EF1α*-F	5'-GTCATTGATGCTCCTGGTCA-3	Elongation factor	Dey *et al.* 2014	House-keeping gene
	Hv*EF1α*-R	5'-CTGCTTCACACCAAGAGTGA-3'			
2	Hv*ERF*-like-F (24530)	5´-CCGTACTTCTTCTACGAACA-3´	Ethylene-responsive factor-like transcription factor IPR016177	Dey *et al.* 2014	Target
	Hv*ERF*-like-R (24530)	5'-CGGTTCAGATCCAGATCAAA-3'			
3	Hv*WRKY22*-F (45055)	5'-AGAGCACTACCCGTTCTCCA-3'	WRKY TF 22 IPR003657 (DNA-binding WRKY)	Dey *et al.* 2014	Target
	Hv*WRKY22*-R (45055)	5'-GACACCACCTCGTCCAACTC-3'			
4	Hv*PR1*-F	5'-GGACTACGACTACGGCTCCA-3	Pathogenesis-related protein	Shrestha *et al.* 2019	Target
	Hv*PR1*-R	5'-GGCTCGTAGTTGCAGGTGAT-3			
5	Hv*PR17b*-F	5'-CGAGGTTCCTCGACTACTGC-3	Pathogenesis-related protein	Shrestha *et al.* 2019	Target
	Hv*PR17b*-R	5'-ATCACATTCAGCCTCCGAAC-3			
6	MLOC10956-F	5'-GCCAGAAGCCATATCTGCAC-3	UDP-glycosyl-transferase-like protein (UGT)	Han *et al.* 2016	Target
	MLOC10956-R	5'-GCAGAAAAACTCACCGGAGC-3			

6.6.1 RNA extraction and RT-qPCR conditions

For RNA extraction, 50 mg of frozen shoot material from two biological replicates were pooled for a total of 100 mg. Three pooled samples, containing six biological replicates, were grinded in liquid nitrogen. RNA was isolated using the RNeasy Plant Mini Kit (Qiagen), following the manufacturer's protocol, and was quantified with a NanoDrop spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). Subsequently, cDNA was synthesized from 1.5 µg RNA by reverse transcription with SuperScript II (Thermo Fisher Scientific, Germany).

Relative quantitative PCR (qPCR) was performed with three technical replicates per sample according to the mix preparation protocol of the SensiMix SYBR Low ROX Kit (Bioline). Each qPCR reaction contained 10 µL of 2 X Power SYBR Green Master Mix (LifeTechnologies, Darmstadt, Germany), 0.5 µM primers (Metabion International AG, Germany) and 25 ng of cDNA in 20 µL reaction volume. The qPCR conditions consisted of a pre-step at 50 °C for 2 min and denaturation at 95 °C for 10 min, followed by 40 cycles at 95 °C for 15 sec, 60 °C for 1 min with data sampling.

6.6.2 Calculation of gene expression differences

The qPCR threshold cycle (Ct) values of technical replicates were averaged. The ΔCt value was calculated by subtracting the Ct value of the reference housekeeping gene *EF1α* from the Ct value of the gene of interest. The relative gene expression differences ΔΔCT were determined by normalizing each treatment to the control treatment (Livak & Schmittgen, 2001). Gene expression differences were depicted as logarithmically transformed fold change. Calculations were performed for each sample individually, so that the standard error of the mean included three pooled replicates.

6.7 Detection of bacterial colonization on barley roots

In most experiments, bacterial strains were inoculated that have been GFP-labelled previously to this work (see 6.1 for detailled description). Unlabelled strains required further staining with fluorescence *in situ* hybridization (FISH) as described below (see 6.7.1). For the sake of best visability, however, only micrographs of GFP-labelled cells are displayed in this thesis.

6.7.1 Fluorescence *in situ* hybridization

For the detection of unlabelled strains, fluorescence *in situ* hybridization (FISH) was performed following a protocol of Alquéres *et al.* (2013). Chemicals were obtained from AppliChem, Germany. Fixed root pieces were treated in an increasing ethanol series of 50 %, 80 % and 96 % [vol/vol] for 3 min each for desiccation. Subsequently, roots were incubated in 50 µL hybridization buffer (0.9 M NaCl, 0.01 % sodium dodecyl sulfate (SDS), 10 mM Tris-HCl (pH 8.0), 35 % deionized formamide) with 15 pmol of the fluorescently labelled probes. The probes EUB338I-III labelled with fluorescein (FITC, Metabion International AG, Germany) stained all eubacteria (Amann *et al.* 1990, Daims *et al.* 1999) while ACISP145 labelled with Cy3 (Thermo Scientific, Germany) was specific for *A. radicis* and Rhi1247-Cy3 for *R. radiobacter*. Samples were hybridized for 1.5 h at 46 °C. Probe sequences are listed in Table 6.6.

Table 6.6 Probe specification for Fluorescence *in situ* hybridisation (FISH).

Probe	**Specificity**	**Binding position**	**Sequence 3'-5'**	**Formamide conc. [%]**	**Reference**
EUB338I	eubacteria	16S rRNA transcript 338-355	GCTGCCTCC CGTAGGAGT	var.	Amann *et al.* 1990
EUB338II	eubacteria	16S rRNA transcript 338-355	GCAGCCACC CGTAGGTGT	var.	Daims *et al.* 1999
EUB338III	eubacteria	16S rRNA transcript 338-355	GCTGCCACC CGTAGGTGT	var.	Manz *et al.* 1992
Rhi1247	*Rhizobium sp.*	16S rRNA transcript 1247-1252	TCGCTGCC CACTGTG	35	Sharma *et al.* 2008
ACISP145	*Acidovorax sp.*	16S rRNA transcript 145-162	TTTCGCTCC GTTATCCCC	35	Schmid & Rothballer, unpulished

6.7.2 Microscopic detection via CLSM

Fixed or stained roots were cut to pieces of ca. 1 cm, placed on black coated microscopic slides and embedded in CitiFluor (AF1, Glycerol/PBS). Root samples were investigated with a confocal laser scanning microscope LSM880 (Zeiss, Oberkochen, Germany) with argon laser excitation at 488 nm (emission filter BP 495-550 + LP 570), DPSS laser excitation at 561 nm (BP 570-620 + LP 645) and helium neon laser excitation at 633 nm (BP 570-620 + LP 645) as control channel. Cells were observed with a 64 x C-Apochromat water immersion objective leading to 640 x total maginifaction. Micrographs were taken with the software Zen Black Edition (Zeiss, Oberkochen, Germany).

For quantification of bacterial colonization patterns, fresh root samples were used without fixation or storage to avoid cell and GFP degradation. Root pieces were embedded in Citifluor immediately after harvesting and analyzed by microscopy on the same day. From each treatment, at least 24 representative fields of view were randomly selected where bacteria were expected (root surface with emerging root hairs) and adjusted to a maximum of visible cells. Cells on micrographs were counted with the help of artifical brightness regulation.

6.7.3 CFU counting

For CFU counting, roots were harvested, shortly washed in 1x PBS and measured as described above (see 6.4.8). For handling reasons, these steps could not be performed in a fully sterile manner, however, used material was thoroughly sterilized between bacterial treatments. Since from some roots several pieces were cut off for microscopic analysis, the final biomass was measured again immediately before grinding. The root was homogenized in a sterilized mortar in 1 mL 1x PBS (corresponding to dilution 10^0). From this suspension, a dilution series in 10-fold steps was established up to 10^{-4}. From selected dilutions (usually 10^{-1} and 10^{-2} for ArN35e and ArR_{neg}, 10^{-3} to 10^{-4} for ArI_{neg} and $ArR_{neg}I_{neg}$), 100 µL were plated out on NB agar plates containing the respective antibiotics for the expected bacteria. For comparable results, at least three biological replicates were examined in three techniqual replicates for each dilution step. In AE4 and AE5, 50 µL were additionally dropped on agar plates without plating. The needed space on agar plates was here extremely reduced so that three technical replicates and two dilution steps could be transferred to one single plate, saving time and material. Control plants were treated similarly and spotted on plates with all four antibiotic combinations.
Plates were incubated at 30 °C for three days (drops) and four days (plated), pictures were taken with a photostation and CFU were counted on selected plates that optimally contained 10-200 colonies.

6.7.4 Diagnostic PCR

For standard preparation, the differently GFP-labelled *A. radicis* reference strains ArN35e and ArI_{neg} were cultivated on NB agar plates for two days and resuspended in 1x NB medium. Subsequently, gDNA was extracted from pure cultures with the DNeasy UltraClean Microbial Kit (Qiagen, Hilden, Germany). Finally, DNA was eluted in 50 µL NE buffer and DNA concentration was verified with a NanoDrop One (Thermo Fisher Scientific, Waltham, MA, USA). A stock dilution of 20 ng/µL was established. From this stock, a 1:2 dilution series was established in NFW for each diagnostic PCR ranging from 10, 5, 2.5, 1.25, 0.625, 0.3125, 0.156 to 0.078 ng/µL.
As positive controls served the GFP-containing plasmids with which the two strains were labelled initially: pJBA28 (carrier plasmid for mini-Tn5-Km-PA1/04/03-RBSII-gfpmut3*-T^0-

T^1, Anderson *et al.* 1998) as reference for ArN35e and ArR_{neg}; pBBR1MCS-2-gfpmut3* for ArI_{neg} and $ArR_{neg}I_{neg}$ (Rothballer *et al.*, unpublished). *Escherichia coli* carrying the respective plasmids was cultivated in 5 mL liquid LB medium over night and washed twice with 1x PBS. Plasmids were extracted with the NucleoSpin Plasmid Mini Prep Kit.

From frozen root samples, DNA was extracted and quantified as described in the library preparation step for Illumina amplicon sequencing (see 6.5.1). Similarly, an uninoculated plant from the axenic experiment AE2 served as negative control. Sample concentration was not adjusted but 1 µL was directly inserted in triplicates into each PCR reaction for volumetric tracing of *A. radicis* DNA while keeping the proportions to other bacterial DNA.

Primer for diagnostic PCR targeting the GFP gene were GFPmut3star15-F (5‘-TGCGTAA-AGGAGAAGAAC-3‘) and GFPmut3star725-R (5‘-GTATAGTTCATCCATGCC-3‘). Other primer pairs were tested but not further used due to unspecificity. The full sequence of the targeted GFP gene is provided in the supplement (Figure S20).

PCR followed the standard DreamTaq protocol (see 6.10) with 25 cycles. Primer annealing took place at 52 °C for 30 sec and elongation at 72 °C for 50 sec. Unknown samples were visualized next to the respective DNA standard series and controls on 1 % agarose gels. A NEBNext protocol was tried for higher polymerase specificity but this led to overamplification of the standard DNA and was not further used.

6.8 Liquid biosensor assays for AHL detection

In order to evaluate AHL concentrations in the plant root surrounding, a liquid plate-format assay with the AHL-sensitive biosensor strain *A. tumefaciens* A136 was developed. Liquid Hoagland’s solution was recovered from axenic glass tubes by inserting a long syringe needle. The liquid sample was stored in glass flasks and either immediately processed or stored at -20 °C up to two days. Homoserine lactones degrade rapidly to homoserine over time. One week after sampling, usually no AHL remained detectable with the applied biosensor strain.

Agrobacterium tumefaciens A136 contains two *lacZ*-fused plasmids so that it turns over X-gal into a blue colour in the presence of most AHLs (Stickler *et al.* 1998). To keep the biosensor conditions comparable, *A. tumefaciens* cryostock aliquots of 100 µL were generated. For each trial, a separate inoculum was thawed and added to 7 mL NB medium (conditions and

antibiotics see Table 6.1) and pre-cultured for 2 h to approx. OD_{600} = 0.2. Subsequently, 40 µg/mL of X-Gal was added to the culture. The biosensor solution was pipetted in a translucent flat-bottom 96-well plate (Greiner) and mixed 1:1 with the samples in triplicates.

To confirm that only biologically active molecules are detected by the biosensor, sample AHLs were artifically hydrolyzed in test runs. To cleave the lactone ring, 60 µL NaOH (1 M) was added to 480 µL sample and incubated for 15 min on a plate shaker following Frommberger *et al.* (2005). The sample was neutralized with 60 µL HCl (1 M). The same procedure was performed with the standard series. Comparing the blue colouration of hydrolyzed versus untreated samples gives the amount of active AHL molecules. Since hydrolyzed samples never showed any blue colouration in the trials of this work, the original sample value was considered to reflect active AHL amounts only.

For the standard series, dilutions of synthetic OH-C10-HSL and C6-HSL were established from stock solutions (1 mg/mL) in sterile MilliQ in the concentrations 50000, 10000, 5000, 1000, 500, 100, 50, 10, 1, 0.1, 0.01 and 0 ng/mL. This approximately covers the range between 200 µM and 50 pM. The biosensor was sensitive down to 0.004 µM OH-C10-HSL and 0.25 µM C6-HSL. In synthetic AHL treatments, 10 µM of synthetic AHL was added to the nutrient solution what is equivalent to 2700 ng/mL OH-C10-HSL and 2000 ng/mL C6-HSL. Standards and blanks were present on each plate. AHL-containing media were always handeled with low binding pipet tips.

Biosensor assay plates were incubated at 30 °C shaking at 150 rpm. Colour change was evaluated visually and captured as image after 10 h when the assay sensitivity and colour intensity remained constant. With a plate reader (SpectraMax iD3, Molecular Devices), absorption was measured at 492 nm and 630 nm. However, absorption values did not not reflect the actual colour sufficiently, so that a manual estimation of AHL concentration was prefered. Doing this, sample well colour was compared to the well colours of the respective AHL standard and transformed into approximated AHL concentration values.

6.9 ELISA for AHL detection

For a more precise AHL detection, also a sandwich coating antigen enzyme-linked immunosorbent assay (ELISA) was tried in this work but not further applied. It comprised HSL2-BSA as a protein carrier (HSL conjugated to bovine serum albumin) and a secondary anti-AHL monoclonal antibody HSL1-1A5, both developed in-house (Chen *et al.* 2010). Unfortunately, the Hoagland sample properties after a long cultivation period repeatedly interfered with the competetive antibody binding. The standard series were not reliable for AHL concentration calculation. Therefore, a liquid biosensor assay was developed, detecting reasonable AHL amounts in the plant compartment (see 6.8).

