

Impaired microbial *N*-acyl homoserine lactone signalling increases plant resistance to aphids across variable abiotic and biotic environments

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Abstract

Beneficial bacteria interact with plants using signalling molecules, such as *N*-acyl homoserine-lactones (AHLs). Although there is evidence that these molecules affect plant responses to pathogens, few studies have examined their effect on plant-insect and microbiome interactions, especially under variable soil conditions. We investigated the effect of the AHL-producing rhizobacterium *Acidovorax radicans* and its AHL-negative mutant (does not produce AHLs) on modulating barley (*Hordeum vulgare*) plant interactions with cereal aphids (*Sitobion avenae*) and earthworms (*Dendrobaena veneta*) across variable nutrient soils. *Acidovorax radicans* inoculation increased plant growth and suppressed aphids, with stronger effects by the AHL-negative mutant. However, effects varied between barley cultivars and the presence of earthworms altered interaction outcomes. Bacteria-induced plant defences differed between cultivars, and aphid exposure, with pathogenesis-related and *WRKY* pathways partly explaining the ecological effects in the more resistant cultivars. Additionally, we observed few but specific indirect effects via the wider root microbiome where the AHL-mutant strain influenced rare OTU abundances. We conclude that bacterial AHL-signalling disruption affects plant-microbial interactions by inducing different plant pathways, leading to increased insect resistance, also mediated by the surrounding biotic and abiotic environment. Understanding the mechanisms by which beneficial bacteria can reduce insect pests is a key research area for developing effective insect pest management strategies in sustainable agriculture.

KEYWORDS

AHL-signalling, earthworms, ecological interactions, herbivores, microbiome, plant defence, plant resistance, rhizobacteria, soil nutrients

Oriana Sanchez-Mahecha and Sophia Klink contributed equally to this study.

[Correction added on 8 August 2022, after first online publication: The second affiliation has been updated in this version.]

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1 | INTRODUCTION

Plants live in close association with multiple soil microorganisms that can enhance plant growth and disease resistance (Berendsen et al., 2012). When under stress, plants release exudates from their roots, which have been hypothesized to serve as a cue to actively recruit bacteria from the surrounding bulk soil (Friman et al., 2020b; Pineda et al., 2012; Rolfe et al., 2019). Different plant species and genotypes within species have been shown to recruit different microbial communities (Marques et al., 2014; Xiong et al., 2021). During this process, plants and bacteria must communicate with each other (Hartmann & Schikora, 2012). Many Gram-negative bacteria produce signalling molecules such as *N*-acyl-homoserine lactones (AHLs) in the process of quorum sensing (QS) that allow bacteria to communicate with each other. Several studies have reported that plants also respond to synthetic AHL application (Mathesius et al., 2003; Schuhegger et al., 2006; Veliz-Vallejos et al., 2020). AHLs therefore might play an essential role in bacteria-plant interactions that lead to improved plant health and tolerance to biotic and abiotic stress (Hartmann et al., 2014; Schikora et al., 2016). For example, the application of *N*-3-oxo-hexanoyl homoserine lactones promoted primary root growth of *Arabidopsis thaliana* (Zhao et al., 2015), while short and long-chain AHLs enhanced the shoot growth and lateral root formation of barley plants (Rankl et al., 2016). Comparable effects have been reported after inoculation with beneficial bacteria that produce AHLs, for model and crop plants, including *Medicago truncatula*, *Oryza sativa*, and *Hordeum vulgare* (Figueiras et al., 2020; Han et al., 2016; Veliz-Vallejos et al., 2014). It is thought that AHL-producing bacteria benefit the plant by altering plant nutrient levels and inducing plant defences (Hartmann et al., 2021).

Interacting microorganisms, as well as feeding herbivores, are able to induce plant defences. These defences are mainly governed by the phytohormones jasmonic acid (JA), ethylene (ET), salicylic acid (SA), and abscisic acid (ABA) (Pieterse et al., 2009; Thaler et al., 2012; Vlot et al., 2021). Many phytohormones are induced by insect feeding or pathogen infection, with JA defences more often attributed to chewing herbivore defence while SA is linked to pathogen infection or feeding by sucking herbivores (Pieterse et al., 2009; Friman et al., 2020a). Beneficial bacteria have been shown to enhance plant defence responses against herbivores and pathogens through the coordination of similar phytohormonal pathways (Erb, 2019; Pieterse et al., 2014). However, microbial-induced resistance (MiR) against herbivores is still not well-understood, especially for sucking herbivores (Nalam et al., 2019; Rashid & Chung, 2017). MiR occurs when plant defence responses to insects are enhanced through associations with microbes (e.g., root-associated rhizobacteria). The mechanisms involved are diverse and may include a strengthening of the plant immune system through priming and induced systemic resistance (ISR), increased attraction of pest natural enemies through altering the herbivore-induced plant volatiles emission (HIPVs), and will likely expand to other novel pathways not yet uncovered (Trivedi et al., 2020; Zehnder et al., 2001). Recent work has shown that *Bacillus spp.* induced plant

resistance against sucking herbivores by stimulating the SA- and JA-dependent pathways in rice (Rashid et al., 2018), tomato (Valenzuela-Soto et al., 2010), and wheat (Veselova et al., 2019), leading to reduced herbivore growth. Similarly, in barley plants, systemic resistance in response to bacterial pathogen infection has been associated with JA/ABA or *WRKY* and ethylene response factors (Dey et al., 2014). *WRKY* appears to be associated with stress tolerance responses related to JA or ABA signalling pathways, or functions on the interface between JA and SA signalling (Dey et al., 2014; Morkunas et al., 2011). One study in *Arabidopsis* plants revealed that *WRKY* transcription factors were also induced in response to aphid attack (Kuśnierczyk et al., 2008). This suggests that there are multiple hormones and nodes regulating the signalling hormone networks involved in MiR against insects. It also highlights the complexity of such resistance and underlines the necessity to study plant-microbial signalling factors, like AHLs, that might represent important signals for the plant.

Given that AHL release by bacteria during quorum sensing is a fundamental signal for bacterial and bacteria-plant communication, it is plausible that AHL production by rhizobacteria could mediate their effects on the plant (Hartmann et al., 2021; Schikora et al., 2016). This includes changing the ability of a bacterium to promote plant growth or induce plant defences with subsequent effects on feeding herbivores. For example, plants might perceive a bacterium that produces AHLs as a 'friend', minimizing a plant pathogen response (Pieterse et al., 2014). In contrast, when a bacterial strain is no longer producing AHLs, it might be recognized differently, with the plant responding to it as a pathogen (an 'enemy') (Beneduzi et al., 2012; Dey et al., 2014; Vlot et al., 2009). Supporting this, barley plants inoculated with a no AHL-producing strain of *Acidovorax radialis* N35e, showed a stronger related flavonoid biosynthesis defence response pathway compared to the AHL-producing wildtype strain (Han et al., 2016). In contrast, Shrestha et al. (2019) found that barley plants inoculated with an AHL-producing *Ensifer meliloti* strain showed increased expression of pathogenesis-related genes (*PR1* and *PR17b*) compared to a non-producing strain, and was associated with higher plant resistance against the fungus *Blumeria graminis*. There is increasing evidence that microbial-AHLs also affect the plant response to herbivores (Heidel et al., 2009). Moreover, a recent study reported that AHL-producing bacteria modulate plant responses to insects dependent on plant genotype (Wehner et al., 2021). Therefore, further studies are needed to unravel the mechanistic role that AHLs may play in mediating microbe-induced resistance for having a better prediction for the outcome of beneficial bacteria effects on the plant.

Whether microbial signalling molecules, such as AHLs, influence the microbe-plant interaction with third parties like herbivores is unknown, and bacterial effects can be inconsistent, making their effects difficult to predict (D'Alessandro et al., 2014; Pangesti et al., 2015). This is especially challenging for implementing beneficial organisms in natural conditions where multiple factors are present, including a diverse microbial community and a soil-borne fauna, that might modulate the inter-kingdom signal exchange (Çakmakçı et al.,

2006; Hol et al., 2013; Pineda et al., 2013). Soil microbial communities are highly diverse and many members will sense the AHLs produced by inoculated bacteria, with consequences for soil community composition and functioning (Rodriguez et al., 2019). Simultaneously, other microorganisms in the rhizosphere can also produce their own AHLs and the enzymes to degrade them (e.g., lactonases), influencing the AHL-signalling of inoculated bacteria. By these indirect microbiome-mediated effects of bacterial inoculation, the presence or absence of AHLs could indirectly affect plant resistance to herbivores. Furthermore, larger invertebrate organisms can play an important role in shaping soil communities and soil-plant interactions. Within natural soils, earthworms are important ecosystem engineers, and themselves can affect plant growth and resistance against herbivores (Xiao et al., 2018) and alter the relative abundance of specific bacteria (Braga et al., 2016; Medina-Sauza et al., 2019). Importantly, previous work demonstrated that earthworms can increase microbe-induced plant resistance to aphids (Zytyńska et al., 2020). The earthworm effect on microbial communities has also been shown to depend on other abiotic factors, such as low-nutrient conditions (Koubová et al., 2015). Since plant-beneficial microbes are already known to have benefits for plants under abiotic stress (Berendsen et al., 2012; Carvalhais et al., 2013), it is necessary to consider all these factors simultaneously to get a full understanding of the role of microbes in inducing plant resistance to herbivores.

