

# Uptake, degradation and chiral discrimination of *N*-acyl-D/L-homoserine lactones by barley (*Hordeum vulgare*) and yam bean (*Pachyrhizus erosus*) plants

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**Abstract** Bacterial intraspecies and interspecies communication in the rhizosphere is mediated by diffusible signal molecules. Many Gram-negative bacteria use *N*-acyl-homoserine lactones (AHLs) as autoinducers in the quorum sensing response. While bacterial signalling is well described, the fate of AHLs in contact with plants is much less known. Thus, adsorption, uptake and translocation of *N*-hexanoyl- (C6-HSL), *N*-octanoyl- (C8-HSL) and *N*-decanoyl-homoserine lactone (C10-HSL) were studied in axenic systems with barley (*Hordeum vulgare* L.) and the legume yam bean (*Pachyrhizus erosus* (L.) Urban) as model plants using ultra-performance liquid chromatography (UPLC), Fourier transform ion cyclotron resonance mass spectrometry (FTICR-MS) and tritium-labelled AHLs.

Decreases in AHL concentration due to abiotic adsorption or degradation were tolerable under the experimental conditions. The presence of plants enhanced AHL decline in media depending on the compounds' lipophilicity, whereby the legume caused stronger AHL decrease than barley. All tested AHLs were traceable in root extracts of both plants. While all AHLs except C10-HSL were detectable in barley shoots, only C6-HSL was found in shoots of yam bean. Furthermore, tritium-labelled AHLs were used to determine short-term uptake kinetics. Chiral separation by GC-MS revealed that both plants discriminated D-AHL stereoisomers to different extents. These results indicate substantial differences in uptake and degradation of different AHLs in the plants tested.

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## Introduction

Only a few decades ago [1] bacteria were discovered to possess the ability to sense their population density and regulate gene expression in a process later termed quorum sensing [2]. Bacteria constitutively produce small signal compounds, which leave the cell by diffusion or active efflux [3] and accumulate in the vicinity while the number of bacteria increases. Once a threshold concentration is exceeded, those signal compounds re-entering the cell bind to a transcription factor [4], which stimulates transcription of its biosynthetic genes and the transcription of certain target genes [5]. Thus, the quorum sensing system allows bacteria to regulate the expression of genes responsible for various functions such as production of antibiotics and virulence factors, swarming, plasmid conjugal transfer and many more [6]. In Gram-negative bacteria, a wide range of quorum sensing systems are based on *N*-acyl-homoserine lactone structures [7], referred to here as AHLs. Most recently it was suggested that AHL autoinduction also functions as efficiency and diffusion sensing in addition to its role in intraspecies and interspecies signalling [8]. This signal is not restricted to bacteria, but actually also affects roots of plant species in the rhizosphere, a soil compartment representing a rather densely populated microbial habitat, where AHLs may act as a signal to the plant [9], too. For instance, it has been shown that the *Medicago truncatula* root proteome undergoes a change in over 150 proteins when exposed to certain AHLs [10]. In tomato, AHL treatment leads to systemic induction of defence genes [11]. In turn, plants have evolved mechanisms to interfere with and disrupt quorum sensing. The macroalga *Delisea pulchra*, which inhibits AHL-dependent swarming motility in a strain of *Serratia liquefaciens* by production of halogenated furanones [12], is just one example of a reverse reaction. The abovementioned legume *Medicago* interferes with bacterial quorum sensing by production of substances that stimulate or inhibit responses in reporter strains [13].

Though there have been reports on the influence of AHLs on plants, on signal inactivation and the production of structural mimics affecting bacterial communities, the fate of the signal compound itself in plants has up to now scarcely been traced. Regarding our model plants barley (*Hordeum vulgare*) and yam bean (*Pachyrhizus erosus*), according to our present knowledge, it was unknown whether signal molecules could be taken up and transported into and inside the plant. Based on this question, the AHL depletion in growth media, AHL uptake and translocation in these two relatively distant related species and AHL

depletion in growth media were examined. Legumes represent interesting model dicots, since they are naturally exposed to multiple AHLs in their symbiosis with legume-nodulating rhizobia [14], as opposed to monocots like barley which have no comparable symbiotic interactions with bacteria. In these experiments, barley and the tropical legume yam bean were grown as individual plants and directly compared regarding their uptake and translocation of three AHLs: *N*-hexanoyl- (C6-HSL), *N*-octanoyl- (C8-HSL) and *N*-decanoyl- (C10-HSL) homoserine lactone, which all represent naturally occurring signals in Gram-negative bacteria [15, 16]. AHLs with unsubstituted acyl chains were chosen, firstly because of higher stability relative to oxo-, hydroxy- and other derivatives, and also to check the importance of lipophilic features in connection to AHL–plant interaction. Aspects of abiotic processes were thereby taken into account as well as the fact that the AHLs used were racemic and might be discriminated during uptake by the plants.