6.10 PCR protocols

Unless further specified, bacterial DNA was amplified in a conventional DreamTaq PCR system. For each reaction, 3 µL of DreamTaq Buffer (Thermo Fisher Scientific, Germany) was mixed with 3 µL dNTPs (2.5 mM working solution) and 3 µL of the forward and reverse primers, respectively (10 µM working solution). The final volume was adjusted with NFW to 29 µL. Lastly, 0.2 µL of DreamTaq Polymerase (Thermo Fisher Scientific, Germany) was added to the mix. Each reaction was complemented with 1 µL DNA (50-100 ng/µL, when above this range only 0.5 µL DNA was inserted). Samples were amplified at least in triplicates with a no-template-control. All handling steps were performed on ice.
The conventional DreamTaq PCR protocol comprised an initial 10 min denaturation step at 94 °C, 30 cycles of DNA separation at 94 °C for 30 sec, primer annealing at variable temperatures for 30 sec and elongation at 72 °C for 1 min per kb. Final elongation took place at 72 °C for 5 min and storage at 8 °C.

For cloning, when the sequence needed to be crucially accurate, a more sensitive KOD PCR system was applied. For each reaction, 3 µL of 10x KOD Buffer (Sigma-Aldrich, Germany) was mixed with 1.8 µL $MgSO_4$ (25 mM working solution) and 3 µL dNTPs (2.5 mM working solution), 1.5 µL DMSO, 5.2 µL NFW and 12 µL Betain. From each primer, 1 µL (5 µM working solution) was used. Finally 0.5 µL KOD Polymerase (Sigma-Aldrich, Germany) was

added to the mastermix. In a volume of 29 µL, 1 µL DNA was inserted (concentration ca. 100 ng/µL). Samples were amplified at least in triplicates with a no-template-control. All handling steps were performed on ice.
The conventional KOD PCR protocol comprised an initial 5 min denaturation step at 95 °C, 30 cycles of DNA separation at 95 °C for 20 sec, primer annealing at variable temperatures for 20 sec and elongation at 70 °C for 15 sec per kb. Final elongation took place at 70 °C for 5 min and storage at 8 °C.

6.11 Statistical analysis

Normal distribution of data was checked for all parameters in all datasets previous to analysis. In case of other distributions, e.g., for aphid counts, data were log-transformed on a trial basis. Shoot, root and aphid data were analyzed with standard linear models in RStudio 1.2.1335 with R version 3.6.0 (packages: lm, lmer). In the big soil experiments SE1 and SE2, all models contained the main effects, their interactions and, as blocking factor, experimental run nested within harvest day to control for variations between temporal blocks and sampling dates. In all other experiments, run was added as conventional blocking factor. Mixed effect models counted in barley cultivar, earthworm and aphid treatments as putative interacting factors which were systematically removed from the model if no significant influence on the bacterial main effect was revealed. This way, models were simplified by backwards stepping until the minimal adequate model was reached. Datasets were reduced to the respective subsets for pairwise comparison of treatments or in respect to the baseline control. The significance level was set to 5 %.

6.11.1 Statistics on plant immune gene expression data

Differences in plant gene expression were analyzed similarly to the statistics described above (see 6.11). Mixed effect models counted in barley, earthworm and aphid treatments as interacting factors which influences were systemically checked in the described backwards stepping approach.

6.11.2 Statistics on microbiome data

Statistics on microbiome results were performed in RStudio 1.2.1335 with R version 3.6.0 using the Rhea pipeline (Lagkouvardos *et al.* 2017). Group differences were evaluated with the generalized UniFrac method which is a balanced version between unweighted and weighted UniFrac, less sensitive to differing OTU abundances (Chen *et al.* 2012). Results of the distance matrix were presented in multidimensional scaling (MDS) plots. Permutational multivariate analysis of variance using distance matrices (vegan::adonis) was performed to determine significant separation of groups and sample pairs (Anderson 2001). All statistics and vizualisations were implemented in the Rhea pipeline and used with default settings.
For correlation analysis, genera were selected that showed a significant aphid effect or a significant change in relative abundance across all bacteria. Rhea automatically calculates the Pearson's correlation coefficients and visualizes them as coloured dots in matrix format.
In experiment SE2, taxa were defined as common when the mean relative abundance across all samples was >1 % and as rare when this value was <1 %. Genera with changing relative abundance were identified using a MANOVA across all bacterial treatments taking multiple genera of the same abundance group as dependent response variables. Due to restrictions in the number of response variables per model, medium and low abundant genera were devided into smaller groups. Final P-values were determined by a MANOVA including all identified genera as dependent response variables. For the sake of simplicity, the model omitted additional interactions.

6.11.3 Power analysis

In order to estimate the minimal needed sampling size for axenic aphid experiments including all AHL mutant strains, a power analysis was conducted based on values from previous axenic trials. Two common calculation methods were chosen and compared 1) Lehr's rule of thumb (Lehr 1992, see Equation 1 below) and 2) the online ClinCalc Sample Size Calculator (https://clincalc.com/stats/samplesize.aspx). Empirical mean and standard deviations were determined for each bacterial treatment within the respective previous experiment and compared pairwise. The sample size estimation is based on a two-sided 5 % significance level with a power of ca. 80 %. Exemplary calculations are depicted in the supplement in Table S1.

$$n = 2 * (1.96 + 0.84)^2 * \frac{\sigma^2}{d^2}$$

Equation 6.1 Lehr's rule of thumb for sample size estimation.
Minimal needed sample size (n) is calculated based on empirical values from previous experiments. Two-sided significance level of 5 % and power of 80 % give a co-factor of approx. 16. The squared estimated variance σ between two treatment groups is devided by the squared estimated effect size *d* i.e., the difference between two treatment group means. Sample size is slightly overestimated in this equation according to Lehr (1992).

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Supplement

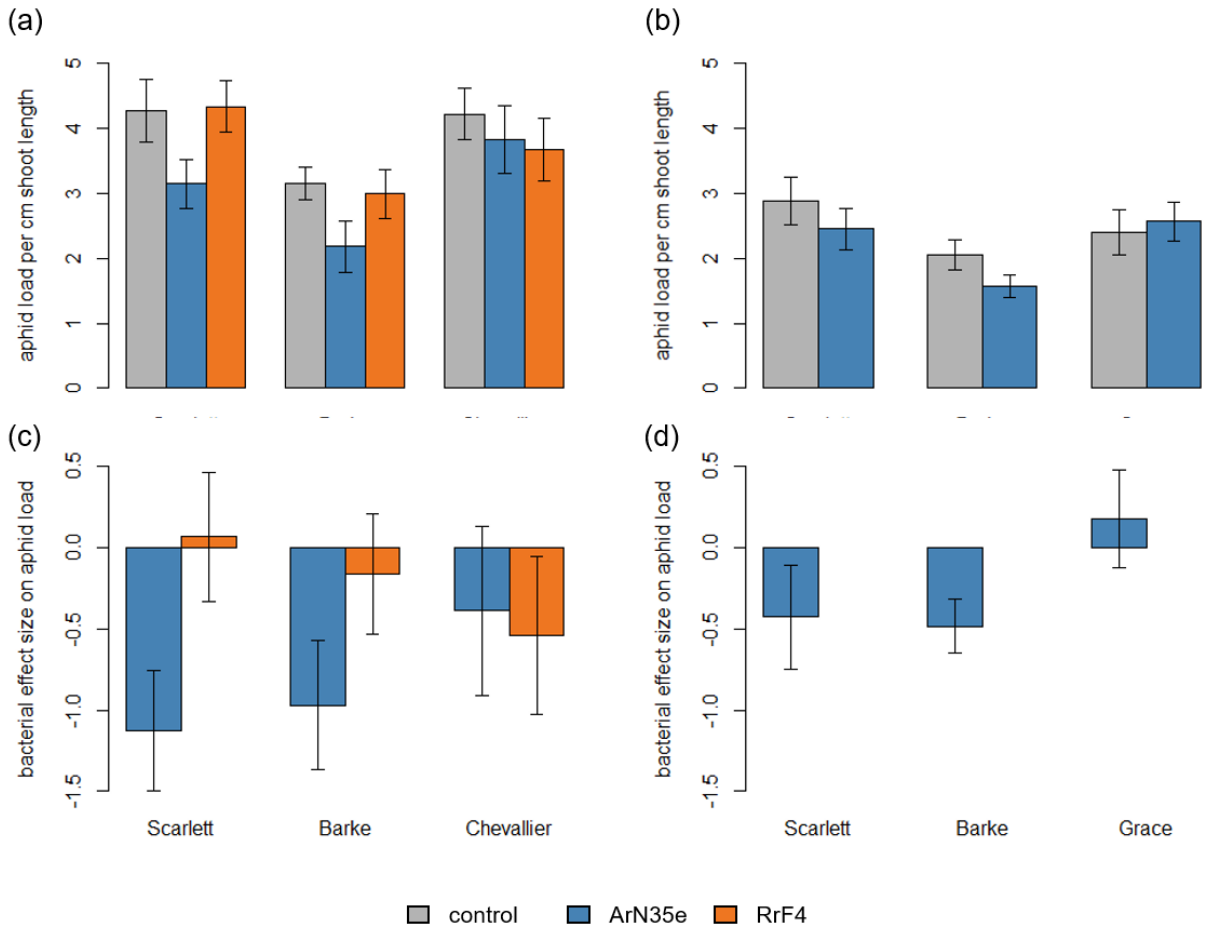


Figure S1 Comparison of the effect of bacterial test strains on aphid suppression in soil.
Depicted are relative and absolute aphid suppression data complementary to Figure 2.1 *Acidovorax radicis* N35e repeatedly reduced aphid load in three independent experiments while *Rhizobium radiobacter* F4 did not. a) and c) absolute and relative aphid suppression data of soil experiment SE1 (aphid genotypes Sickte and Fescue combined, Earthworms and NoEarthworms combined) including barley genotype Chevallier. b) and d) absolute and relative aphid suppression data of soil experiment SE2 (aphid genotype Fescue, Earthworms and NoEarthworms combined) including barley genotype Grace. Depicted are the absolut data and the bacterial effect size on aphid number per cm shoot length normalized by control plants within each experiment on day 21 post inoculation. Error bars ± 1 SE, a) 30-40. b) n = 30-40.

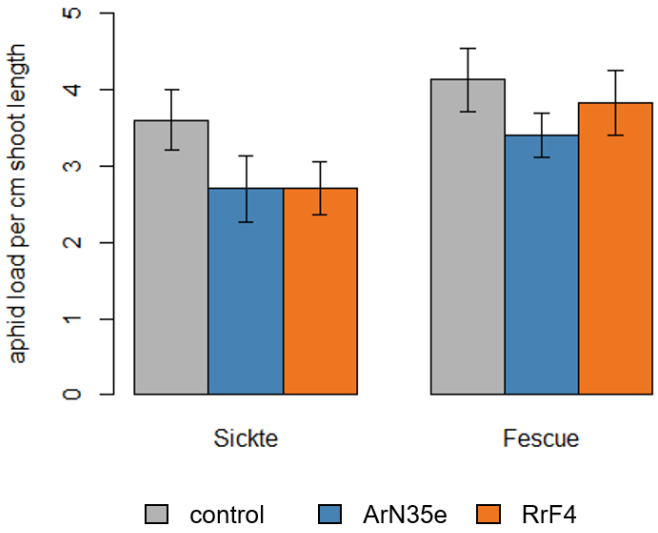


Figure S2 Aphid reduction of bacterial test strains in aphid genotype Fescue and Sickte.
Depicted are relative and absolute aphid suppression data complementary to Figure 2.1. *Acidovorax radicis* (ArN35e) repeatedly reduced aphid load in three independent experiments while *Rhizobium radiobacter* (RrF4) did only in aphid genotype Sickte. Aphid genotype main effect p = 0.0015 **, ArN35e reduced aphids slightly but not significantly vs. control (Sickte: n = 0.231, Fescue: p = 0.128). RrF4 reduced aphids more prominently in Sickte (p = 0.232) than in Fescue (p = 0.66) vs. control. Experiment SE1 only. Earthworms included, barley cultivars Scarlett, Barke and Chevallier included. n = 30-40.

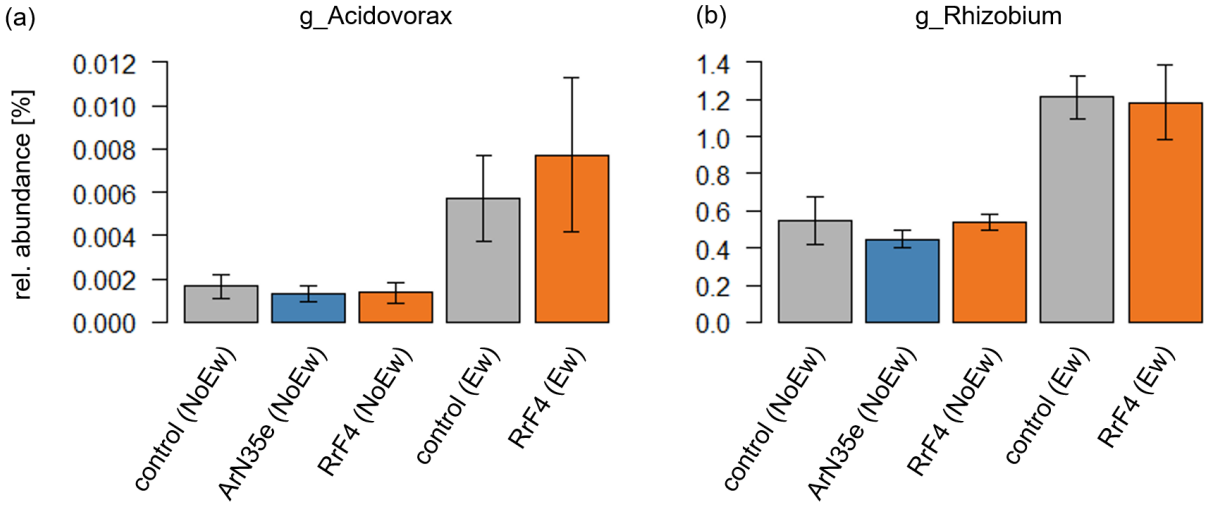


Figure S3 Abundances of the genera *Acidovorax* and *Rhizobium* in the plant rhizosphere.
Depicted are the rel. abundances of a) the genus *Acidovorax* and b) the genus *Rhizobium* identified by 16S rRNA gene amplicon sequencing in rhizosphere samples after 21 days from soil experiment SE1. Abundances did not differ between samples inoculated with *Acidovorax radicis* (ArN35e) or *Rhizobium radiobacter* (RrF4). Earthworms (Ew) increased the rel. abundance of both genera in comparison to NoEarthworms (NoEw). Both genera did not re-appear in the microbiome analysis of SE2.