In this study, we inoculated barley plants with the aphid-suppressing bacterium *Acidovorax radicis* N35e (Zytyńska et al., 2020) using the wildtype strain (ArWT) and its AHL-mutant strain (not producing AHLs; ArAHL-). We evaluated if the presence/absence of microbial AHLs influences the effect of *A. radicis* reducing cereal aphid (*Sitobion avenae*) growth rates on three different barley cultivars, across earthworm treatments (*Dendrobaena veneta*) and soil nutrient levels. Moreover, we analysed the expression of target plant response genes and the rhizosphere microbiome to disentangle direct and indirect AHL effects on the plant-microbe-insect interaction.

We hypothesized that: (1) *A. radicis* inoculation increases plant growth and suppresses aphids, with increased effect under low nutrient conditions and earthworm presence in the soil. (2) Removal of AHLs (ArAHL-) alters the effect of the bacteria on insect growth. (3) *A. radicis* inoculation and aphid infestation alter plant defences, with variable effects depending on the strain inoculated (ArWT or ArAHL-), mediated by earthworms. (4) The effect of bacterial inoculation on aphid suppression occurs in part via indirect effects in the rhizosphere microbiome.

2 | MATERIALS AND METHODS

2.1 | Model system and experiment design

The present study was conducted in climate-controlled chambers at the Technical University of Munich Model EcoSystem Analyser research facility, Germany (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 these where the light was gradually increased/decreased). The model system included (1) three barley (*H. vulgare* L.) cultivars: Barke (Saatzucht Breun GmbH), Grace (Ackermann Saatzeit GmbH), and Scarlett (Saatzeit Breun GmbH); (2) two strains of the plant-beneficial rhizobacterium *A. radicis* (details below) plus a control with no bacterial inoculum; (3) presence/absence of epigeic earthworms *D. veneta* Rosa 1886 (obtained from wurmwelten.de); (4) presence/absence of *S. avenae* L. aphids and, (5) low/high nutrients using low nutrient pot soil (Floradur multiplication substrate, Floragard) supplemented with 25% strength Hoagland nutrient solution for the high nutrient soil or ddH₂O for the low nutrient soil (Hoagland & Aron, 1950).

We used a fully factorial experimental design (72 treatments with eight replicates) that was run across two temporal blocks (four replicates per block). Data collection occurred over four consecutive days within each block, since the experiment was part of a larger design incorporating abiotic effects that are not presented here. Two climate chambers were used in each temporal block (two replicates per chamber) to eliminate any chamber confounding effect; however, a climate chamber malfunction during the second run reduced the total number of replicates to six, for a total of 432 plants. Plants were placed in the chambers in trays (6 pots per tray) separated by bacteria treatment, and trays were randomized within replicates in the chamber.

2.2 | Bacterial treatments

We used *A. radicis* (wildtype strain, produces AHLs; ArWT), and *A. radicis aral::tet* which does not produce AHLs (ArAHL-) and a control (MgCl₂ only solution). The *A. radicis aral* knock-out mutant ArAHL- was generated by inserting a tetracyclin resistance gene into the *aral* gene responsible for AHL synthesis, as described in Han et al. (2016). The ArAHL- mutant was no longer able to produce OH-C10-HSL but still had its AHL sensing capacity. AHL-production was verified for both strains by a cross-streaking test against the AHL-biosensor *Agrobacterium tumefaciens* A136 (following Ravn et al., 2001). For microscopic detection, ArWT was chromosomally GFP-labelled by Li et al. (2012). ArAHL- was labelled via the plasmid pBBR1MCS-2-GFPmut3* by Han et al. (2016).

For inoculum preparation, the strains were grown on NB agar plates with kanamycin (50 µg/ml) and ampicillin (100 µg/ml) for ArWT and kanamycin (50 µg/ml) and tetracyclin (20 µg/ml) for ArAHL-. The bacterial lawn was resuspended in 10 mM MgCl₂ with Tween20 (100 µl/L). The optical density was adjusted to OD₆₀₀ = 1.0 [approx. 10⁸ colony-forming units (cfu/ml)].

2.3 | Insects

Cereal aphids (*S. avenae* L; genotype 'Fescue') were maintained as a lab population on the barley cultivar Kym for 4 years before the experiment. Cereal aphids (Hemiptera: Aphididae) are economically important pests producing high annual plant-yield losses by sucking

the plant phloem sap and transmitting viruses to the plant (Kamphuis et al., 2013). Additionally, they have biological characteristics that contribute to their vast capacity for exponential population growth, such as being parthenogenetic and viviparous organisms allowing them to reproduce without males and conferring the ability to shorten the time between birth and reproductive maturity (they are born with embryos that are complete and ready to be born). These characteristics mean that aphids can double the size of the population in each life cycle (Powell et al., 2007) maximizing their exploitation of the host plant and the multiplicity of the colony.

2.4 | Ecological experiment

Barley seeds were surface sterilized in 4% sodium hypochlorite solution and rinsed with tap water. The seeds were germinated between moist filter paper for 5 days in a dark cabinet at room temperature. After that, barley seedlings from all cultivars were inoculated with each of the three bacterial treatments (1) No inoculum, (2) ArWT, (2) ArAHL- by soaking the roots and the lower shoot part in the corresponding bacterial solution for one hour. The root and shoot lengths of each seedling were recorded and similar-sized seedlings within each bacterial treatment were transplanted directly into 10 cm diameter pots with potting soil, previously fertilized with 30 ml of either low (water) or high (25% Hoagland's solution) nutrients. Plants were watered three times per week, and nutrient solution addition was repeated after 14 days of seedling planting. Two days posttransplantation, plant height was measured from the base of the shoot until the tip of the longest leaf and chlorophyll content was recorded using a chlorophyll measurement device (Konica Minolta SPAD-502).

From Day 6, we added four medium-sized earthworms (combined weight of 1.2–1.8 g) directly in the plant soil and covered the base of the pot with organza mesh secured by an elastic band to prevent earthworm escape. Earthworms were cleaned and were starved for 24h as described in Zytynska et al. (2020). After that, two preadult aphids (4th instar) were added to the base of the plant shoot in the aphid treatment plants. All plants were covered with an air-permeable cellophane bag (HJ Kopp GmbH) secured with an elastic band to prevent aphid movement between pots.

Two weeks after aphid addition (Day 21 post plant transplantation), the total number of aphids and the remaining earthworms were counted and final plant variables were measured: plant shoot length, shoot fresh weight, chlorophyll content, root length (longest root), and root fresh weight. The roots were cleaned using 1X phosphate-buffered saline (PBS) solution per bacterial treatment, then shoot and root samples were quickly frozen in liquid nitrogen and stored at -80°C for further analyses.

2.5 | Plant gene expression analysis

From the initial three cultivars, we selected barley cultivars Barke and Scarlett for gene expression analysis across bacteria, aphid, and

earthworm treatments. These cultivars strongly differed in responses to aphids. Barke was overall resistant to aphids and had fewer numbers compared to Scarlett, which was the cultivar with the highest aphid numbers, but where the bacteria had the strongest effect. We focused on target genes that have been reported to be associated with barley plant defences induced by bacteria and are also relevant for plant-aphid interactions (Delp et al., 2009). For this, we selected two pathogenesis-related genes: *PR1* and *PR17b*, ethylene-responsive factor (*ERF*), *WRKY-TF22*, and *UGT*-related flavonoid biosynthesis genes (for primer sequences, see Table S1). As a calibrator for the gene expression analysis, we used Elongation factor (*HvEF1a*; housekeeping gene).

We pooled shoot tissue from the two biological replicates of the same treatment within each chamber per run from the ecological experiment. Since we had six biological replicates, the pooling step left a final three pooled replicates. The pooled replicates were then homogenized in liquid nitrogen and RNA was isolated using the RNeasy Plant Mini Kit (Qiagen), following the manufacturer's protocol. Subsequently, cDNA was synthesized from 1.5 μg total RNA by reverse transcription with SuperScript II (Thermo Fisher). Relative quantitative PCR (qPCR) was performed with three technical replicates per sample, on a qPCR-machine PeQlab (VWR) according to the mix preparation protocol of the SensiMix SYBR Low ROX Kit (Bioline) with the primers listed in Table S1. The qPCR threshold cycle (*Ct*) values were normalized by averaging the technical replicates. Afterward, ΔCt was calculated by subtracting the *Ct* average values of each gene of interest minus the *Ct* average value of the endogenous control Elongation factor (*HvEF1a*; housekeeping gene) for each sample ($N = 71$, one replicate of Barke cultivar was lost during the experiment due to the death of both biological replicates).