## Experimental

### Chemicals

*N*-Hexanoyl- (C6-HSL), *N*-octanoyl- (C8-HSL) and *N*-decanoyl- (C10-HSL) homoserine lactone were obtained from Sigma-Aldrich (Steinheim, Germany) and kept at  $-20\text{ }^{\circ}\text{C}$ . For plant treatment, each compound was freshly solved in ethanol before use. Stock solutions for standards were prepared by dissolving the substances in acetonitrile at a concentration of  $1,000\text{ mg L}^{-1}$ . The stock solutions were kept at  $-20\text{ }^{\circ}\text{C}$  and could be stored over a 4-week period. Standard solutions were prepared by diluting the stock solutions with the same mineral media as used in the plant system purchased from Duchefa Biochemie BV, The Netherlands. Acetonitrile of “hypergrade” quality for the ultra-performance liquid chromatography (UPLC) analysis as well as ethanol, methanol, dichloromethane and isopropanol were purchased from Merck (Darmstadt, Germany), and hexane from Riedel-de Haen (Seelze, Germany). Water was provided by a Milli-Q system (Millipore Corporation, Billerica, USA). All chemicals used in the experiment were at least of analytical grade.

### Plant growth conditions

Barley seeds (*Hordeum vulgare* L.), cv. “Barke” (Josef Breun GdB, Herzogenaurach, Germany) and yam bean seeds (*Pachyrhizus erosus* (L.) Urban), cv. “EC 550” (Ebenezer Belford, College of Science, Kwame Nkrumah University of Science and Technology, Kumasi, Ghana) were surface sterilized according to a literature protocol [17] using Tween 20 (1% v/v), ethanol (70% v/v), sodium

hypochlorite solution and sterile distilled water. Seeds were then germinated in the dark on nutrient broth agar for 4 days (barley) or 7 days (yam bean); seedlings free of contamination were selected and then incubated in the sterile system with media. The sterile setup consisted of Duran vials (diameter 30 mm) filled with approximately 45 g of glass beads (diameter 1.7–2 mm). All seedlings were grown individually in a separate system to avoid possible interactions between plantlets. AHLs were dissolved in ethanol and added to mineral media at a final concentration of 10  $\mu\text{M}$  (0.1% ethanol). Control plants were supplied with an equal volume of ethanol. Plants received 14 h light per day in Heraeus–Vötsch chambers (PAR at 35  $\text{W m}^{-2}$ ). Barley was grown at 15 °C during the day and 12 °C during the night phase, whereas yam bean as a tropical plant had to be grown in a separate chamber adjusted to 20 °C. Barley was harvested 21 days and yam bean 28 days after surface sterilization and checked for contamination. Plants were separated into roots and shoots and kept at –85 °C. Disparities in germination time and growth conditions were inevitable due to the different physiological development of the two plants and allowed one to compare both species in a nearly equal growth stage.

#### AHL aging and degradation test

AHL stability was tested by filling up vials with glass beads and 10 mL mineral media as mentioned above, but without the plant. The vials were incubated under the same conditions as in the plant experiment and media were afterwards separated from glass beads by using air pressure. Mineral growth media were filtered through 0.22- $\mu\text{m}$  PTFE discs (Merck, Darmstadt, Germany) before measuring on UPLC. Pure mineral media samples did not contain organic compounds interfering with AHL analysis and moreover, the UPLC method was sensitive enough for measurement without further pre-treatment. Additionally, the same experiment was performed without glass beads to check abiotic degradation without the influence of adsorption onto surface of the glass beads. In each sample, pH value was determined before and after the experiments.

#### Plant extraction

A cleanup and pre-concentration procedure had to be applied for the preparation of plant tissue extracts before measurement on UPLC and Fourier transform ion cyclotron resonance mass spectrometry (FTICR-MS). Plant material was therefore ground in liquid nitrogen to a fine powder and then a 1-g aliquot of shoot or 0.5-g sample of root material was homogenised in 10 mL of ice cold 25% (v/v) acetonitrile. After 20 min ultrasonication, samples were centrifuged for 15 min at 5,100 g and the resulting

supernatants were then carefully removed. The average pH value of the supernatants was 6.5 and therefore unproblematic because AHL hydrolysis occurs at alkaline pH. The supernatants were purified by solid-phase extraction (SPE) and afterwards analysed according to a previously published method [9, 18]. Briefly, after conditioning with methanol and water, the extract was introduced onto a MegaBond Elute cartridge (Varian, Darmstadt, Germany). The cartridge was then washed with a methanol/water mixture, dried under vacuum and then the solutes were eluted with a 2-propanol/hexane mixture (85:15 v/v). The eluate was dried under a nitrogen stream, redissolved with water containing 30% (v/v) acetonitrile and filtered through a PTFE filter. Plant material and media used in further analysis showed no microbial contamination.