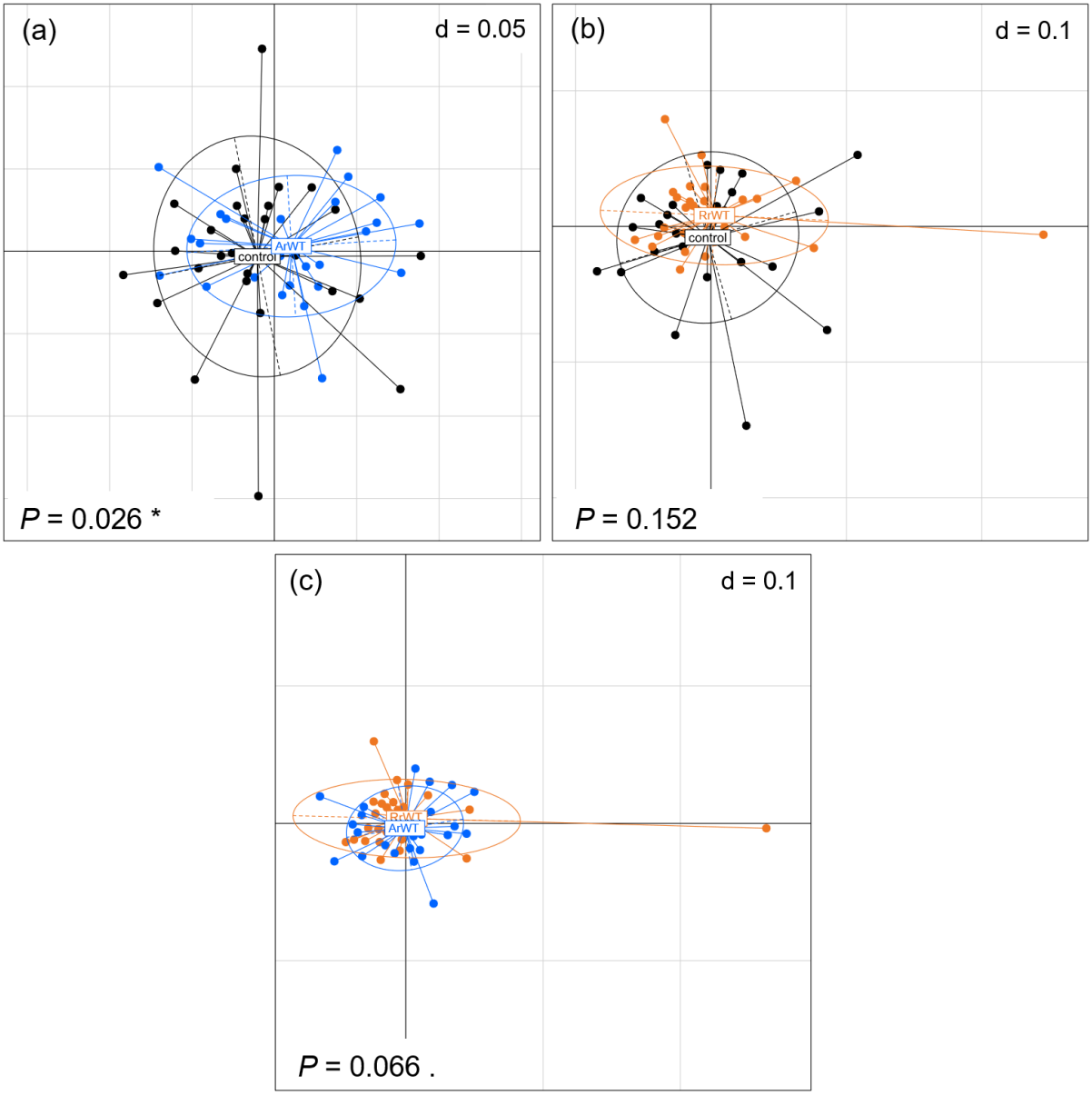


Figure S4 Effect of inoculated bacterial test strains on the microbiome profiles of the barley rhizosphere.
Microbiome analysis of SE1. Depicted are multi-dimensional scaling plots of the beta-diversity in the absence of earthworms. Similarity of microbial profiles was calculated using a generalized UniFrac distance matrix. Permutational multivariate analysis of variance (vegan::adonis) was performed to determine significant separation of bacterial treatment groups. (a) Microbial groups treated with AHL-producing *A. radicis* (ArN35e, blue) did separate significantly from not inoculated control (black). (b) Microbial groups treated with *R. radiobacter* (RrF4, orange) did not separated from control (black). (c) Microbial groups treated with ArN35e (blue) and RrF4 (orange) did not significantly separate from each other.

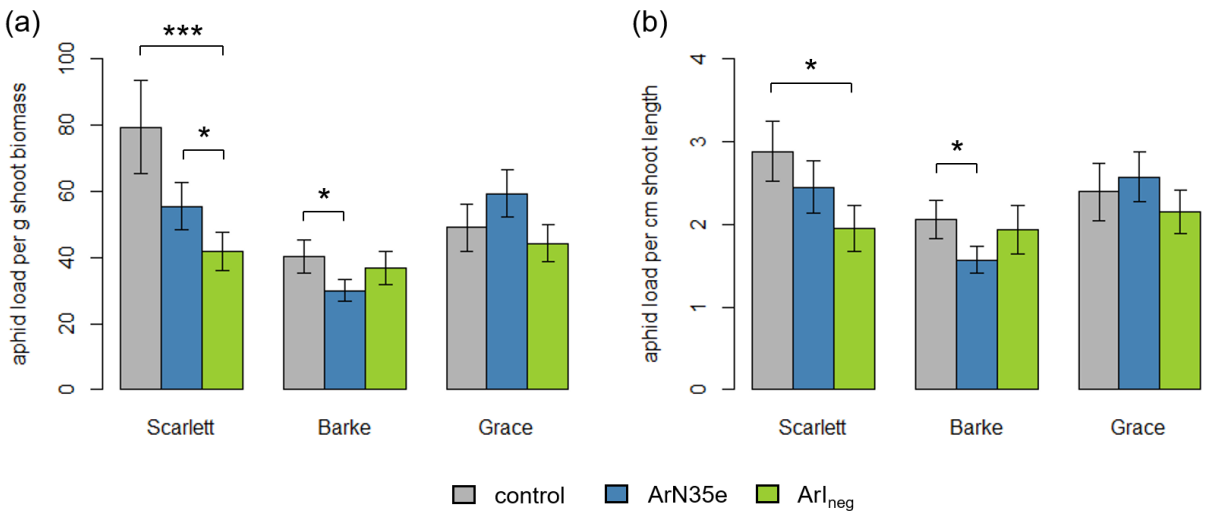


Figure S5 Absolute data on impaired AHL signalling effect on aphid suppression in soil.
Depicted are the absolute aphid suppression data from soil experiment SE2 complementary to Figure 2.5. *Acidovorax radicis* AHL synthesis mutant (ArI_{neg}) decreased aphid loads stronger compared to the AHL-producing strain *A. radicis* (ArN35e) in barley cultivar Scarlett and Barke but not in Grace. a) aphid load calculated after g shoot biomass. b) aphid load calculated after cm shoot length. a) p = 0.0004 ***, p = 0.012 * and p = 0.010 *, b) p = 0.018 * and p = 0.026 *. ArN35e vs. ArI_{neg} in Scarlett p = 0.28. Error bars ± 1 SE. n = 4-8.

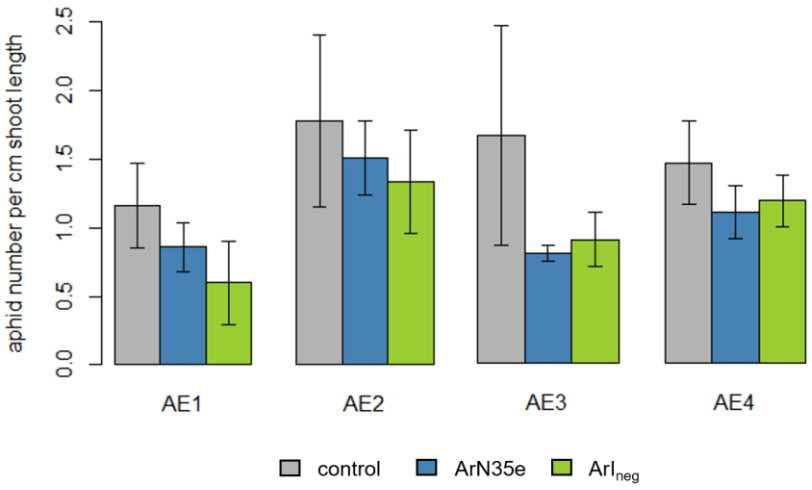


Figure S6 Absolute data on impaired AHL signalling effect on aphid suppression in axenic experiments.
Depicted are the absolute aphid suppression data split for all axenic experiment AE1-AE4 complementary to Figure 2.5. The tendency that *Acidovorax radicis* AHL synthesis mutant (ArI_{neg}) decreased aphid loads stronger compared to the AHL-producing *A. radicis* strain (ArN35e) is not visibly so strongly in axenic experiments than in soil experiments. Differences between ArN35e and ArI_{neg} in AE1: p = 0.51, AE2: p = 0.56, AE3: p = 0.86, AE4: p = 0.76. Error bars ± 1 SE. n = 4-24.

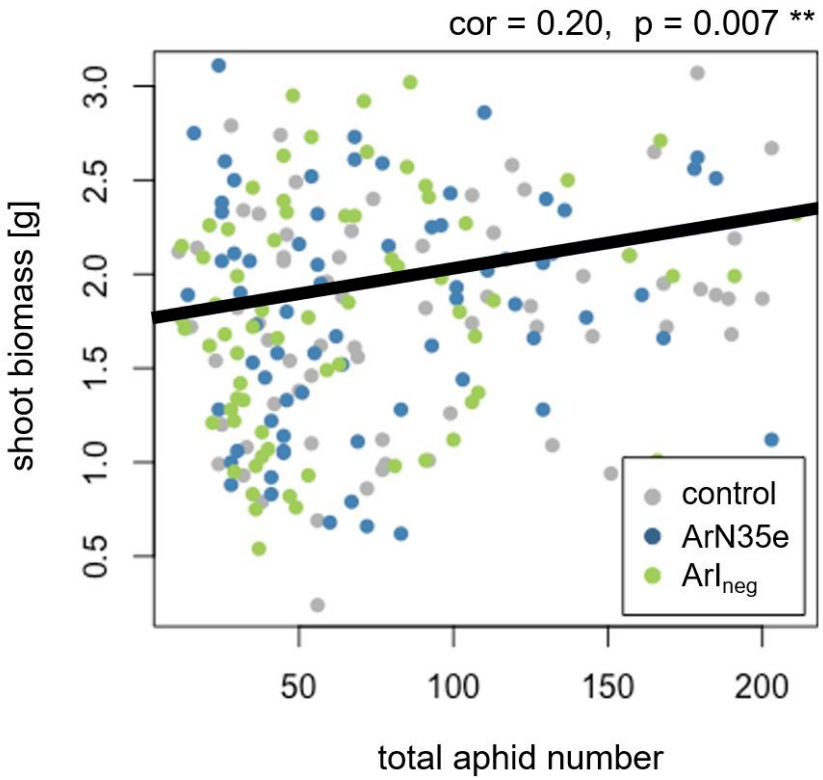


Figure S7 Correlation of total aphid numbers with shoot biomass in soil experiment SE2.
Data re-visualized after Sanchez-Mahecha *et al.* (2022). Total aphid numbers significantly correlated with shoot biomass across all tested bacterial inoculations: the AHL-producing *A. radicis* strain (ArN35e), its AHL synthesis mutant (ArI_{neg}) and the uninoculated control. For aphid suppression analysis in SE2, the calculation of aphid load by g shoot biomass was preferred over the usual calculation by cm shoot length due to a significant correlation between these parameters (see Figure 2.5).

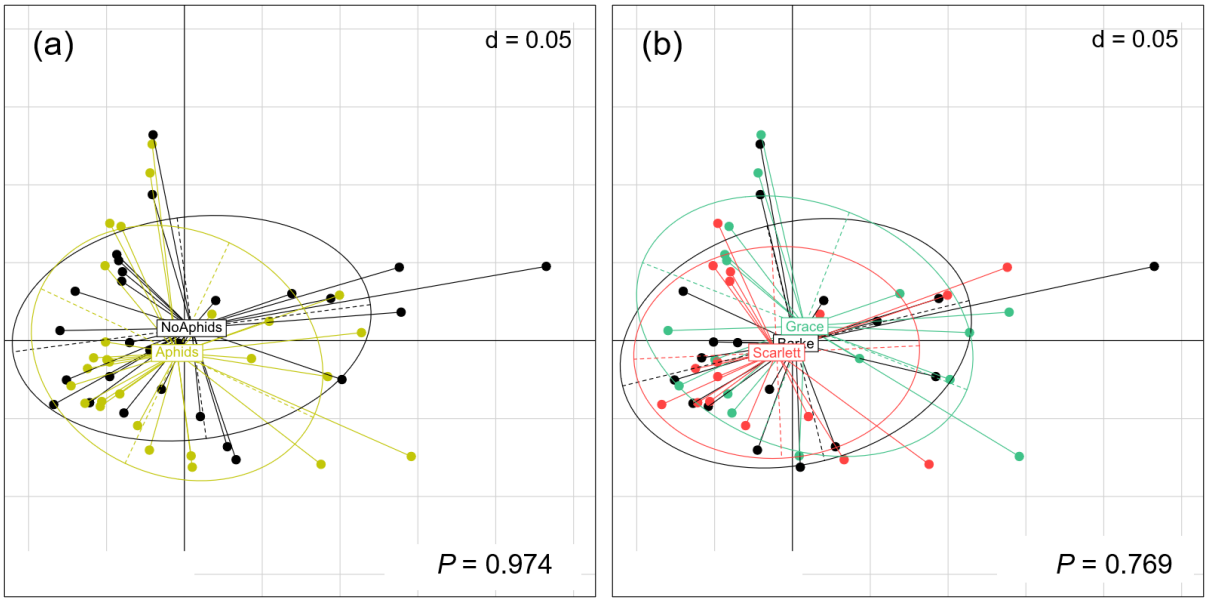


Figure S8 Effect of aphid presence and barley cultivar on the microbiome profile of the barley rhizosphere.