2.6 | Microbiome

To analyse the rhizosphere community composition, we selected three root samples of each barley cultivar across bacteria, aphid, and earthworm treatments ($N = 108$ samples). Microbial DNA was extracted from 0.2 to 0.4 g roots with closely attached soil (FastDNA SPIN Kit for Soil, MP Biomedicals). Extracted material comprised DNA from the root-rhizosphere complex including the endosphere and will be termed 'rhizosphere' in the following. The V4-V5 region of the 16S rRNA gene was amplified with the primers 515-F and 806-R (Table S1). Illumina MiSeq Sequencing resulted in 20.7 Mio total paired-end reads and 12.4 Mio paired-end reads after quality filtering. The retrieved data were processed with a Perl-based remultiplexor and the IMNGS platform (Lagkouravdos et al., 2016) using the UPARSE algorithm (Edgar, 2013). An initial cutoff threshold of 0.001% relative abundance was applied and resulted in 639 classified OTUs with 181 known and 108 unknown genera. Group comparison, diversity, and correlation analysis was done with the Rhea pipeline for R (Lagkouravdos et al., 2017). The data set used in this study is available through GenBank (accession numbers: OM238298-OM240180).

2.7 | Temporal patterns in *A. radialis* colonization on barley roots

To detect the inoculated *A. radialis* strains on the roots, two separate and independent time series experiment were conducted. Barley seeds of cultivar Scarlett were sterilized, germinated and inoculated with bacterial treatments as described above. Plants were grown in phytochambers in trays (23°C, 12:12 h day-night cycle, 55% relative humidity). Roots of each bacterial treatment were harvested and washed in 1x PBS on Days 4, 7, 10, 14, 18, and 21. For microscopy, root pieces of 1 cm length were cut from various positions, fixed in 1:1 (v/v) EtOH-PBS, and stored at -20°C until use. Microscopic pictures were taken with a confocal laser scanning microscope (Zeiss LSM 880) using the software ZEN black 2.3 SP1. GFP-labelled bacteria were visualized by argon laser excitation at 488 nm (emission filter BP 495-550 + LP 570). DPSS and helium-neon laser lines at 561 nm (BP 570-620 + LP 645) and at 633 nm (BP 570-620 + LP 645) were used as control channels.

2.8 | Statistical analysis

2.8.1 | Aphid and plant data performance analysis

The analysis was performed in RStudio version 1.4.1106 using R version 4.0.5 and data was analysed through standard linear models, where the model plant response ($N = 423$, 5-6 replicates per treatment), were: (1) chlorophyll at Day 21, (2) shoot fresh weight (g), (3) root fresh weight (g). For the insect response ($N = 210$, 5-6 replicates per treatment), aphid load per gram (total number of aphids divided by the plant shoot fresh weight) was used to control the plant size effect on the aphids since we observed more aphids on larger plants (Figure S1). All full models contained the main effects (barley, nutrient, bacteria, earthworm, and aphid) and their interactions with the experimental run nested within the harvest-day (2 days: early and late harvest) as blocking factors to control the variation across the temporal blocks and the variation across the days in the aphid/earthworm addition and data collection. Model simplification was done using the backward stepwise method and we present the minimal adequate model results (Table 1). After running the models, we found strong main effects that were visualized using raw data; while for the interactions, plant and insect data relative to the control plants (without bacteria) within harvest, barley genotype, earthworm, and aphid (just for the plant variable) was used. This was used to first control for strong main effects of the models and second, to further, explore and elucidate the bacteria interactions across biotic and abiotic treatments (i.e., the context-dependency of the effects).

2.8.2 | Gene expression analysis

Significance differences in the gene expression of the plants inoculated with *A. radialis* strains compared to control plants (without

bacteria) across the treatments (barley, aphid, earthworm) were determined by mixed effect models (performed with RStudio 1.4.1106, using R version 4.0.5, Packages: car, lmer). The response variable of the model was the normalized log 2-fold change ($2^{-\Delta\Delta Ct}$) values of each gene (relative effect compared to control) with run as a random effect (temporal block), and the fixed factors were the barley cultivar, bacteria treatment (ArWT vs ArAHL-), earthworm treatment and aphid treatment. The normalized log 2-fold change was calculated by subtracting the ΔCt of the plants inoculated with *A. radialis* strains to the ΔCt of the control plants from each treatment group (with/without aphids, with/without earthworms). The resulting $\Delta\Delta Ct$ value was used for the fold change calculation $\log(2^{-\Delta\Delta Ct}; 2)$.

2.8.3 | Microbiome analysis

Microbiome results were statistically evaluated in RStudio 1.2.1335 with R version 3.6.0 using the Rhea pipeline (Lagkouravos et al., 2017). Taxa were classified as common when the relative abundance mean across all samples was >1% and as rare when <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. Medium and low abundant genera were split into smaller groups due to restrictions in the number of response variables per model. Final p -values were calculated by a MANOVA including all identified genera as dependent response variables. The model omitted additional interactions. For correlation analysis, those genera were selected that showed either a significant change ($p < 0.05$) in relative abundance across all bacteria and in both earthworm treatments independently, or a significant aphid effect. Pearson's correlation coefficients were calculated and visualized with the Rhea pipeline for R.

3 | RESULTS

3.1 | Effect of bacterial inoculation, earthworms and its interactions on plant and aphid growth

Bacterial inoculation influenced plant biomass (Figure 1a; Table 1). Barley cultivars differed in weight with smaller Scarlett plants than the others and, as expected, earthworms strongly increased plant biomass while aphids decreased it (Figure 1a) and nutrient addition had no effect. Importantly, the strength of these main effects was context-dependent, resulting in a four-way interaction on shoot biomass (barley cultivar \times bacteria \times earthworm \times aphid: Figure 1b, Table 1). Specifically, the variation in effect sizes across different treatments differed between cultivars and was stronger for Scarlett than Barke. Overall, on Scarlett, we observed increased plant growth when earthworms were present for both *A. radialis* strains irrespective of aphid presence but this was lost when earthworms were absent, except when aphids were also present on ArAHL- plants (Figure 1b). For Barke, we observed fewer growth-promotion effects and these

TABLE 1 Summary of plant and aphid responses to barley cultivar, soil nutrients, plant bacterial inoculation, earthworms, and aphids

	d.f.	Shoot biomass		Root biomass		Chlorophyll		Aphid load	
		F	p	F	p	F	p	F	p
Blocking factor	6	120.86	<0.001	27.34	<0.001	9.63	<0.001	15.73	<0.001
Cultivar	2	23.35	<0.001	4.93	0.008	44.24	<0.001	5.29	0.006
Nutrient	1	1.29	0.257	0.15	0.703	3.71	0.055	-	-
Bacteria	2	5.70	0.004	2.50	0.084	0.58	0.563	7.00	0.001
Earthworm	1	692.78	<0.001	49.67	<0.001	60.67	<0.001	16.06	<0.001
Aphid	1	21.45	<0.001	0.71	0.401	0.12	0.726	na	na
Cultivar × nutrient	2	5.01	0.007	1.62	0.200	-	-	-	-
Cultivar × bacteria	4	2.10	0.080	-	-	1.98	0.096	3.81	0.005
Nutrient × bacteria	2	1.47	0.231	-	-	0.73	0.482	-	-
Cultivar × earthworm	2	2.28	0.103	0.15	0.863	-	-	-	-
Nutrient × earthworm	1	0.04	0.848	2.81	0.095	-	-	-	-
Bacteria × earthworm	2	0.68	0.508	2.16	0.117	2.69	0.069	-	-
Cultivar × aphid	2	0.63	0.535	0.67	0.510	-	-	na	na
Nutrient × aphid	1	0.05	0.824	0.00	0.947	0.07	0.796	na	na
Bacteria × aphid	2	1.56	0.211	-	-	1.29	0.278	na	na
Earthworm × aphid	1	0.52	0.471	0.77	0.381	-	-	na	na
Cultivar × nutrient × earthworm	2	2.74	0.066	0.05	0.955	-	-	-	-
Cultivar × bacteria × earthworm	4	1.53	0.192	-	-	-	-	-	-
Nutrient × bacteria × earthworm	2	2.02	0.134	-	-	-	-	-	-
Cultivar × nutrient × aphid	2	2.70	0.068	0.30	0.743	-	-	na	na
Cultivar × bacteria × aphid	4	1.73	0.143	-	-	-	-	na	na
Nutrient × bacteria × aphid	2	-	-	-	-	4.67	0.010	na	na
Cultivar × earthworm × aphid	2	0.96	0.385	1.26	0.285	-	-	na	na
Nutrient × earthworm × aphid	1	-	-	0.44	0.509	-	-	na	na
Bacteria × earthworm × aphid	2	0.42	0.659	-	-	-	-	na	na
Culti. × nutr. × worm × aphid	2	-	-	3.30	0.038	-	-	na	na
Culti. × bact. × worm × aphid	4	2.89	0.022	-	-	-	-	na	Na
Residuals		371		392		398		197	

Note: Significant effects in bold ($p < 0.05$). Terms not included in mode (na) and terms removed from minimal adequate model after model simplification (-).

were not affected by earthworm treatments but differed between aphid treatments (Figure 1b). Plants inoculated with ArAHL- showed some increased growth only when there were no aphids, while ArWT plants showed reduced growth compared to control plants. Finally, on Grace we observed no consistent bacterial inoculation patterns on shoot biomass as effects strongly differed across the aphid and earthworm treatment combinations (Figure 1b). Root biomass was increased by earthworms, varied with barley cultivar and the other factors had no effects (Table 1).