Figure adapted after Sanchez-Mahecha *et al.* (2022). Depicted are multi-dimensional scaling plots of the beta-diversity. Similarity of microbial profiles was calculated using a generalized UniFrac distance matrix. Permutational multivariate analysis of variance (vegan::adonis) was performed to determine significant separation of treatment groups. (a) Microbial profiles for Aphids (yellow) vs. NoAphids (black) treatments showed no significant differences. (b) Microbial profiles for barley cultivars Barke (black) vs. Grace (green) vs. Scarlett (salmon) showed no significant differences.

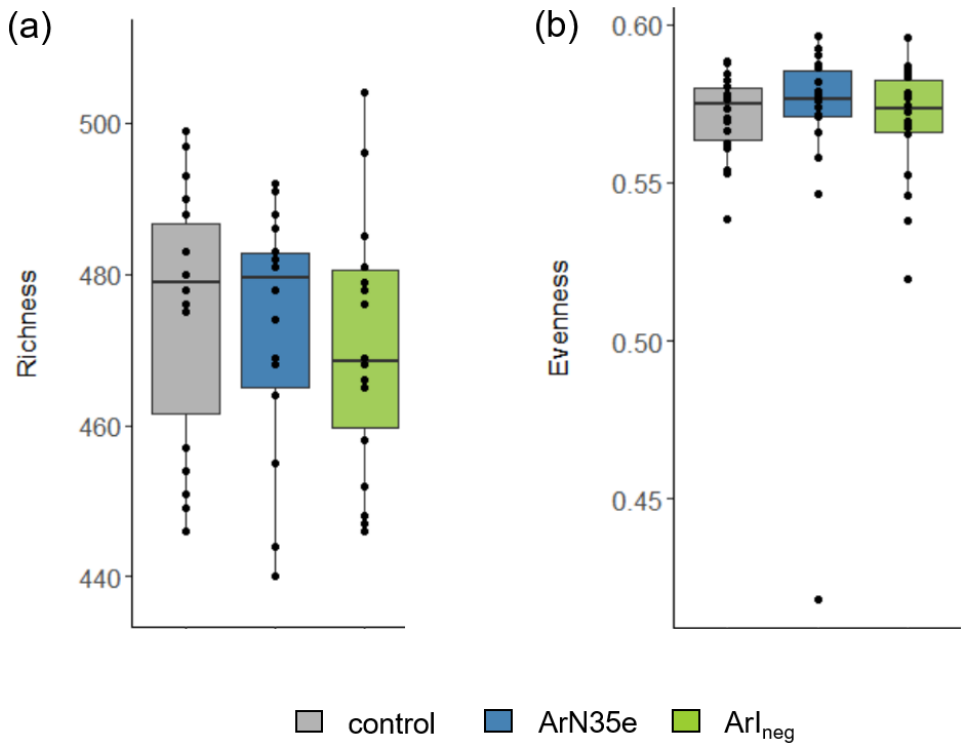
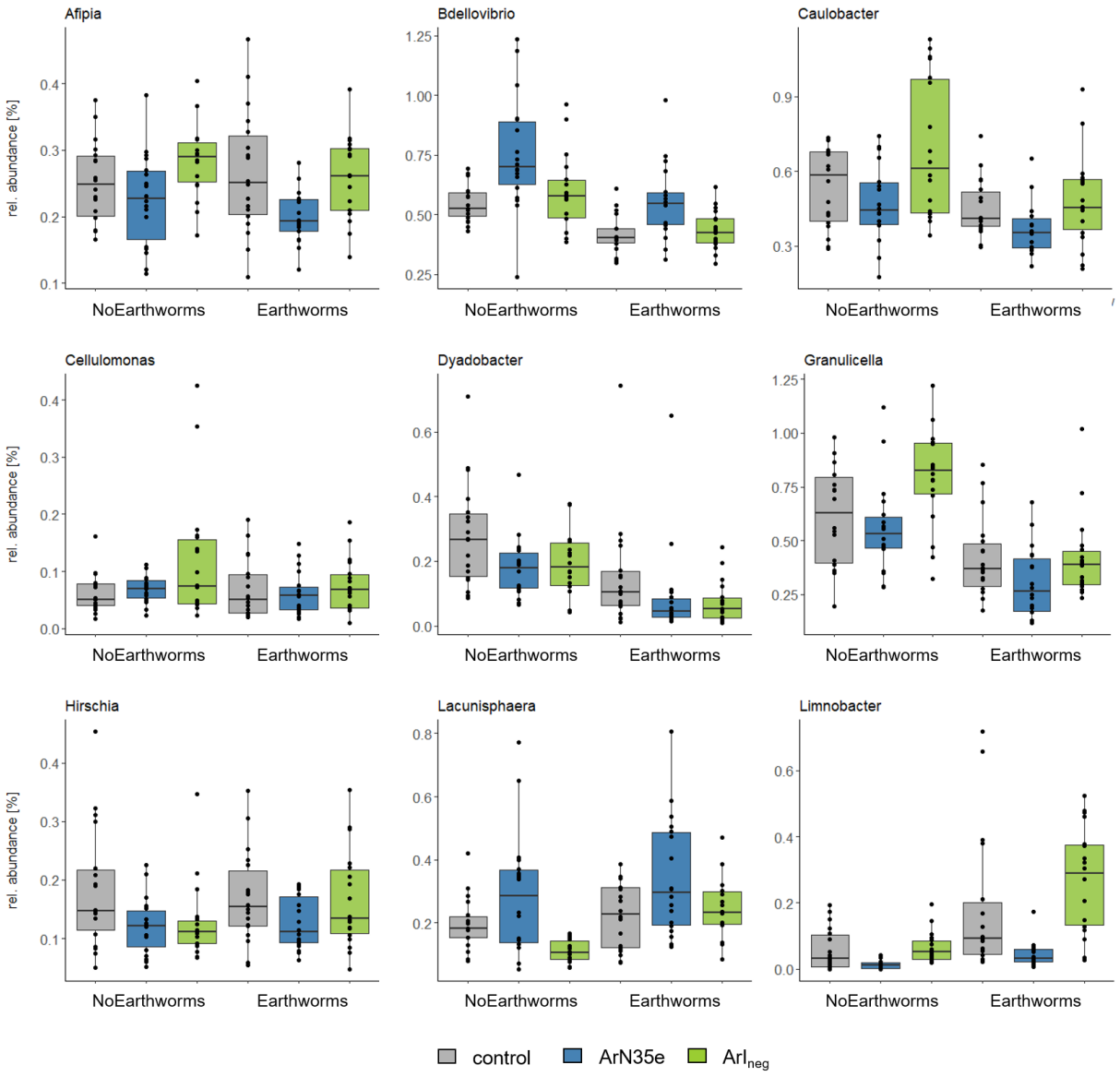


Figure S9 Richness and evenness of the barley rhizosphere microbiome.
Figure adapted after Sanchez-Mahecha *et al.* (2022). Diversity of rhizosphere microbiome samples from soil experiment SE2 treated with *A. radicis* N35e and AHL signalling mutant. (a) Species richness per bacterial treatment. (b) Species evenness per bacterial treatment. Shown are the effects of the not inoculated control (grey), the AHL-producing strain *A. radicis* (ArN35e, blue) and the *A. radicis* AHL mutant (ArI$_{neg}$, green), respectively. There was no significant variance between the treatment groups.



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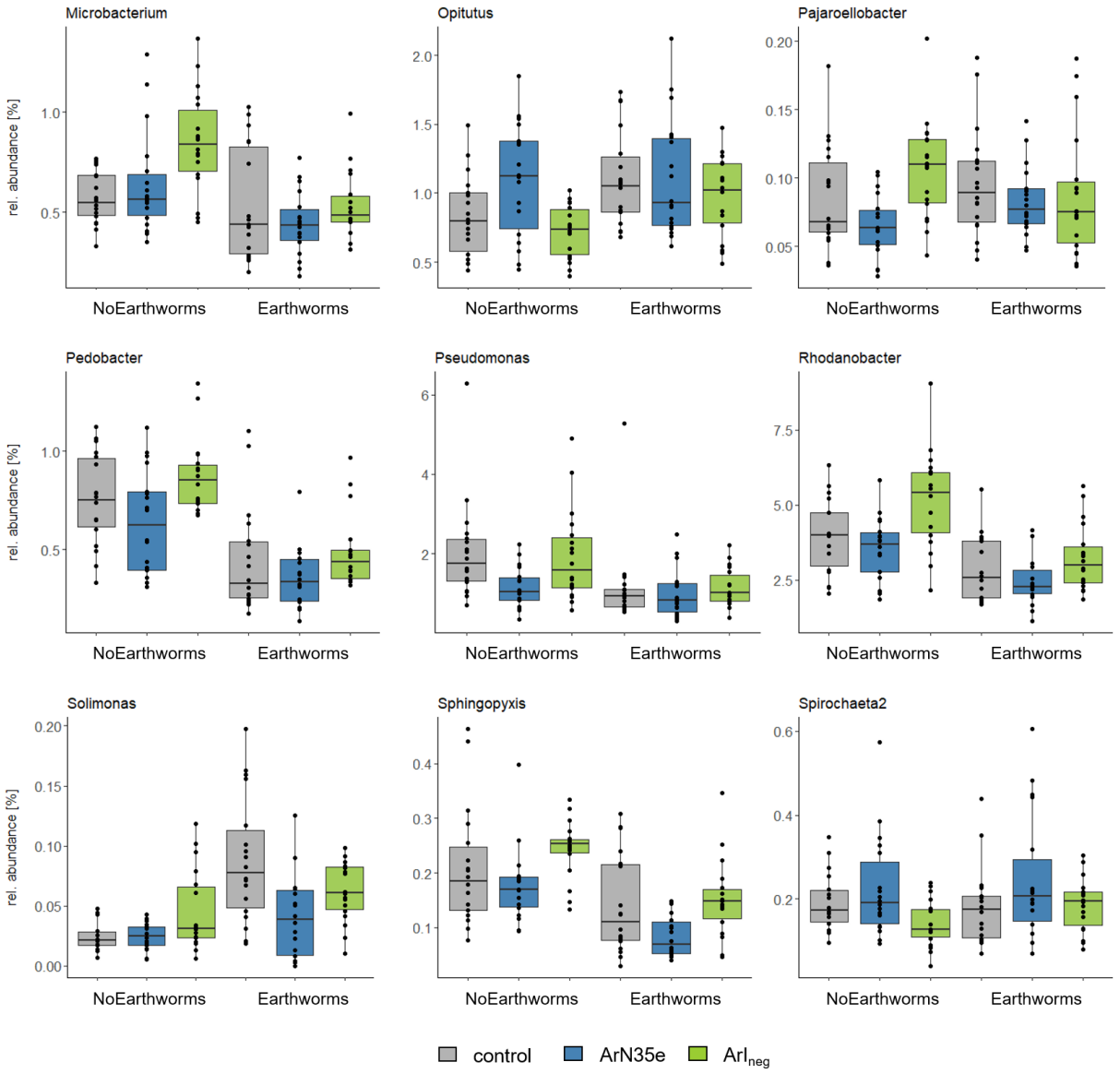

Figure S10 Relative abundances of all changing genera in the barley rhizosphere microbiome. Displayed are the eighteen genera changing significantly in their relative abundance upon inoculation of AHL-producing *A. radicis* (ArN35e) and/or the *A. radicis* AHL synthesis mutant (ArI_{neg}) compared to the uninoculated control (grey). Relative abundances are shown across NoAphid and Aphid treatments, split for Earthworm treatment to illustrate tendencies independent of aphid and earthworm presence. Significant main effects are shown in Table 2.1. n = 16.