Leaf chlorophyll content varied across plant cultivars, with the highest chlorophyll observed in Scarlett, followed by Barke and Grace and was increased by earthworms (Figure 1c). Bacteria, aphids, and

nutrient addition had no main effects on the chlorophyll content; however, their effects depended on each other (nutrient × bacteria × aphid; Figure 1d, Table 1). Specifically, inoculation of bacteria was affected by aphid presence; however, this effect was specific for the bacteria-nutrient combination. Overall, an increase in chlorophyll content was observed in ArAHL- inoculated plants in both nutrient levels but was specifically reduced when aphids were present together with low nutrient soil conditions. A similar effect was found on ArWT with the difference that chlorophyll content was reduced specifically on high nutrient soil conditions in combination with aphid presence.

Aphid load (number of aphids per gram of plant 14 days after addition) was reduced on bacteria inoculated plants compared to

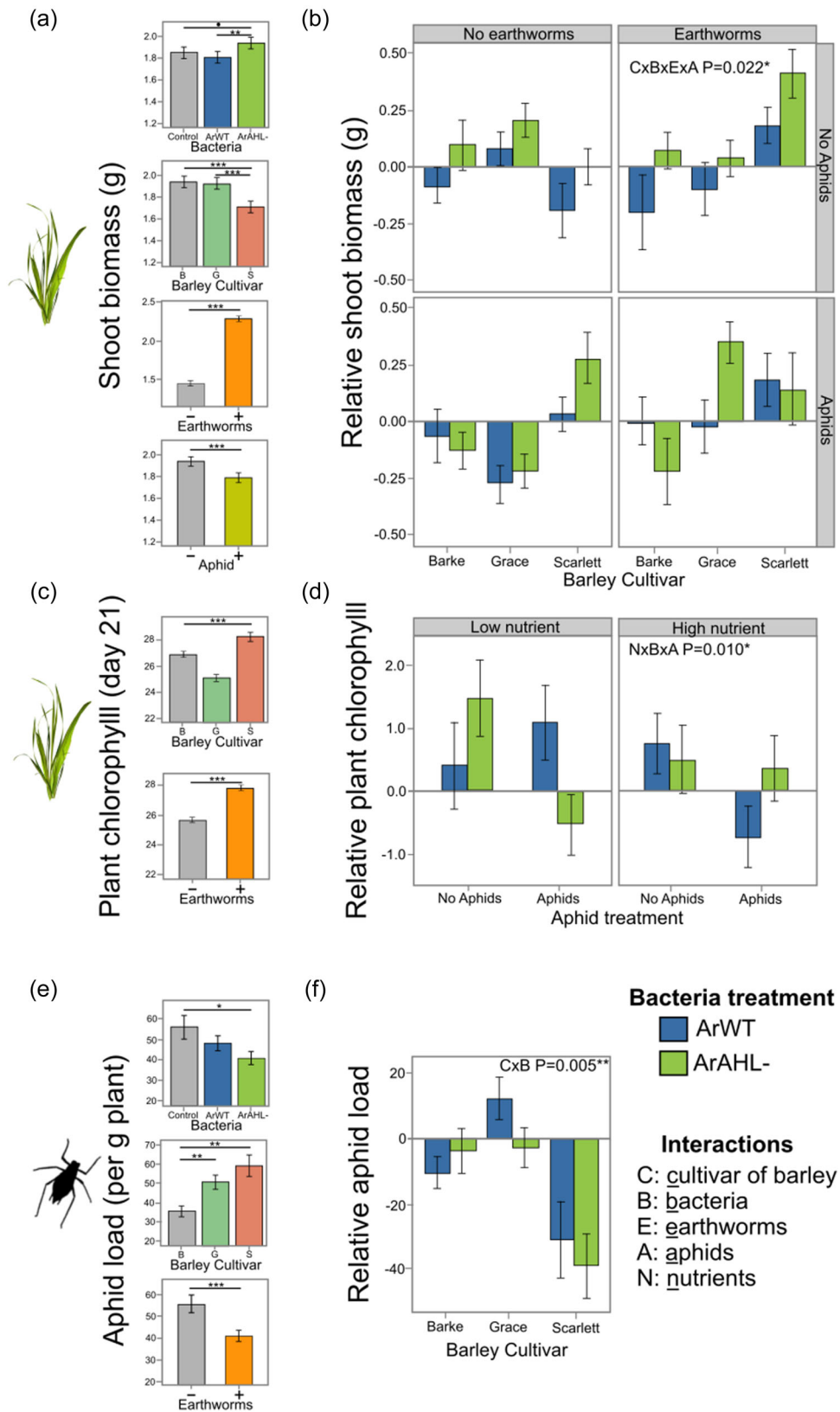


FIGURE 1 (See caption on next page)

control plants (Figure 1e, Table 1). Aphid load varied across barley cultivars with a lower aphid load on Barke compared with Grace and Scarlett (Figure 1e). Importantly, bacterial inoculations interacted with barley cultivar (barley cultivar \times bacteria; Figure 1f, Table 1), with stronger suppression on Scarlett by both strains while ArWT induced suppression in Barke but not Grace plants. Earthworms also decreased aphid load across all bacteria and barley cultivars (Figure 1e), while nutrient addition had no main effect. Similar patterns were found for aphid number (Figure S2) while aphid load is presented here as it controls for the plant growth effects of bacterial inoculation (Figure 1a) since larger plants tended to host large population of aphids irrespective of bacterial treatment (Figure S1).

3.2 | Effect of bacterial inoculation and earthworms on defence-related gene expression responses to aphids

To investigate how beneficial bacteria mediated plant defences response to aphid exposure across earthworm presence and absence, a transcriptional profile of five genes were made from plants inoculated with *A. radialis* strains: ArWT (produces AHLs) and ArAHL- (does not produce AHLs) relative to control plants (without bacteria). Here we compared responses in the aphid-susceptible cultivar Scarlett (stronger microbe-induced resistance to aphids) to those in the more resistant cultivar Barke (weaker microbe-induced resistance). For the two pathogenesis-related (*PR*) genes, the relative expression strongly differed between barley cultivars (Figure 2a,b; Table 2) with an overall higher relative expression of the *PR* genes in Barke and lower relative expression in Scarlett. Importantly, bacterial inoculation effects on *PR1* gene expression differed between cultivars (cultivar \times bacteria; Figure 2a), with a higher relative expression of *PR1* in plants inoculated with ArWT and ArAHL- in Barke, and lower relative expression in Scarlett inoculated with ArAHL-. Across both cultivars, a stronger response was observed in plants inoculated with ArAHL- (Figure 2a,b). For both *PR* genes, aphid presence also affected the relative expression of the plants (Figure 2a,b; Table 2). Furthermore, for both genes, the difference in the response of the cultivars depended on earthworms and aphid presence (cultivar \times earthworm \times aphid; Figure 2a,b). On Barke, the relative expression of *PR1* and *PR17b* was higher when aphids were present in the absence of earthworms, but when earthworms were present, there was minimal change in gene expression compared to control plants. On the contrary, for Scarlett bacterial inoculation resulted in a lower relative gene expression when aphids were

present, while the relative expression of *PR1* was increased when aphids and earthworms were present (Figure 2a), with no difference to control plants for *PR17b* (Figure 2b).

The relative expression of the ethylene-responsive factor-*ERF* was significantly affected by the bacteria inoculations (Figure 2c; Table 2) but did not differ between cultivars. With a higher relative expression in ArAHL- inoculated plants and lower relative expression in ArWT inoculated plants compared to control plants (Figure 2c). However, the relative expression of *WRKY-TF22* differed between cultivars (Figure 2d); with a higher relative expression observed in ArAHL- inoculated Barke plants compared to Scarlett. Furthermore, *ERF* expression was affected by aphid presence (Table 2), while no main effect of aphids was observed on *WRKY-TF22* relative expression. Importantly, the *ERF* and *WRKY-TF22* expression across barley cultivars depended on aphid and earthworm presence (Table 2). Specifically, on Barke higher relative expression of *ERF* and *WRKY-TF22* was observed on plants with aphids and without earthworms with opposite effects when earthworms were present (Figure 2c,d). However, on Scarlett reduced expression of *ERF* and *WRKY-TF22* was observed when aphids were present without earthworms that changed when earthworms were present to a minimal change on the relative gene expression or similar expression compared to control plants (Figure 2c,d).

Additionally, relative *UGT*-related flavonoid biosynthesis gene expression did not differ between *A. radialis* strains or between barley cultivars (Table 2). However, a tendency of higher gene expression was observed on ArAHL- in Barke and in ArWT and ArAHL- Scarlett inoculated plants (Figure 2e). Moreover, *UGT* relative expression was consistently higher when aphids were present (Table 2). However, its expression across cultivars was context-dependent on aphid and earthworm presence (cultivar \times earthworm \times aphid; Figure 2e, Table 2). When aphids were absent, there was no difference between inoculated and control plants with earthworms but when earthworms were absent, relative expression was reduced on Barke and increased on Scarlett (Figure 2e).

3.3 | Temporal patterns in *A. radialis* colonization on barley roots

In two additional sets of plants, both *A. radialis* strains were clearly present on barley roots 4 days post bacterial inoculation (Figure 3). After 7 and 10 days, ArWT cells were no longer present in visible quantities (potentially an artefact due to less stable labelling, see Figure S3a,b) while fewer cells of ArAHL- could still be detected

FIGURE 1 Effects of bacterial inoculation, barley cultivar, earthworms and aphids on (a) plant biomass (main effects), (b) relative plant biomass (bacteria to control) and interactions, (c) plant chlorophyll (main effects), (d) relative plant chlorophyll (bacteria to control) and interactions, (e) aphid load using the number of aphids per gram of fresh plant biomass (main effects) and (f) relative aphid load (bacteria to control). The relative effects are bacteria inoculated plants compared to control plants within barley cultivar, earthworm, and aphid treatment, per harvest block within the experimental run (see methods). Error bars \pm 1SE ($n = 6$ replicates). *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$, • $p < 0.1$.

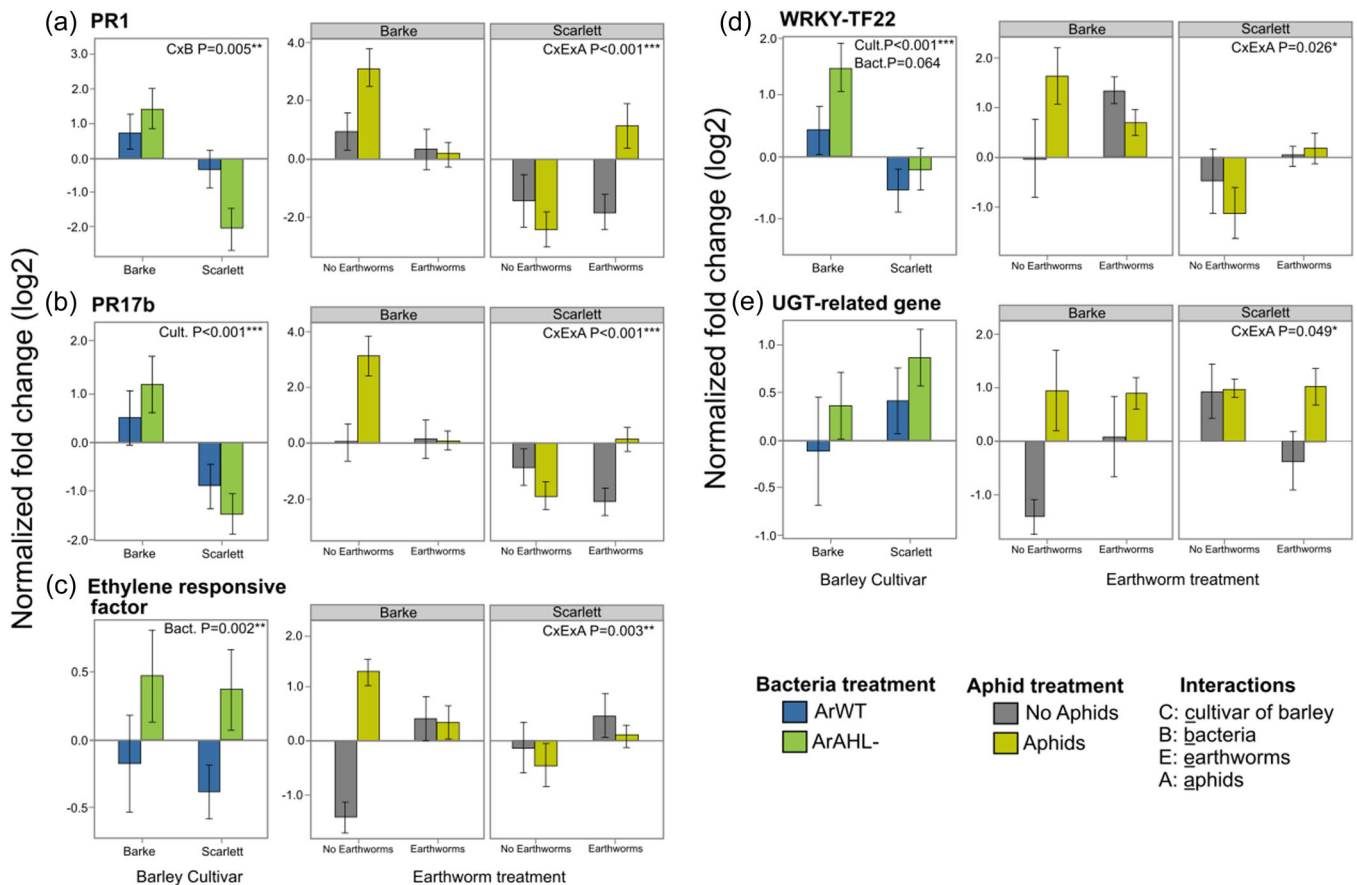


FIGURE 2 Relative gene expression of plants inoculated with *Acidovorax radicis* wildtype (ArWT) or AHL-negative mutant (ArAHL-) compared to control plants, across aphid and earthworm treatments. Expression levels across bacteria strains by cultivar, and across earthworm and aphid treatments by cultivar, for (a) PR1, (c) PR17b, (e) Ethylene responsive-factor (g) WRKY-TF22, (i) UGT-related gene. Values given as the normalized log 2-fold change ($2^{-\Delta\Delta Ct}$). Ct values from genes were normalized to the housekeeping gene EF1 α , and $\Delta\Delta Ct$ values were calculated from plants inoculated with relevant *A. radicis* strain relative to control plants (see Section 2), graphs for the effect of earthworm and aphids average across bacteria strain (no interaction with bacteria, Table 2). Error bars \pm 1SE. $n = 3$ replicates, each replicate is a pool of two biological replicates (within-chamber experimental replicates). [Color figure can be viewed at wileyonlinelibrary.com]

(Figure S3c,d). However, a diagnostic PCR targeting the GFP gene also revealed the presence of ArWT cells up to Day 10 (data not shown). From Day 14 onwards, both *A. radicis* strains could no longer be detected by microscopic measures nor by diagnostic PCR.

3.4 | Effect of *A. radicis* on the microbial community composition

A. radicis wildtype (ArWT) inoculation did not change the barley rhizosphere community composition (PERMANOVA, $p = 0.26$, Figure 4a) while in *A. radicis* AHL-mutant treatments (ArAHL-) the microbiome separated significantly from ArWT ($p = 0.009$, Figure 4b) and the control ($p = 0.0495$, Figure 4c). The relative abundance of rare OTUs (<1%) were more affected than common OTUs (>1%) ($p = 0.001$ vs. $p = 0.489$; Figure 5a,b). Species richness and evenness were consistent across the bacterial treatments (Figure S4). Earthworm treatment strongly changed the microbial community ($p = 0.001$; Figure 4d). In contrast,

barley cultivars and aphid treatments did not have a significant impact on the rhizosphere microbiome ($p = 0.769$ and $p = 0.974$ respectively; Figure 4e, Figure S5).

Eighteen genera showed a significantly altered relative abundance in the rhizosphere when inoculated with either one of the two *A. radicis* strains including most prominently the genera *Afipia*, *Bdellovibrio*, *Lacunisphaera*, *Limnobacter*, *Rhodanobacter*, and *Sphingopyxis* (bacterial main effect, $p < 0.05$, Table 3). Sixteen of these genera also showed a significantly different response between ArWT and ArAHL- treatments (AHL effect). Eleven of 16 genera increased in relative abundance when treated with ArAHL- (e.g., *Rhodanobacter*, *Pseudomonas*, *Microbacterium*, *Caulobacter*, *Granulicella*, and *Pedobacter*), while only a few genera decreased (*Lacunisphaera*, *Spirochaeta 2*). Some genera increased (*Opiritutus*, *Bdellovibrio*) and decreased (*Limnobacter*) with ArWT treatment. The majority belong to rare OTUs while only *Pseudomonas*, *Rhodanobacter* and *Opiritutus* were common community members. In total, three genera were significantly changing upon aphid treatments: *Dyadobacter*, *Hirschia*, and

TABLE 2 Summary of plant gene expression levels for five defence-related plant genes across barley cultivar, bacterial inoculation, earthworm, and aphid treatments

	d.f.	PR1		PR17b		Ethylene responsive factor		WRKY-TF 22		UGT-related gene	
		χ^2	<i>p</i>	χ^2	<i>p</i>	χ^2	<i>p</i>	χ^2	<i>p</i>	χ^2	<i>p</i>
Cultivar	1	27.48	<0.001	27.30	<0.001	0.49	0.485	13.18	<0.001	2.00	0.157
Bacteria	1	1.56	0.211	0.02	0.891	9.65	0.002	3.43	0.064	1.62	0.203
Earthworm	1	0.06	0.810	1.90	0.168	5.12	0.024	2.58	0.108	0.01	0.910
Aphid	1	5.23	0.022	7.43	0.006	4.60	0.032	0.16	0.691	9.73	0.002
Cultivar × bacteria	1	7.73	0.005	2.55	0.110	0.06	0.813	0.88	0.349	0.00	0.983
Cultivar × earthworm	1	15.03	<0.001	5.78	0.016	0.07	0.792	0.97	0.325	3.41	0.065
Bacteria × earthworm	1	1.00	0.317	0.06	0.812	0.02	0.887	1.61	0.205	1.13	0.287
Cultivar × aphid	1	0.00	0.980	1.43	0.231	13.45	<0.001	1.18	0.277	1.40	0.237
Bacteria × aphid	1	0.39	0.534	0.11	0.745	0.61	0.434	0.31	0.578	0.47	0.493
Earthworm × aphid	1	0.84	0.359	0.00	0.946	9.34	0.002	1.17	0.280	0.02	0.881
Cultivar × bacteria × earthworm	1	0.04	0.852	0.04	0.843	0.12	0.728	0.06	0.804	0.01	0.920
Cultivar × bacteria × aphid	1	0.02	0.895	0.00	0.995	0.35	0.557	1.13	0.287	0.53	0.469
Cultivar × earthworm × aphid	1	12.87	<0.001	16.92	<0.001	8.90	0.003	4.96	0.026	3.87	0.049

Note: Significant effects in bold ($p < 0.05$).