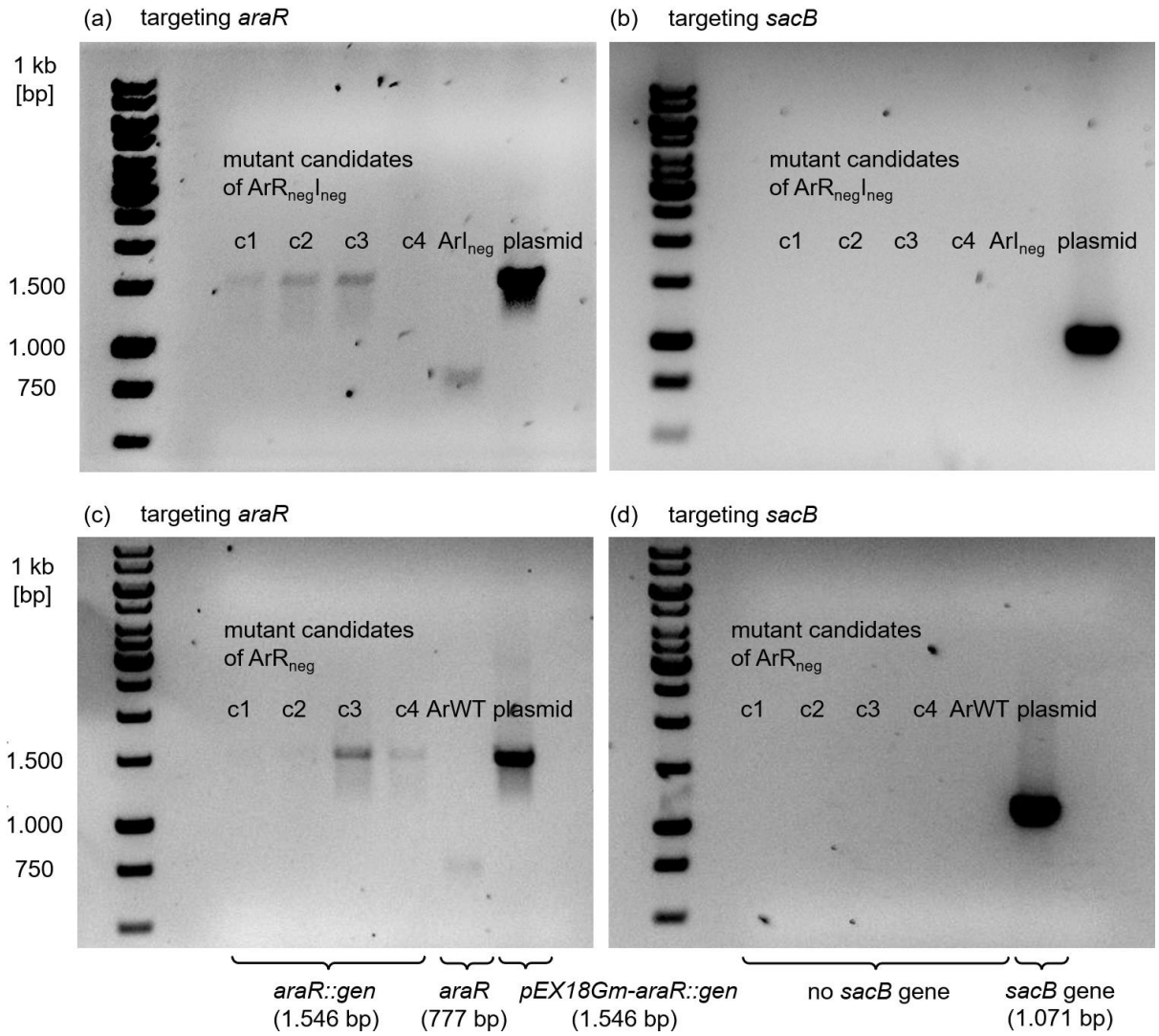

Figure S11 Mutant verification of *Acidovorax radicis araR* gene knockout by PCR.
Successful *araR* gene knock-out was evaluated for a-b) *A. radicis* double mutant (ArR$_{neg}$I$_{neg}$) and c-d) *A. radicis* sensing mutant (ArR$_{neg}$) targeting the *araR* and the *sacB* gene. Gel electrophoresis reveal amplification of the interrupted version *araR::gen* (777 bp + 769 bp = 1.546 bp) in mutant candidates. As positive controls, the normal *araR* (777 bp) was amplified from genomic DNA from the basis strains ArN35e and ArI$_{neg}$. The pEX18-Gm-*araR::gen* gene replacement vector showed the longer *araR* version. In correct mutants, no *sacB* gene (1.071 bp) should be present after successful counterselection. The pEX18-Gm-*araR::gen* gene replacement vector showed the *sacB* (1.071 bp). The verification PCR was repeated two times.

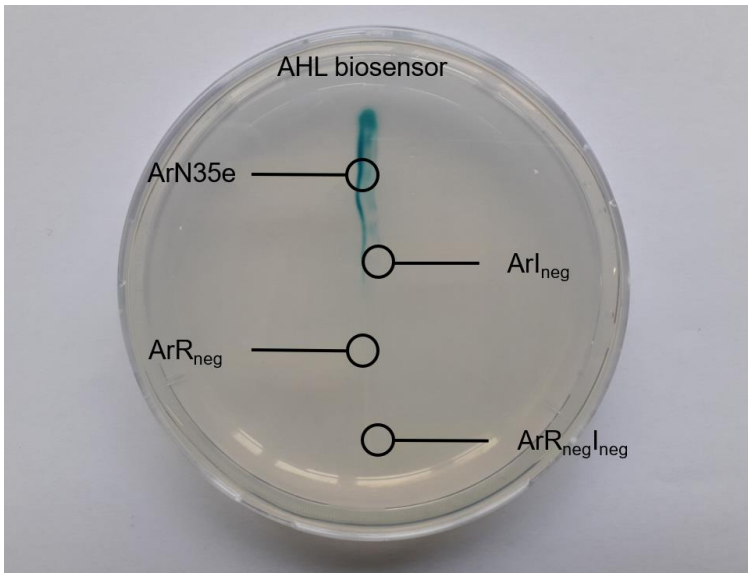

Figure S12 AHL production analysis of *A. radicis* mutants by cross-streak biosensor assay. AHL production of *Acidovorax radicis* N35e strains was tested with a plate cross-streak assay against the AHL biosensor strain *Agrobacterium tumefaciens* A136 bearing *traR*- and *traI-lacZ* fusion genes (Stickler *et al.* 1998). The test strain *Acidovorax radicis* N35e (ArN35e) showed AHL production (blue colour) after 24 h while all other strains – *A. radicis* synthesis mutant (ArI$_{neg}$), *A. radicis* AHL sensing mutant (ArR$_{neg}$) and *A. radicis* AHL double mutant (ArR$_{neg}$I$_{neg}$) – did not show AHL production. NB agar plate containing 40 µg/mL X-gal following a protocol of Ravn *et al.* (2001).

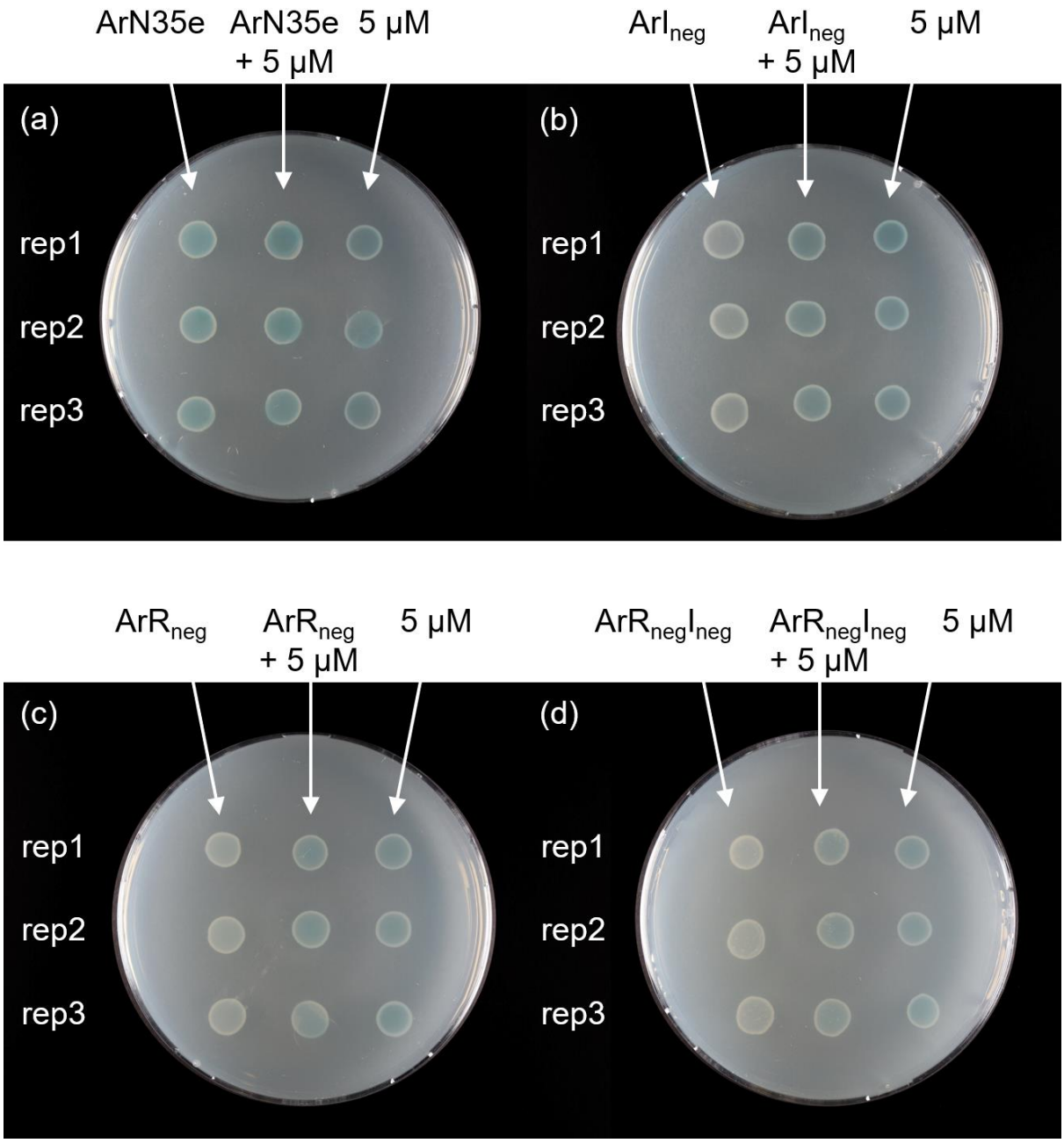

Figure S13 AHL production analysis of *Acidovorax radicis* mutants by biosensor drop assay. AHL production test of *A. radicis* mutant strains complemented with synthetic AHL. Strains under investigation were mixed with the biosensor strain *Agrobacterium tumefaciens* A136 and eventual 5 µM (final concentration) of synthetic OH-C10-HSL. Mixed samples were dropped in triplicates on agar plates. Droplets contained 40 µg/mL X-Gal. After 48 h, only a) test strain *A. radicis* N35e (ArN35e) showed AHL production (blue colour) in the absence of additional AHL while the other mutant strains – b) *A. radicis* synthesis mutant (ArI$_{neg}$), c) *A. radicis* AHL sensing mutant (ArR$_{neg}$) and d) *A. radicis* AHL double mutant (ArR$_{neg}$I$_{neg}$) – did not show AHL production. The biosensor method was not sensitive enough to vizualise eventually added up AHL concentration when microbial AHL production is turned on by synthetic AHL.

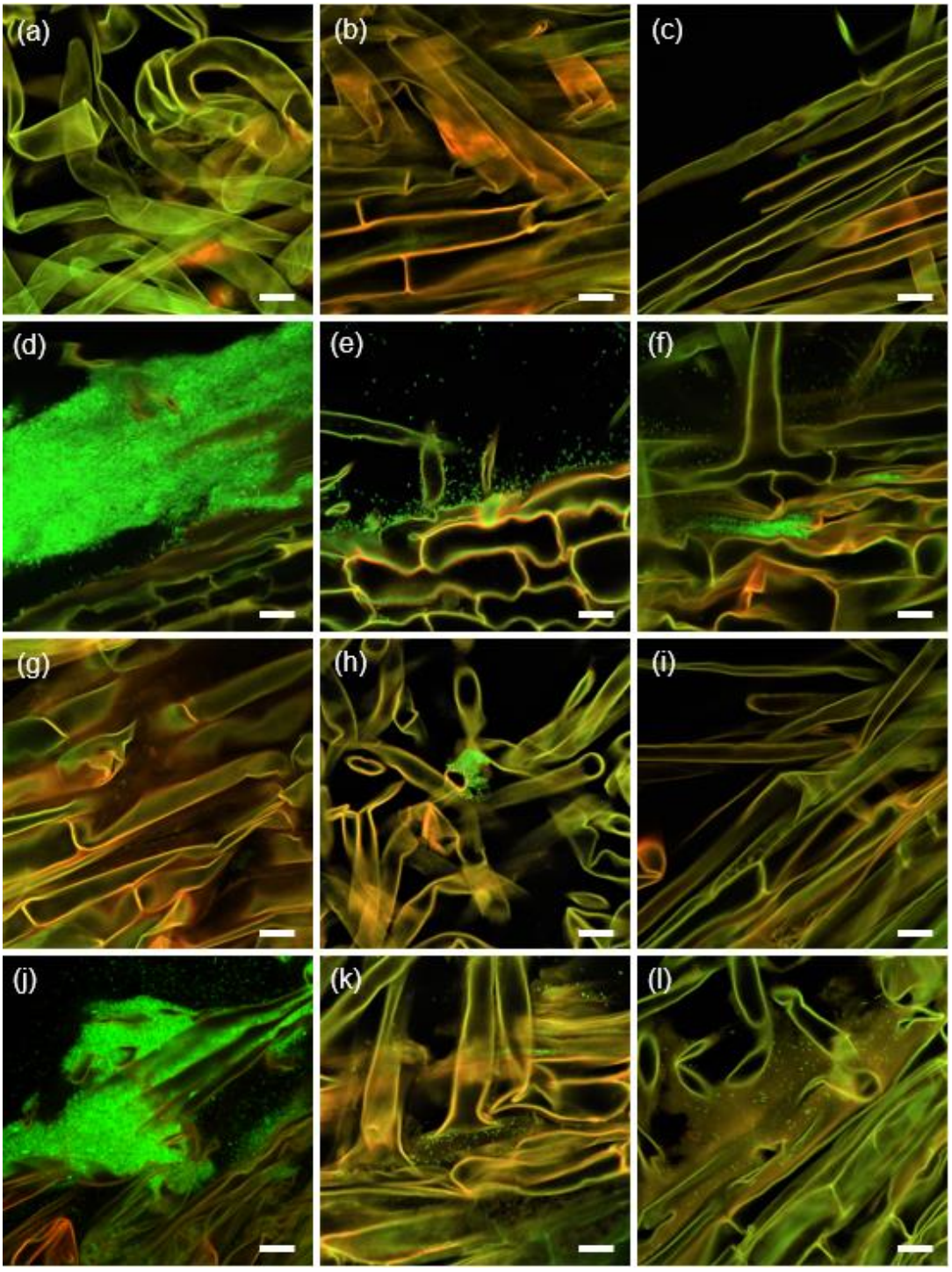

Figure S14 Root colonization pattern of AHL sensing and signalling mutants over time.
Additional micrographs to Figure 2.18. a-c) ArN35e**,** d-f) ArI_{neg}, g-i) ArR_{neg} and j-l) $ArR_{neg}I_{neg}$ colonization after 4, 7 and 10 days. AHL-producing *A. radicis* (ArN35e) was hardly detectable and showed very weak GFP labelling (arrowheads in a and c). *A. radicis* AHL synthesis mutant (ArI_{neg}) was visible in very high quantities. *A. radicis* AHL sensing mutant (ArR_{neg}) was detectable in low quantities. *A. radicis* AHL double mutant ($ArR_{neg}I_{neg}$) was visible in high quantities. All bacterial strains were GFP labelled and are visible in green, root background in yellow. Fresh root material from AE6 was analyzed by Confocal Scanning Laser Microscopy without fixation or staining. Scale bar = 10 µm.