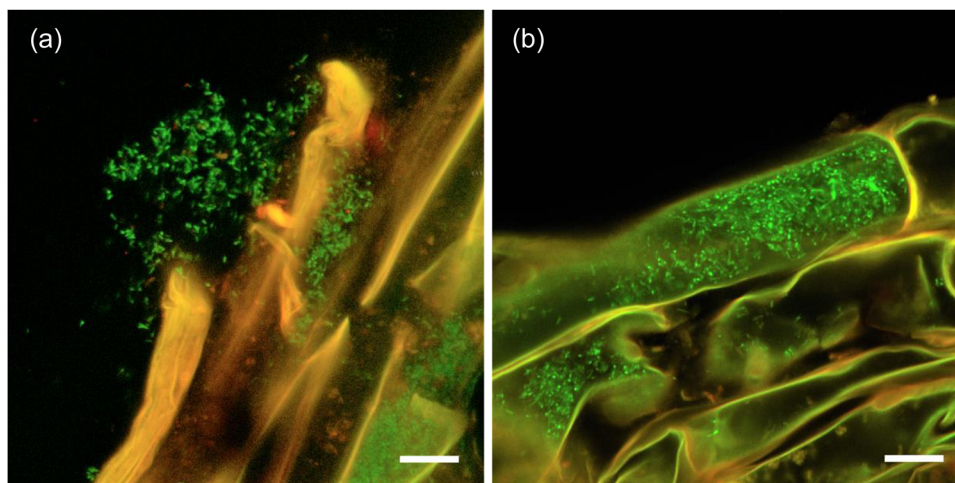


FIGURE 3 Confocal micrographs of *Acidovorax radicis* colonization on barley (*Hordeum vulgare*) roots. Four days after inoculation, (a) *A. radicis* wildtype (ArWT) cells and (b) *A. radicis* AHL-negative mutant (ArAHL-) cells were detectable in the rhizosphere in high quantities. ArWT cells were chromosomally GFP-labelled. ArAHL- cells were GFP-labelled on plasmid level. GFP-labelled cells are shown in green, root background is shown in yellow, soil particles appear in red. Micrographs were taken from independent time series experiments in soil. Scale bar = 10 μ m. [Color figure can be viewed at wileyonlinelibrary.com]

Stenotrophomonas (Aphid effect). Relative abundances of all known genera are depicted in Table S2.

To examine the role of these changing genera on aphid suppression, we correlated bacterial relative abundances and aphid loads (Figure 5c). One notable positive association (aphid growth facilitation) was with *Bdellovibrio* in control treatments, which became less positive for plants inoculated with either *A. radicis* strain, suggesting reduced aphid growth

facilitation. Negative associations indicate a role in aphid suppression, e.g., if aphid loads are low while bacterial abundances are high. However, we observed few consistent effects that would suggest clear microbiome-wide effects on aphid suppression through *A. radicis* inoculation. Most interestingly, *Solimonas*, *Sphingopyxis* and *Hirschia* turned from positive associations in ArWT samples to negative in ArAHL- samples. Yet, this was only when there were no earthworms;

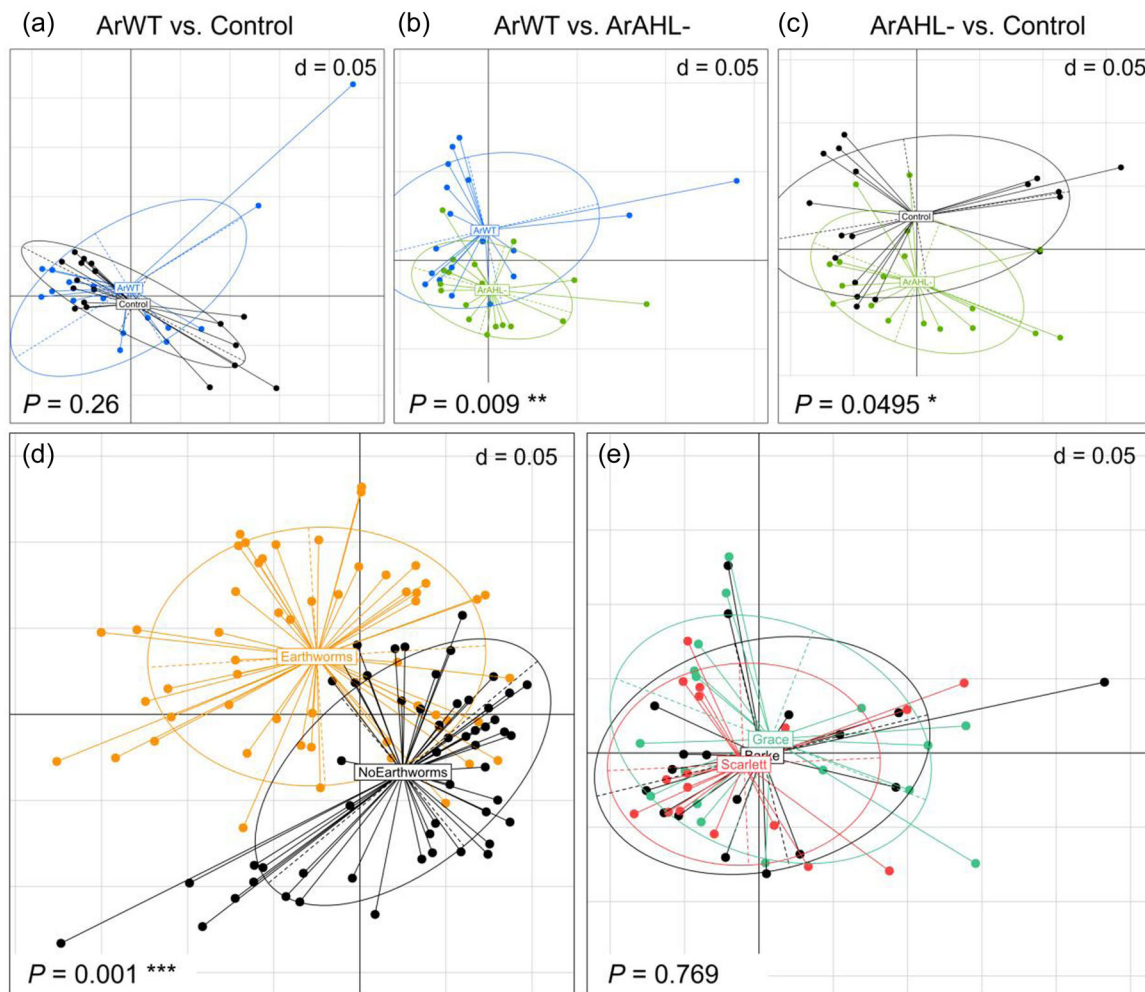


FIGURE 4 Microbiome profiles of the barley (*Hordeum vulgare*) rhizosphere after treatment with bacteria (*Acidovorax radicus*), aphids (*Sitobion avenae*) and barley cultivars in the absence of earthworms (*Dendrobaena veneta*). Depicted are multi-dimensional scaling plots. Similarity of microbial profiles 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 groups treated with AHL-producing *A. radicus* wildtype (ArWT, blue) did not separate significantly from not inoculated groups (Control, black). (b) Microbial groups treated with the *A. radicus* AHL-mutant (ArAHL-, green) separated significantly from ArWT (blue). (c) Microbial groups treated with ArAHL- (green) separated significantly from the Control (black). (d) Microbial profiles differed significantly between Earthworms (orange) and NoEarthworms (black) treatment. (e) Microbial profiles for barley cultivars Barke (black) versus Grace (green) versus Scarlett (salmon) showed no significant differences. [Color figure can be viewed at wileyonlinelibrary.com]

when earthworms were present the association was consistent across bacterial strains. Lastly, we observed the tendency that correlations were less pronounced in ArAHL- treatments compared to ArWT and the control, e.g., in *Caulobacter*, *Cellulomonas*, *Opitutus* and *Rhodanobacter*. The relative abundances of all changing genera are depicted in the supplemental material comparing all bacterial effects (Figure S6) and AHL effects (Figure S7).

4 | DISCUSSION

Our study shows that the effect of the beneficial rhizobacterium *Acidovorax radicus* on plant growth and aphid suppression was altered by the ability of the inoculated bacteria to produce AHL signalling

molecules, dependent on the plant cultivar. Averaged across all plant cultivars and environmental treatments, inoculation with the AHL-negative mutant (ArAHL-) strain increased aphid suppression compared with the AHL producing wildtype strain (ArWT). This effect was strongly dependent on the plant cultivar indicating genetic diversity in responses to AHL-bacterial production. Our work confirms previous findings that beneficial rhizobacteria largely negatively affect herbivores' performance across variable environments (Heinen et al., 2018; Xi & Zytynska, 2022; Zytynska et al., 2020). We also hypothesized that plant growth and aphid suppression effects would be enhanced under low nutrient conditions but found few effects of soil nutrients. Based on previous work (Zytynska et al., 2020) we also hypothesized that effects would be increased with earthworm presence in the soil, and this was largely

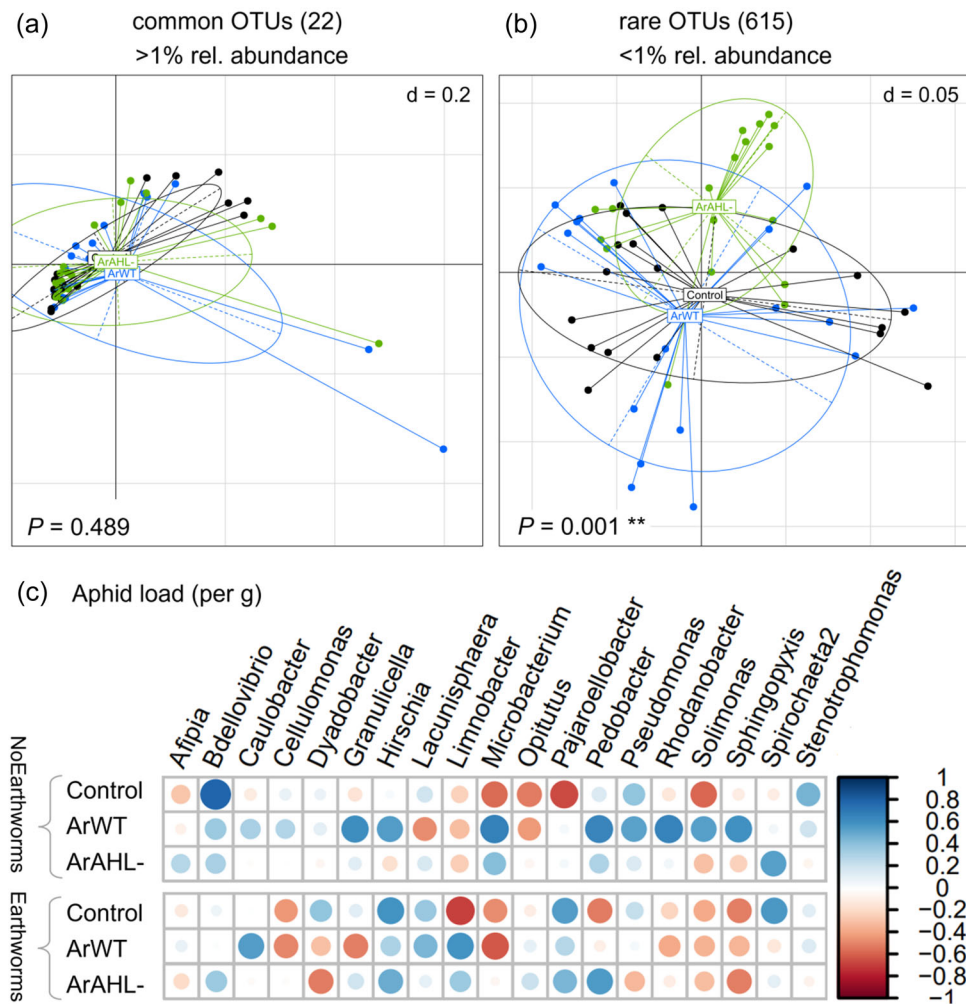


FIGURE 5 Microbiome profiles of OTU groups and aphid-bacteria correlations in the barley rhizosphere (*Hordeum vulgare*). (a and b) Depicted are multi-dimensional scaling plots of the beta-diversity in the absence of earthworms (*Dendrobaena veneta*). Similarity of microbial profiles was calculated using a generalized UniFrac distance matrix. Permutational multivariate analysis of variance (vegan::adonis) was performed to determine significant separation of bacterial treatments per OTU group. OTUs were classified as common or rare based on the relative abundance mean across all samples of one treatment. Groups are separated for *Acidovorax radicis* wildtype (ArWT, blue), *A. radicis* AHL-negative mutant (ArAHL-, green) and no inoculum (Control, black). (c) Correlation plot showing negative (red) and positive (blue) correlations between aphid load and the abundance of genera. Genera with significantly changing bacterial and aphid main effect were selected. 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 without earthworms (Pearson's *R* = 0.800, *p* = 0.01). [Color figure can be viewed at wileyonlinelibrary.com]

confirmed only for plant growth. We further hypothesized that inoculated bacteria induce plant defences against feeding aphids that act to reduce aphid reproduction. Specifically, we found that the two *A. radicis* strains altered the expression of plant defence genes differently, with strong differences between the aphid susceptible (Scarlett) and relatively resistant (Barke) plant cultivar. Moreover, the disruption of AHL production of inoculated *A. radicis* affected the wider rhizosphere community with a stronger effect on rare OTUs; additionally, many genera were affected by earthworms and a few by aphid feeding. However, these microbiome changes were not highly associated with aphid suppression, suggesting that direct interactions of *A. radicis* with the plant underlie the aphid suppression effect rather than indirect effects due to changes in the wider microbiome.

A. radicis inoculation increased plant biomass and chlorophyll content even when plants were under herbivory in some cultivars, and especially when earthworms were present, confirming previous studies (Han et al., 2016; Li et al., 2012; Zytynska et al., 2020). Interestingly, we found that the ArAHL- mutant had a stronger plant growth-promoting effect, suggesting that AHLs may actually reduce the beneficial properties. The increase in the beneficial effect of the AHL-mutant strain was unexpected since, on one hand, various previous studies reported stronger beneficial effects of bacterial inoculation on plant growth when AHLs are produced or added (Filgueiras et al., 2020; Schenk et al., 2012; Schuegger et al., 2006; Zhao et al., 2015). On the other hand, Han et al. (2016) could not find any change in plant biomass after two weeks in a comparable soil

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

Abbreviation: AHLs, N-acyl homoserine-lactones.

Note: Eighteen genera changed in their relative abundance with $p < 0.05$ for at least one factor. Genera are sorted by decreasing mean relative abundance (% abundance across all treatments). n.s., not significant.

*** $p < 0.001$; ** $p < 0.01$; * $p < 0.05$.

* $p < 0.1$.

experiment with both *A. radialis* strains. Only after two months, they reported plant growth promotion but this was elicited by both *A. radialis* strains. One explanation for the plant growth differences in the present study could be that in certain cases microbial AHL production negatively regulates plant growth promotion, for example via the production of indole-3-acetic acid (Müller et al., 2009). According to this, some genes could be fully upregulated when the microbial-AHLs are not produced, leading to increased plant growth.

Our study provides new insight into how microbial signalling molecules affect plant responses to aphids. Both *A. radialis* strains showed strong aphid-suppressive effects in Scarlett plants, the cultivar that is most susceptible to aphids. In Barke plants, which were the most resistant to aphids, there was a weaker but significant aphid reduction primarily by the wild-type strain that produces AHLs. However, in Grace (mid-way between susceptibility of Barke and Scarlett) there was even some increased aphid growth rates by the wild-type *A. radialis*. These results are similar to previous studies, with some general effects across plant cultivars but also many differences, highlighting the genetic determination of these interactions (Friman

TABLE 3 Summary of significant effects of bacterial inoculation, aphids, earthworms, and AHLs on other genera of bacteria in the rhizosphere microbial community of barley plants

et al., 2020a; Martinuz et al., 2012; Pangesti et al., 2015; Wehner et al., 2021). The potentially stronger effects of the AHL-mutant need to be further examined, however, since most previous studies reported that bacterial AHL production enhanced plant responses to biotic stresses compared to AHL-negative strains (Hartmann et al., 2014; Schikora et al., 2016). For example, AHL-producing *Ensifer meliloti* increased barley resistance against aphids, but similar to our findings only in some cultivars (Wehner et al., 2021). One explanation might be that AHLs mitigate plant responses to microbes, with production of AHLs reducing pathogen-like responses to the bacteria itself, leading to reduced overall defence induction. Yet, when AHL production is impaired, this might interfere with the plant–bacteria recognition so that the bacteria are perceived as invasive. Thus, invasive organisms could represent stressful stimuli for the plant that might plausibly trigger that the plant compensates growth and set seeds earlier to avoid the harmful organism (Dietrich et al., 2005; Järemo et al., 1996). Hence, increased shoot biomass and fewer aphid numbers on plants inoculated with the AHL-mutant could point to an alert immune state in response to the missing AHL

stimulus (Mauch-Mani et al., 2017; Pieterse et al., 2012). The *A. radialis* effect on plant growth and suppressing aphids was stronger under suboptimal conditions in our study, that is, low-nutrient soil or absence of earthworms. This follows the hypothesis that plants recruit more useful bacteria or more readily establish beneficial interactions under suboptimal conditions (Berendsen et al., 2012; Bonneau et al., 2013; Carvalhais et al., 2013).