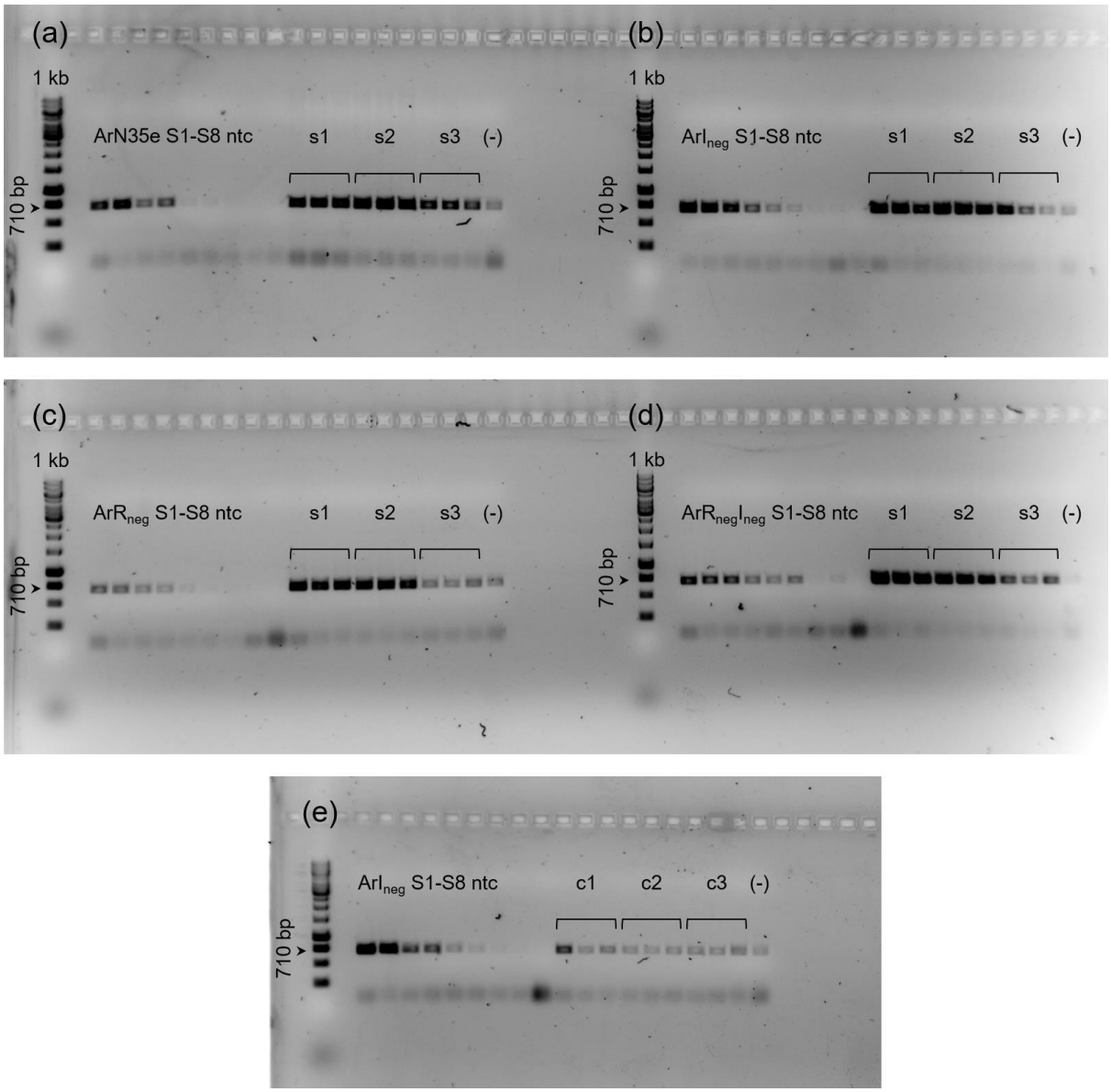

Figure S15 Molecular detection of *A. radicis mutant* strains in barley rhizosphere samples via diagnostic PCR targeting GFPmut3*.
Examplary result from axenic experiment AE4. Agarose gels reveal amplification of a 710 bp long DNA fragment in all unknown rhizosphere samples inoculated with a) *Acidovorax radicis* N35e (ArN35e), b) *A. radicis* synthesis mutant (ArI$_{neg}$), c) *A. radicis* sensing mutant (ArR$_{neg}$), d) *A. radicis* double mutant (ArR$_{neg}$I$_{neg}$) and e) uninoculated control. The slight amplification in control samples and (-) indicate unspecific amplification from random bacteria, which could not be eliminated by PCR optimization. Bands are therefore mainly considered as false positives. Still, the band thickness assumes the presence of actual GFP-labelled cells. For quantification, a standard series of pure chromosomal DNA from the respective *A. radicis* strain is presented on the left gel side. Standards S1-S8 ranged from 10, 5, 2.5, 1.25, 0.625, 0.3125, 0.156 to 0.078 ng/µL. ntc = no template control in the last position. s1-3 = randomly chosen unknown barley rhizosphere samples with inoculated bacteria. c1-3 = randomly chosen unknown barley rhizosphere samples without bacterial inoculation. (-) = uninoculated negative control sample from sterile roots from axenic experiment AE2.

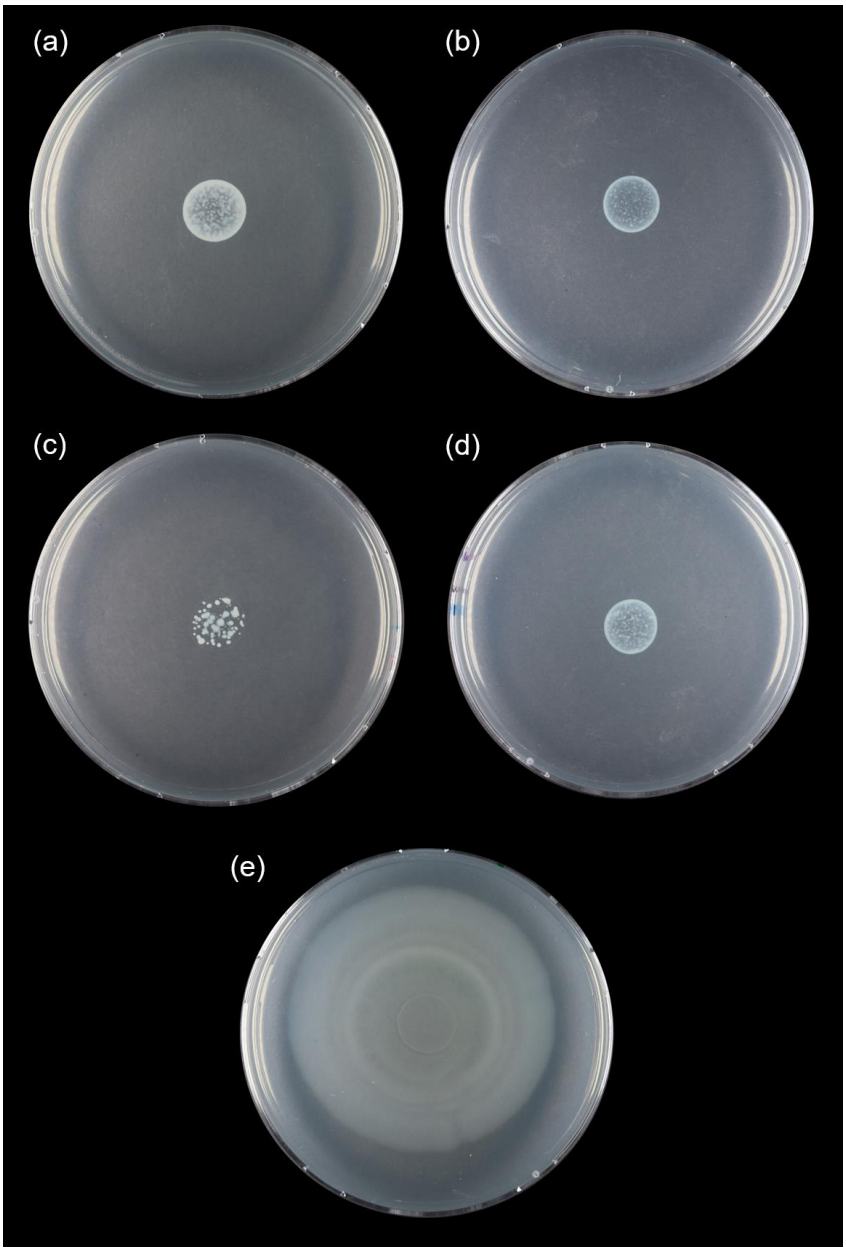

Figure S16 Swarming capacity test of *Acidovorax radicis* mutants.
Halo formation after 48 h on semi-solid agar plates. *Acidovorax radicis* showed no swarming capacity in all mutants in this assay. Cell swarming was only visible in the positive control i.e., colony spreads out over the plate. Overnight liquid cultures were spotted as one 10 µL droplet on semi-solid agar plates (0.5 % (w/v) agar) containing the respective antibiotics (see Table 6.1, protocol after Caiazza *et al.*, 2005). a) *Acidovorax radicis* N35e (ArN35e), b) *A. radicis* AHL synthesis mutant (ArI_{neg}), c) *A. radicis* AHL sensing mutant (ArR_{neg}), d) *A. radicis* AHL double mutant ($ArR_{neg}I_{neg}$), e) positive control *Pseudomonas sp.* SCA7 (Kuhl-Nagel *et al.* 2022). The swarming assay was performed in duplicates and repeated three times independently.

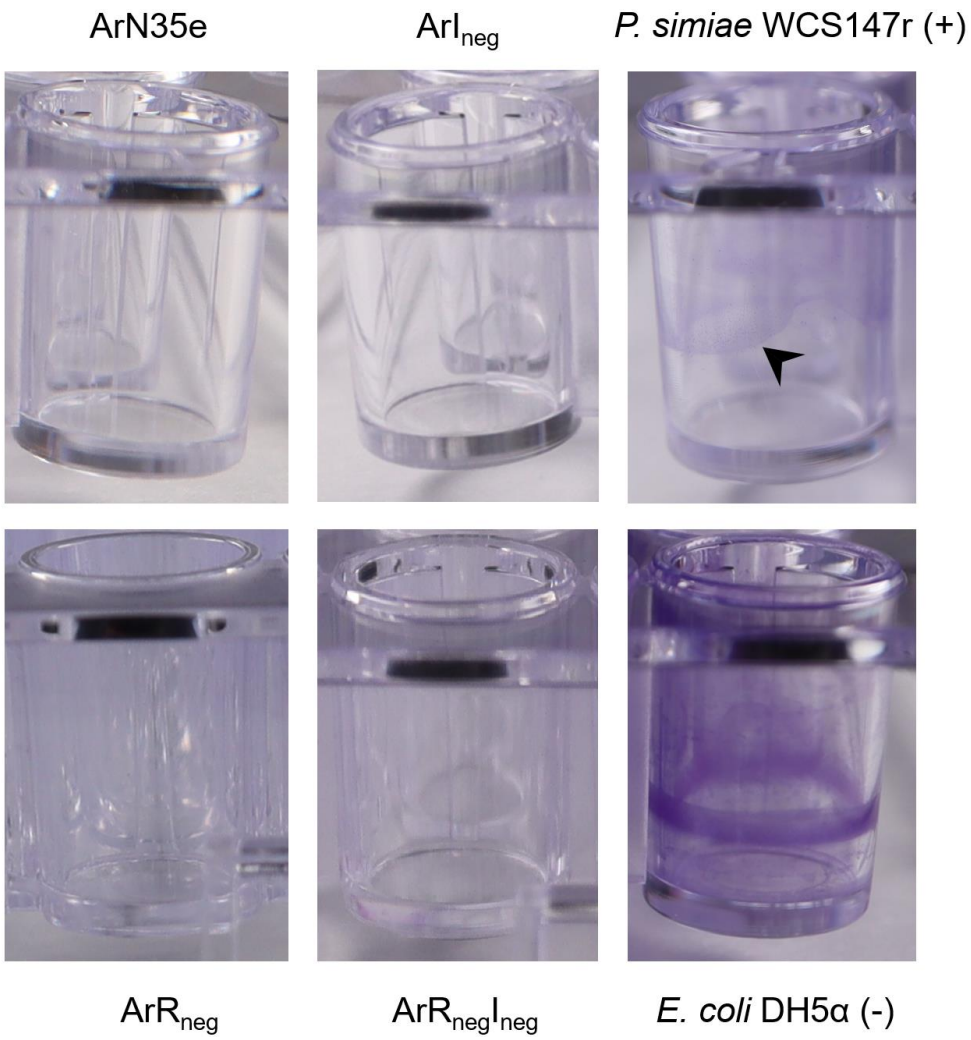

Figure S17 Biofilm formation assay of *Acidovorax radicis* mutants.
Biofilm formation was tested in a microtiter format following a protocol of O'Toole (2011). Crystal violet staining was performed after 24 h. AHL-producing *A. radicis* N35e (ArN35e) and *A. radicis* sensing mutant (ArR$_{neg}$) showed no biofilm formation while the biofilm-forming positive control *Pseudomonas simiae* WCS417r showed a slight violet ring-shaped matrix encirceling the wells (arrowhead). *A. radicis* synthesis mutant (ArI$_{neg}$) and *A. radicis* double mutant (ArR$_{neg}$I$_{neg}$) showed no biofilm formation while the negative control *Escherichia coli* DH5α showed strong violet rings. Biofilms were afterwards solubilized in 30 % acetic acid for better visibility and absorption measurements. The experiment was repeated 4 times.

Figure S18 Sequence of the *araR* gene from *Acidovorax radicis* N35e

>AHL receptor gene *araR* from *Acidovorax radicis* N35e, complete cds
CATCAGCCCAGCAACCCCAGCATGGCGGCACGGACGGTGGCGGCCGTCTTGTTGGTGGT
TTGCAGCTTGGTCACGGCATTCTTGACATGGAAGTTCACGGTGTTTTCCGACACATCCAG
CAGCGAGGAGATGTCGGCAGAGGTCTTGCCGTCGGCGGTCCATTTGAGCACCTCGATTT
CTCTGGCCGTCAGATGCGGCTGCATCAGTTCGGCCTGGCGGGTCGTAAAGATGCGCGAC
AGGATCACGTGCGCAATGTGCGCCAGCCAGCGCATCTTCATCTCACTGTGGGCAATCTCT
TCGGGTGTCAGGGCCTCGGACGGACGCGCCAGCGTCAGCATGCCACGCACCCCGCCGCC
GTCAATGCACGATTGCGCCCAACCAAAACGCAATCCGAACGATCGTGCGTCTTCCCAGA
AATTCTGGGCCGATGCAAACACATCGTCCGACCAGACCAGCGGGGTCTGCTTGCGGCAT
CCGTGGAGCACCGTCGGATCGATCTGCACATAGCCCTGTTGCGCATACCGTTCCTGCAGG
CCAAGGGGTAGTTGTTTAACAAAATGGTCTTTGGGTTGGACAAGGGCATGGGTACCCGC
AGGCCATAGGCGCAGTACTCAAACCCCAGAGCGTGCGCAGCGAGCTCGATTTTGCAAAA
AACCTCTTGCTCTGACTGCGCGCGGTCCATCACCCCCAAAAGATCTTCTTGCCAGCTTGT
CATAGGACTCCAACACCGAGCACCACCCCACAGAGACTGACCATCCGCACGCGAATTAC
GCGCCAT

Figure S19 Sequence of the gentamicin resistance gene used for *araR* disruption

> Gentamicin restistance gene from pEX18-Gm, complete cds
TTGACATAAGCCTGTTCGGTTCGTAAACTGTAATGCAAGTAGCGTATGCGCTCACGCAAC
TGGTCCAGAACCTTGACCGAACGCAGCGGTGGTAACGGCGCAGTGGCGGTTTTCATGGC
TTGTTATGACTGTTTTTTTGTACAGTCTATGCCTCGGGCATCCAAGCAGCAAGCGCGTTAC
GCCGTGGGTCGATGTTTGATGTTATGGAGCAGCAACGATGTTACGCAGCAGCAACGATG
TTACGCAGCAGGGCAGTCGCCCTAAAACAAAGTTAGGTGGCTCAAGTATGGGCATCATT
CGCACATGTAGGCTCGGCCCTGACCAAGTCAAATCCATGCGGGCTGCTCTTGATCTTTTC
GGTCGTGAGTTCGGAGACGTAGCCACCTACTCCCAACATCAGCCGGACTCCGATTACCTC
GGGAACTTGCTCCGTAGTAAGACATTCATCGCGCTTGCTGCCTTCGACCAAGAAGCGGTT
GTTGGCGCTCTCGCGGCTTACGTTCTGCCCAGGTTTGAGCAGCCGCGTAGTGAGATCTAT
ATCTATGATCTCGCAGTCTCCGGCGAGCACCGGAGGCAGGGCATTGCCACCGCGCTCATC
AATCTCCTCAAGCATGAGGCCAACGCGCTTGGTGCTTATGTGATCTACGTGCAAGCAGAT
TACGGTGACGATCCCGCAGTGGCTCTCTATACAAAGTTGGGCATACGGGAAGAAGTGAT
GCACTTTGATATCGACCCAAGTACCGCCACCTAA

Figure S20 Sequence of the GFP gene integrated in *Acidovorax radicis* strains

>KU248761.1 Synthetic construct GFPmut3 gene, complete cds
GAGGAGAATTAAGCATGCGTAAAGGAGAAGAACTTTTCACTGGAGTTGTCCCAATTCTT
GTTGAATTAGATGGTGATGTTAATGGGCACAAATTTTCTGTCAGTGGAGAGGGTGAAGG
TGATGCAACATACGGAAAACTTACCCTTAAATTTATTTGCACTACTGGAAAACTACCTGT
TCCATGGCCAACACTTGTCACTACTTTCGGTTATGGTGTTCAATGCTTTGCGAGATACCC
AGATCATATGAAACGGCATGACTTTTTCAAGAGTGCCATGCCCGAAGGTTATGTACAGG
AAAGAACTATATTTTTCAAAGATGACGGGAACTACAAGACACGTGCTGAAGTCAAGTTT
GAAGGTGATACCCTTGTTAATAGAATCGAGTTAAAAGGTATTGATTTTAAAGAAGATGG
AAACATTCTTGGACACAAATTGGAATACAACTATAACTCACACAATGTATACATCATGG
CAGACAAACAAAAGAATGGAATCAAAGTTAACTTCAAAATTAGACACAACATTGAAGA
TGGAAGCGTTCAACTAGCAGACCATTATCAACAAAATACTCCAATTGGCGATGGCCCTG

TCCTTTTACCAGACAACCATTACCTGTCCACACAATCTGCCCTTTCGAAAGATCCCAACG
AAAAGAGAGACCACATGGTCCTTCTTGAGTTTGTAACAGCTGCTGGGATTACACATGGC
ATGGATGAACTATACAAATAA

Table S1 Power analysis of axenic aphid experiments.
Minimal sample size (n) was determined for AE4 based on empirical values from previous experiment AE3. The analysis revealed that hundreds of true replicates would be needed for valuable statistical statements, as examplarily shown for shoot length and aphid load. Sampling size was calculated after Lehr's rule of thumb and compared to the online ClinCalc Sample Size Calculator (see 6.11.3). Both calculations are based on a two-sided significance level of 5 % and power of 80 % (Lehr 1992). n = minimal sample size, s = standard deviation, μ = mean, σ = variance.

	ArN35e vs. control		ArI_{neg} vs. control		ArN35e vs. ArI_{neg}	
	shoot length	**aphid load per g shoot biomass**	**shoot length**	**aphid load per g shoot biomass**	**shoot length**	**aphid load per g shoot biomass**
μ1	24.76	0.34	24.76	0.34	24.33	0.29
μ2	24.33	0.29	24.90	0.28	24.90	0.28
s1	2.75	0.44	2.38	0.44	2.75	0.14
s2	2.38	0.14	3.86	0.19	3.86	0.19
σ	2.6	0.3	3.2	0.3	3.4	0.2
n (Lehr)	**569**	**538**	**7946**	**441**	**538**	**4356**
n (ClinCalc)	**574**	**565**	**8201**	**394**	**559**	**6279**

Table S2 Significance values for the effect of bacterial test strains on plant growth.
Values correspond to Figure 2.3. Inoculation of bacterial test strains were compared for their effect on plant growth 21 dpi under axenic cultivation conditions (experiments AE1, AE2, AE3, AE4) and soil (experiments SE1, SE2, SE3). Barley cv. Barke, Scarlett and Grace were treated with AHL-producing *A. radicis* N35e (ArN35e), *R. radiobacter* (RrF4) and a control inoculum in SE2. In SE4, RrF4 was inoculated in high, medium and low inoculation doses to barley cv. Barke. a-d) n = 76, e-h) = 8-32, i-l) n = 16. Only groups with at least one significant value are listed.

SE2		**p-value**
(e)	ArN35e – control	0.0924 .
(e)	ArN35e – RrF4	0.0001 ***
(f)	RrF4 – control	<0.0001 ***
(f)	ArN35e – RrF4	0.0001 ***
(g)	ArN35e – RrF4	0.0106 *
(h)	ArN35e – control	0.0227 *
(h)	RrF4 – control	0.0049 **
(h)	ArN35e – RrF4	0.0001 ***

SE4		**p-value**
(i)	RrF4 medium – control	0.0033 **
(j)	RrF4 medium – control	0.0437 *
(k)	RrF4 medium – control	0.0831 .
(k)	RrF4 high – control	0.0221 *
(l)	RrF4 medium – control	0.0006 ***
(l)	RrF4 high – control	0.0198 *

Table S3 Significance values for relative immune response gene expression of barley plants upon impaired AHL signalling.
Values correspond to Figure 2.6. A qPCR analysis revealed the log2-fold expression change of selected barley immune response genes (independent analysis of SE2 and AE2). Barley leaves of the cultivars Barke and Scarlett were treated with AHL-producing *A. radicis* N35e (ArN35e), *A. radicis* synthesis mutant (ArI_{neg}) and a control inoculum under soil and axenic cultivation conditions. n = 3-12. True replicate number n = 3 while each replicate is a pool of two biological replicates. Only groups with at least one significant value are listed.

	Treatment comparison	Cultivar and condition	Immune response gene	p-value
(b)	ArI_{neg} – control	Barke (soil)	*PR1*	0.0261 *
(c)	ArI_{neg} – control	Barke (soil)	*PR17b*	0.0451 *
(d)	ArI_{neg} – control	Barke (soil)	*WRKY22*	0.0038 **
(d)	ArN35e – ArI_{neg}	Barke (soil)	*WRKY22*	0.0807 .
(f)	ArN35e – ArI_{neg}	Scarlett (soil)	*ERF-like*	0.0473 *
(g)	ArI_{neg} – control	Scarlett (soil)	*PR1*	0.0085 **
(g)	ArN35e – ArI_{neg}	Scarlett (soil)	*PR1*	0.0343 *
(h)	ArI_{neg} – control	Scarlett (soil)	*PR17b*	0.0298 *
(l)	ArN35e – control	Scarlett (axenic)	*PR1*	0.0079 **
(l)	ArN35e – ArI_{neg}	Scarlett (axenic)	*PR1*	0.0900 .
(m)	ArN35e – control	Scarlett (axenic)	*PR17b*	0.0008 ***
(o)	ArI_{neg} – control	Scarlett (axenic)	*UGT*	0.0310 *
(o)	ArN35e – ArI_{neg}	Scarlett (axenic)	*UGT*	0.0887 .

Table S4 Significance values for impaired AHL synthesis effect on plant growth.
Values correspond to Figure 2.7. Bacterial AHL synthesis mutants are compared for their effect on plant growth 21 dpi under axenic cultivation conditions (experiments AE1, AE2, AE3, AE4) and in soil (experiments SE1, SE2, SE3). Barley cv. Barke, Scarlett and Grace were treated with AHL-producing *A. radicis* N35e (ArN35e) and *R. radiobacter* F4 (RrF4), AHL synthesis mutant *A. radicis* (ArI_{neg}) and *R. radiobacter* (RrI_{neg}) and a control inoculum. n = 69-76, e-h) n = 12-36, i-l) n = 8-23. Only groups with at least one significant value are listed.

SE2		p-value
(e)	ArN35e – control	0.0924 .
(e)	ArN35e – ArI_{neg} (Scarlett)	0.0780 .
(e)	ArN35e – ArI_{neg} (Barke)	0.0265 *
(g)	ArI_{neg} – control	0.0870 .
(h)	ArN35e – control	0.0227 *
(h)	ArN35e – ArI_{neg}	0.0011 **

SE3		p-value
(j)	RrF4 – control	<0.0001 ***
(j)	RrF4 – RrI_{neg}	<0.0001 ***
(l)	RrF4 – control	0.0013 **
(l)	RrF4 – RrI_{neg}	0.0002 ***

Table S5 Relative abundances of all known genera in the rhizosphere microbial community. Listed genera were identified by 16S Amplicon sequencing from soil experiment SE2. Sorted by decreasing mean rel. abundance across all treatments. The barley rhizosphere was inoculated with AHL-producing *A. radicis* (ArN35e), *A. radicis* AHL synthesis mutant (ArI_{neg}) and a control inoculum.

	rel. abundance [%] in bacterial treatment		
Genera	**control**	**ArN35e**	**ArI_{neg}**
Rhodanobacter	3.998	3.538	5.186
Devosia	3.992	3.704	3.600
Streptomyces	3.829	3.229	3.096
Cellvibrio	3.555	3.826	2.687
Cytophaga	3.430	2.814	2.720
Flavobacterium	2.623	2.066	2.759
Asticcacaulis	2.130	1.735	2.279
Rhodopseudomonas	1.625	1.597	1.956
Pseudomonas	2.033	1.144	1.939
Fluviicola	1.473	1.246	1.362
Pseudolabrys	1.185	1.453	1.208
OM27 clade	1.240	1.216	1.163
Ramlibacter	1.138	1.194	1.103
Rhodobacter	1.156	1.277	0.891
Luteolibacter	0.924	1.017	1.077
Pseudarthrobacter	0.866	0.775	1.165
Opitutus	0.828	1.095	0.722
Sporocytophaga	0.586	1.057	0.680
Pedobacter	0.752	0.637	0.870
Planctopirus	0.628	0.751	0.791
Kosakonia	1.135	0.197	0.820
Myxococcus	1.213	0.639	0.257
Microbacterium	0.573	0.641	0.850
P3OB-42	0.533	0.735	0.752
Granulicella	0.601	0.558	0.801
Bdellovibrio	0.546	0.753	0.595
Chthoniobacter	0.550	0.735	0.576
Caulobacter	0.536	0.472	0.692
Acidothermus	0.501	0.558	0.631
Massilia	0.553	0.389	0.741
Aquicella	0.574	0.755	0.275
Mesorhizobium	0.516	0.467	0.549
Mycobacterium	0.468	0.486	0.491
Edaphobaculum	0.484	0.474	0.448
Hydrogenophaga	0.473	0.441	0.395
BIyi10	0.419	0.419	0.426
Peredibacter	0.416	0.423	0.421
Parafrigoribacterium	0.390	0.390	0.465
Flavisolibacter	0.371	0.374	0.427
Pseudoxanthomonas	0.395	0.371	0.376
Novosphingobium	0.337	0.361	0.400
Mucilaginibacter	0.382	0.335	0.375
Haliangium	0.323	0.344	0.385
Flavitalea	0.268	0.287	0.316
Chitinophaga	0.223	0.185	0.460
Emticicia	0.328	0.301	0.236
Nocardioides	0.271	0.288	0.299
Pirellula	0.367	0.276	0.202
Rhodococcus	0.294	0.285	0.260