We observed that the plant defence responses differed between plants inoculated with ArWT and ArAHL-, and the bacterial effect in most of the genes differed between cultivars. For example, in Barke (the less susceptible cultivar to aphids), upregulation of the *PR1* and *PR17b* genes was observed on plants inoculated with *A. radialis* and on plants with aphids. These pathogenesis-related genes are generally associated with defences against insects and biotrophic pathogens (Delp et al., 2009; Leybourne et al., 2019). Similarly, we observed an upregulation of *WRKY-TF22* in this cultivar, which is related to enhanced systemic barley immunity against pathogenic bacteria (Dey et al., 2014; Liu et al., 2014). In contrast, we observed an opposite gene expression in Scarlett for *A. radialis* inoculated plants or plants exposed to aphids. Scarlett was the most susceptible aphid cultivar and also where the bacteria had the strongest suppressive effect. Surprisingly, however, instead of having an upregulation of the genes, as observed in Barke plants, we observed downregulation of the pathogenesis-related genes and *WRKY-TF22*. In line with our results, Shrestha et al. (2019) found that beneficial bacteria-induced barley resistance against the fungus *Blumeria graminis* was associated with plant genetic differences between barley cultivars. One explanation could be that the selected cultivars in this study are inherently different in resistance against pathogens and aphids, likely as a result of selective breeding. Traditional breeding methods focus on enhanced plant yield or disease resistance rather than insect resistance, and different selection criteria may, in turn, impair a plant's defence response in different areas. Given that biotrophic pathogens and aphids often trigger similar defences (i.e., salicylate pathway, Pieterse et al., 2014), it is likely that selection for pathogen resistance also affects aphids. In our study, aphid numbers were lower in Barke than in Scarlett cultivar, even without any bacterial inoculation, suggesting that Barke has a stronger constitutive level of defences against aphids, which align with our observed patterns in gene expression (Leybourne et al., 2019; Mehrabi et al., 2014). Importantly, our results suggest that in Scarlett, rhizobacterial effects on aphid defences are regulated through different genetic pathways than *PR* genes, and that, potentially, suppression of these genes may result in the best defence phenotype in this cultivar. It is possible that *A. radialis* can counter ineffective aphid-induced mechanisms to boost plant defences and induce other more effective pathways (that are yet to be studied).

Both cultivars showed similar defence responses in the ethylene response factor (*ERF*) gene. We observed upregulation caused by the ArAHL- mutant and downregulation in the ArWT strain inoculated plants on both barley cultivars. We suspect that the absence of AHLs could induce plant responses because the lack of this signalling molecule might interfere with plant-bacteria recognition and thus

enhance plant defences. However, it is likely that the *ERF* gene expression might not explain the aphid resistance between the cultivars mediated by *A. radialis* strains. Instead, it is likely that the plant defence enhancement in Scarlett inoculated plants could be related to plant secondary metabolism, specifically flavonoids (Han et al., 2016). We observed a stronger tendency of bacterial-induced expression of the UDP-glucuronosyl transferase (*UGT*) gene, a transcript important for flavonoid biosynthesis. Flavonoids are phenolic compounds associated with plant defence against biotic stresses like feeding insects (Rashid et al., 2018; Treutter, 2005). These flavonoid-related pathways may partly explain the increased aphid suppression in Scarlett and provide additional pathways related to the aphid resistance of Barke plants. This study brings new insights into possible gene pathways enhanced by beneficial bacteria against sustained insect feeding (after 14 days). However, further molecular studies should be implemented in combination with biochemical profiles exploration to discover novel pathways related to plant-microbe insect interaction considering short and long ecological time frames. AHLs play a central role in biofilm formation (Miller & Bassler, 2001), and therefore it is plausible that the colonization ability differs between AHL-producing and no AHL-producing strains. In our study, microscopic visualization pointed towards a more persistent root colonization of the AHL-mutant compared to the wildtype. It has previously been shown that *A. radialis* is able to build biofilm-like structures on roots and colonizes endophytically (Li, 2011). In a previous study, both *A. radialis* WT and the AHL-mutant colonized well in axenic trials but the AHL-mutant was mainly present as single cells while the wildtype formed denser micro-colonies (Han et al., 2016). Furthermore swimming motility has been shown to differ in other *Acidovorax* sp. AHL-mutant strains (Fan et al., 2011) and could influence the localization of inoculated cells on the root surface. More dispersed colonization of the *A. radialis* AHL-mutant could thus open up a niche facilitating the growth of other microbial taxa (Venieraki et al., 2016) and/or could influence the interaction mode with the root. The unexpected higher persistence of the AHL-mutant strain compared to the wildtype found in this study is difficult to explain if genes involved in colonization are expected to be positively regulated by AHL-mediated quorum sensing. However, it might be explained by a QS-dependent negative regulation of some genes important for root adherence that would be upregulated when AHL is missing. Some examples for this negative regulation mechanism can be found, e.g., in *Pantoea stewartii* (Minogue et al., 2002) and *Pectobacterium carotovorum* (Burr et al., 2006). A detailed study on *A. radialis* gene expression by RNAseq and targeted RT-qPCR analysis of QS-dependent genes might elucidate this further. Moreover, AHL-synthesis mutants can still sense and use AHLs as resources while needing less energy for AHL production. With this, they can have an advantage over the AHL-producing wildtype strain as recently demonstrated in *Pseudomonas aeruginosa* populations (Mund et al., 2017). Differential root colonization might also have caused the stronger plant growth promotion effect that we observed for the AHL-mutant strain. In general, in our soil system, the inoculated bacteria appeared to be outcompeted by the prevailing

rhizosphere microbiome within several days but still leads to long lasting plant effects.

Indirect effects on the plant defence response can also be caused by the rhizosphere microbiome that is shaped by signalling molecules. In our study, the presence or absence of AHLs resulted in different rhizosphere microbiome compositions. This outcome was expected, as bacterial strains that do not produce AHLs might influence the abundance and interaction of other taxa, particularly those that depend on AHL signalling. While we found stronger effects on rare OTUs through bacterial inoculation, changes in specific genera were not consistently or clearly related to aphid suppression. Without earthworms, only three genera, *Solimonas*, *Sphingopyxis*, and *Hirschia*, changed from a positive to a slightly negative association with aphid loads when inoculated with *A. radicum* lacking AHLs, which could indicate a causal connection between their relative abundance and aphid suppression. *Solimonas terrae* has recently been found responsive to plant growth stimulation (Tamošiūnė et al., 2020) and microbially induced plant defence (Sommer et al., 2021). When earthworms were present, *Dyadobacter* and again *Sphingopyxis* showed the most negative effect on aphid numbers when AHL signalling was impaired. In addition, *Stenotrophomonas* significantly changed with aphid numbers independently of the bacterial treatment. Several strains of *Stenotrophomonas* have already been reported to be enriched in insect-infested plants and protect them from insects (Jabeen et al., 2018; Kong et al., 2016). All five mentioned bacteria might therefore be interesting candidates to be tested in future inoculation studies. Independent of the AHL effect, earthworms (known ecosystem engineers) altered the microbial community composition. This could be attributed to more aeration, drainage or surrounding soil mixing (Edwards, 2004). We did not find that barley cultivar or aphid feeding shaped the rhizosphere microbiome, in contrast to other studies (Bulgarelli et al., 2015; Friman et al., 2021; Zytynska et al., 2020). Finally, it remains to be investigated if AHLs themselves are the causal signal for the plant resulting in the observed plant-insect responses (direct AHL effect) or if these are rather due to a changed bacterial phenotype as a result of an altered AHL-controlled gene expression (indirect AHL effect). Future experiments with synthetic AHLs and mutants that do not perceive AHLs could elucidate these mechanisms more clearly.

We conclude that the disruption of AHL-signalling by soil bacteria alters bacterial-plant-insect interactions, likely by inducing variable plant defences, and potentially via few but specific interactions with the wider plant-associated microbiome. Yet, these effects were strongly impacted by the plant cultivar, indicating genetic diversity in plant responses to inoculated bacteria, and also across the different abiotic (nutrient) and biotic (earthworm) environments. Our study further highlights how plant defence gene expression can be strongly context dependent influenced by the biotic and abiotic environment, and builds upon current work aiming to identify plant defences induced by soil microbes (Berendsen et al., 2012; Pieterse et al., 2009; Thaler et al., 2012; Vlot et al., 2021). Studies that include multiple biotic and abiotic factors simultaneously are fundamental to fully understand the complex nature of

interactions within the plant holobiont (Zytynska et al., 2021). Moreover, an extensive understanding of the microbial-plant interactions across different biotic and abiotic environments can help in selecting appropriate agents that have the potential to reduce yield gaps in low input farming systems and identify appropriate bacteria-plant species combinations for specific agricultural conditions and systems.

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DATA AVAILABILITY STATEMENT

The support data of the present study are available from the corresponding author upon reasonable request. Microbiome data that support the results are available on GenBank (accession numbers: OM238298-OM240180).

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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