Parvibaculum	0.294	0.282	0.226
Afipia	0.252	0.225	0.284
Gemmatimonas	0.211	0.291	0.254
Brevundimonas	0.219	0.267	0.271
Prosthecobacter	0.272	0.208	0.274
Pelomonas	0.356	0.209	0.172
Stenotrophomonas	0.239	0.217	0.270
Phenylobacterium	0.257	0.223	0.213
SH-PL14	0.202	0.242	0.246
Sediminibacterium	0.206	0.281	0.189
Thermomonas	0.225	0.183	0.247
Dyadobacter	0.281	0.179	0.189
Gryllotalpicola	0.194	0.203	0.246
possible genus 04	0.103	0.312	0.226
Sphingopyxis	0.208	0.176	0.244
Isoptericola	0.167	0.200	0.257
Lacunisphaera	0.197	0.292	0.112
Dokdonella	0.180	0.177	0.220
Ciceribacter	0.210	0.224	0.132
Spirochaeta 2	0.192	0.226	0.142
Luteimonas	0.194	0.163	0.203
Allorhizobium-Neorhizobium-	0.161	0.255	0.134
Castellaniella	0.166	0.176	0.198
Bosea	0.166	0.192	0.166
Anaeromyxobacter	0.155	0.197	0.157
Legionella	0.185	0.215	0.093
Sphingomonas	0.156	0.151	0.184
Sulfurovum	0.147	0.208	0.133
Herpetosiphon	0.296	0.142	0.043
Hirschia	0.184	0.121	0.127
Gemmata	0.125	0.198	0.108
Azospira	0.000	0.001	0.424
IMCC26134	0.188	0.164	0.063
Rheinheimera	0.164	0.181	0.063
Occallatibacter	0.113	0.130	0.148
Duganella	0.142	0.056	0.192
Methyloversatilis	0.100	0.129	0.108
Singulisphaera	0.096	0.092	0.113
Paracoccus	0.112	0.091	0.088
Pedomicrobium	0.091	0.094	0.093
YC-ZSS-LKJ147	0.000	0.269	0.000
Burkholderia-Caballeronia-Paraburkholderia	0.065	0.065	0.135
Herminiimonas	0.084	0.081	0.097
Pajaroellobacter	0.085	0.065	0.107
Cellulomonas	0.062	0.069	0.120
Iamia	0.083	0.085	0.081
Conexibacter	0.073	0.077	0.094
Roseiarcus	0.069	0.076	0.097
Rhodopirellula	0.065	0.097	0.078
Marmoricola	0.071	0.076	0.079
Reyranella	0.074	0.077	0.059
Methylophilus	0.061	0.070	0.068
SWB02	0.076	0.076	0.041
Buchnera	0.073	0.115	0.001
Roseomonas	0.057	0.065	0.059
Roseimicrobium	0.067	0.060	0.051
Nakamurella	0.040	0.081	0.053

Bryobacter	0.055	0.058	0.060
Panacagrimonas	0.058	0.056	0.047
Deinococcus	0.046	0.080	0.031
Bacteriovorax	0.040	0.071	0.043
Rubellimicrobium	0.051	0.055	0.044
Azospirillum	0.039	0.020	0.087
Steroidobacter	0.061	0.030	0.050
Schlesneria	0.043	0.042	0.052
Limnobacter	0.058	0.014	0.065
Pseudonocardia	0.062	0.030	0.040
Ferruginibacter	0.029	0.045	0.046
Flavihumibacter	0.035	0.039	0.041
Kaistia	0.055	0.020	0.032
Kocuria	0.017	0.054	0.034
Solimonas	0.025	0.025	0.046
Sanguibacter	0.028	0.035	0.032
Phreatobacter	0.040	0.035	0.016
Pigmentiphaga	0.043	0.018	0.027
Corallococcus	0.036	0.013	0.036
Paenibacillus	0.047	0.008	0.027
Pseudoflavitalea	0.000	0.081	0.000
Pir4 lineage	0.023	0.041	0.016
OLB13	0.061	0.014	0.004
Aquabacterium	0.009	0.048	0.018
Lechevalieria	0.001	0.001	0.072
Nubsella	0.040	0.019	0.014
TM7a	0.027	0.031	0.015
Aeromicrobium	0.019	0.038	0.016
Hydrocarboniphaga	0.040	0.011	0.021
Methylobacterium-Methylorubrum	0.030	0.030	0.012
Domibacillus	0.034	0.022	0.014
Leptolyngbya PCC-6306	0.047	0.014	0.008
FFCH7168	0.005	0.043	0.022
Halomonas	0.004	0.004	0.058
Actinoplanes	0.013	0.023	0.023
Candidatus Solibacter	0.021	0.021	0.016
Taibaiella	0.009	0.012	0.038
Ketobacter	0.025	0.023	0.010
Exiguobacterium	0.014	0.041	0.001
Fimbriimonas	0.012	0.026	0.012
mle1-7	0.014	0.021	0.012
Coxiella	0.007	0.011	0.026
Sandaracinus	0.019	0.020	0.005
Dongia	0.013	0.020	0.010
Chthonobacter	0.014	0.021	0.008
Clostridium sensu stricto 1	0.000	0.000	0.035
Verrucomicrobium	0.008	0.019	0.007
Lachnoclostridium	0.001	0.001	0.028
Lacibacter	0.015	0.010	0.004
Sphaerisporangium	0.009	0.012	0.007
Sorangium	0.000	0.025	0.000
Dinghuibacter	0.008	0.012	0.004
Fibrella	0.006	0.015	0.002
Larkinella	0.015	0.005	0.004
Longimicrobium	0.006	0.007	0.010
Phaeodactylibacter	0.019	0.002	0.000
Clostridium sensu stricto 3	0.000	0.000	0.020

Terrimonas	0.001	0.000	0.017
Acinetobacter	0.004	0.003	0.009
Silvanigrella	0.003	0.009	0.004
Fontimonas	0.005	0.004	0.007
Blastocatella	0.000	0.000	0.015
Niastella	0.003	0.010	0.002
Vogesella	0.003	0.009	0.003
Lactococcus	0.000	0.014	0.001
Candidatus Chloroploca	0.000	0.000	0.012
Polyangium	0.000	0.010	0.000
Tychonema CCAP 1459-11B	0.010	0.000	0.000
Abditibacterium	0.001	0.000	0.008
Hymenobacter	0.000	0.003	0.005
Subgroup 10	0.000	0.006	0.001
Arcicella	0.006	0.001	0.000
Candidatus Ovatusbacter	0.001	0.001	0.005
Adhaeribacter	0.001	0.000	0.005
Zoogloea	0.001	0.003	0.001
Chryseolinea	0.000	0.000	0.002
Unknown	37.964	41.049	38.830

Table S6 Comparison of rel. abundance change and correlation in the barley rhizosphere microbiome (bacterial effect).
Displayed are those genera where rel. abundance change and direction of correlation was associated with reduced aphid load. Only selected genera from the barley rhizosphere were selected that significantly increased or reduced in rel. abundance with *A. radicis* inoculation (ArN35e compared to the inoculated control, bacterial effect, see Figure S10). For changing genera comparing ArN35e and ArI_{neg} treatments (AHL effect) see Table 2.1. Directions of correlation correspond to Figure 2.11.

Changing genera	**rel. abundance**	**correlation**	**aphid load**
Rhodanobacter	reduced	+	reduced
Pseudomonas	reduced	+	reduced
Opitutus	increased	–	reduced
Pedobacter	reduced	+	reduced
Granulicella	reduced	+	reduced
Caulobacter	reduced	+	reduced
Lacunisphaera	increased	–	reduced
Sphingopyxis	reduced	+	reduced
Hirschia	reduced	+	reduced
Solimonas	reduced	+	reduced

Table S7 Significance values for detected AHL concentration.
Values correspond to Figure 2.11. Treatments groups are compared for the detectable concentration of synthetic AHLs with and without bacterial inoculation relative to the NoAHL control in plant growth medium after 21 days (data combined from AE1 and AE2 across 3 independent experimental runs, n = 17-24). Bacterial inoculation comprised *A. radicis* N35e (ArN35e), *A. radicis* AHL synthesis mutant (ArI_{neg}) and a control inoculum. OH-C10-HSL and C6-HSL were detectable in significantly elevated concentrations. Only significant comparisons are listed.

AHL concentration increase	**control**	**ArN35e**	**ArI_{neg}**
OH-C10-HSL – NoAHL	n.s.	0.0517 .	0.0236 *
C6-HSL – NoAHL	0.0035 **	0.0015 **	0.0736 .
OH-C10-HSL – C6-HSL	0.0734 .	n.s.	n.s.
OH-C10-HSL	**control vs. ArI_{neg}:** 0.0665 .		

Table S8 Significance values for impaired AHL sensing and signalling effects complemented with synthetic AHL addition on aphid suppression.
Values correspond to Figure 2.12. Bacterial groups and/or synthetic AHLs are compared for the effect size on aphid number per cm shoot length normalized by control plants within each experiment on 21 dpi. Bacterial inoculation comprised *A. radicis* N35e (ArN35e), *A. radicis* AHL synthesis mutant (ArI_{neg}), AHL sensing mutant (ArR_{neg}) and AHL double mutant ($ArR_{neg}I_{neg)}$ and a control inoculum. Axenic experiments AE1, AE2, AE3, AE4 combined, OH-C10-HSL: n = 21-38 and C6-HSL: n = 11-12. Only groups with at least one significant value are listed.

AHL effect	**OH-C10-HSL vs. NoAHL**	**C6-HSL vs. NoAHL**
with $ArR_{neg}I_{neg}$	0.0146 *	–
Bacterial effect	**with syn. OH-C10-HSL**	**with syn. C6-HSL**
ArN35e – control	0.0283 *	n.s.
ArN35e – ArI_{neg}	n.s.	n.s.
ArN35e – ArR_{neg}	0.0865 .	–
ArN35e – $ArR_{neg}I_{neg}$	0.0014 **	–
ArI_{neg} – control	0.0150 *	n.s.
ArI_{neg} – ArR_{neg}	0.0503 *	–
ArI_{neg} – $ArR_{neg}I_{neg}$	0.0015 **	–

Table S9 Significance values for impaired AHL sensing and signalling effects complemented with synthetic AHLs on plant growth.
Values correspond to Figure 2.13. Bacterial groups and/or synthetic AHLs are compared for the effect size on plant growth parameters normalized by the respective control plants within each experiment on 21 dpi. e) shoot length, f) root length, g) shoot biomass and h) root biomass. Bacterial inoculation comprised *A. radicis* N35e (ArN35e), *A. radicis* AHL synthesis mutant (ArI_{neg}), AHL sensing mutant (ArR_{neg}) and AHL double mutant ($ArR_{neg}I_{neg}$) and a control inoculum. Axenic experiments AE1, AE2, AE3, AE4 combined, OH-C10-HSL: n = 36-63 and C6-HSL: n = 23-27. Only groups with at least one significant value are listed.

AHL effect	**OH-C10-HSL vs. NoAHL**	**C6-HSL vs. NoAHL**
(e) with ArI_{neg}	0.0685 .	n.s.
(e) with $ArR_{neg}I_{neg}$	0.0246 *	–
(f) with ArI_{neg}	n.s.	0.0752 .
(g) with control	0.0766 .	n.s.
(g) with ArI_{neg}	n.s.	0.0101 *
(h) with control	0.0908 .	n.s.
(h) with ArI_{neg}	n.s.	0.0569 .
Bacterial effect	**with syn. OH-C10-HSL**	**with syn. C6-HSL**
(e) ArN35e – control	0.0911 .	n.s.
(e) ArN35e – $ArR_{neg}I_{neg}$	0.0057 **	–
(e) ArI_{neg} – control	0.0085 **	n.s.
(e) ArI_{neg} – $ArR_{neg}I_{neg}$	0.0002 ***	–
(g) ArN35e – control	0.0522 .	n.s.
(g) ArI_{neg} – control	n.s.	0.0354 *
(g) ArI_{neg} – ArN35e	n.s.	0.0012 **
(h) ArR_{neg} – control	0.0203 *	–
(h) ArR_{neg} – ArI_{neg}	0.0921 .	–

Table S10 Significance values for microbial cell numbers quantified by microscopic cell counting.
Values correspond to Figure 2.15. Fresh root samples were taken for a quantitative screen of bacterial colonization in axenic experiment AE6 and analyzed immediately without fixation. With Confocal Scanning Laser Microscopy, representative fields of view were selected randomly and adjusted so that a maximal number of cells was captured on micrographs (n = 24-31). Cells numbers were estimated per field of view (80 µm x 80 µm). On day 4, four data points of more than 50.000 cells in ArI_{neg} and $ArR_{neg}I_{neg}$ treatments were treated as outliers (i.e., NA) for statistics. Pairwise comparison of linear models are therefore corrected but still might have a small bias towards lower significant values. Only groups with at least one significant value are listed.

Bacterial groups	**day 4**	**day 7**	**day 10**
ArI_{neg} – control	0.0001 ***	0.025 *	< 0.0001 ***
ArI_{neg} – ArN35e	0.0005 ***	0.083 .	< 0.0001 ***
ArI_{neg} – ArR_{neg}	0.245	0.217	< 0.0001 ***
$ArR_{neg}I_{neg}$ – control	< 0.0001 ***	0.0001 ***	< 0.0001 ***
$ArR_{neg}I_{neg}$ – ArN35e	< 0.0001 ***	0.0004 ***	< 0.0001 ***
$ArR_{neg}I_{neg}$ – ArR_{neg}	0.054 .	0.002 **	< 0.0001 ***

Table S11 Significance values for microbial cell numbers quantified by CFU analysis.
Values correspond to Figure 2.16. Bacterial groups are compared for their presence in the barley rhizosphere after 4, 7, 10 and 21 dpi. Grinded root samples from independent axenic experiments AE3, AE4 and AE5 were plated out on the respective antibiotic plates. Cell numbers per g root fresh weight were counted back from colony forming units (CFU). (a-c) n = 3, (d) n = 12, (e-g) n = 8, (h) n = 12. Only groups with at least one significant value are listed.

(a) AE3	**day 4**	**day 7**	**day 10**	**day 21**
ArN35e – ArR_{neg}				0.037 *
ArR_{neg} – ArI_{neg}				0.080 *
ArR_{neg} – $ArR_{neg}I_{neg}$				0.002 **
$ArR_{neg}I_{neg}$ – control				0.035 *
(b-d) AE4				
ArN35e – ArI_{neg}		0.032 *	0.083 .	0.003 **
ArN35e – $ArR_{neg}I_{neg}$		0.044 *	n.s.	0.0008 ***
ArI_{neg} – control		0.085 .	n.s.	n.s.
ArI_{neg} – ArR_{neg}		0.080 .	0.083 .	0.003 **
ArI_{neg} – $ArR_{neg}I_{neg}$		n.s.	0.064 .	n.s.
ArR_{neg} – $ArR_{neg}I_{neg}$		n.s.	0.076 .	0.0008 ***
(e-h) AE5				
ArN35e – ArI_{neg}	0.091 .	0.055 .	n.s.	0.063 .
ArN35e – $ArR_{neg}I_{neg}$	0.010 *	n.s.	n.s.	n.s.
ArI_{neg} – control	n.s.	n.s.	0.076 .	n.s.
ArI_{neg} – ArR_{neg}	n.s.	0.06 .	n.s.	0.092 .
ArI_{neg} – $ArR_{neg}I_{neg}$	n.s.	0.007 **	n.s.	n.s.
ArR_{neg} – $ArR_{neg}I_{neg}$	0.020 *	0.077 .	n.s.	n.s.
$ArR_{neg}I_{neg}$ – control	0.050 *	n.s.	n.s.	n.s.

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