#### Quantification of AHL concentrations on UPLC

AHL analysis for quantification of the signal substances [18] was performed on a Waters Acquity UPLC System (Waters Corporation, Milford, USA) equipped with a 2996 PDA detector. The reversed-phase separation was achieved on a BEH  $\text{C}_{18}$  packing with 1.7- $\mu\text{m}$  particle diameter and column dimensions of 100 $\times$ 2.1 mm (Waters Corporation, Milford, USA). The thermostat of the column was set to 60 °C and the autosampler to 27 °C. The flow rate was 0.9  $\text{mL min}^{-1}$  and the injection volume was 20  $\mu\text{L}$ , injected via full loop. A linear mobile phase gradient was applied starting with water with a content of 10% (v/v) acetonitrile to 100% acetonitrile in 1 min. The detection wavelength was set to 197 nm (1.2-nm width) with a scan rate of 20 Hz. The detector resolution was 1.2 nm. The log $P$  values of the analytes were calculated with Pallas 3.1 (CompuDrug International Inc, Budapest, Hungary).

#### AHL identification by FT-ICR/MS

Positive FTICR spectra for qualitative analysis of AHLs were acquired on a Bruker Daltonics (Bremen, Germany) Apex Qe 12 T system equipped with an APOLLO II source and microspray infusion of 120  $\mu\text{L h}^{-1}$  with a scan number of 256. FT-ICR/MS was not coupled to UPLC, since the combination of high resolution separation technology such as UPLC with peak widths around 1 s with a mass spectrometry method having scanning rates higher than 1 s would lead to qualitative loss of one or other technology. Spectra were acquired in broadband mode and were calibrated externally on clusters of arginine (ca. 10  $\text{mg L}^{-1}$  in 50% (v/v) of methanol with 0.1% (v/v) of formic acid) in the required mass range ( $m/z$  175.11895, 349.23062, 523.34230 and 697.45398). Peaks exceeding a threshold signal-to-noise ratio of 4 were exported to peak lists. The resulting text files containing more than 6,500 mass-intensity

pairs were scanned with a software tool written in Python (<http://www.python.org>) for the presence of peaks from a reference file with theoretically possible protonated and Na<sup>+</sup>-cationized homoserine lactone masses. The masses of C6-, C8- and C10-HSL in that order are 200.12812, 228.15942 and 256.19072 in the protonated or 222.11007, 250.14137 and 278.17267 in the Na-cationized form, respectively. The window width for the search was set to 2-ppm mass accuracy, which indicates a relative difference of  $2 \times 10^{-4}\%$ .

#### Preparation of tritium-labelled AHLs, application and scintillation measurements

*N*-(*trans*-2-Octenoyl)-homoserine lactone and *N*-(*trans*-2-decenoyl)-homoserine lactone were prepared according to literature procedures [19, 20]. For the preparation of tritiated product, the method with Wilkinson's homogeneous catalyst [21] for catalytic tritiation with gaseous tritium was replaced by a simpler and more rapid classic tritiation method with palladium catalyst. In this case, T/H exchange also occurs in the neighbouring positions of the original double bond of aliphatic acyl chains [22]. Thereby it is possible to obtain a product of higher specific activity. The radiochemical purity of the labelled compounds was checked by thin-layer chromatography (TLC) using conditions described below and scanning with a TLC scanner (Raytest, Germany).

*N*-(*trans*-2-Octenoyl)-homoserine lactone (6.7 μmol; 1.5 mg) was dissolved in 0.2 mL dry dioxane and 2.2 mg 5% Pd/BaSO<sub>4</sub> was added. The reaction mixture was stirred for 2 h in a tritium atmosphere (approximately 45% [<sup>3</sup>H] isotopic abundance) at 0.075-MPa pressure. After removal of catalyst and labile radioactivity, the final product was dissolved in ethyl acetate.

The radiochemical purity of product was higher than 98.5% according to TLC analysis on (1) silica gel (Merck, Darmstadt, Germany) in CHCl<sub>3</sub>/CH<sub>3</sub>OH (9:1), CHCl<sub>3</sub>/EtOAc (3:1)-developed three times, and on (2) RP-18 (Merck, Darmstadt, Germany) in CH<sub>3</sub>CN/H<sub>2</sub>O (8:2). The specific radioactivity corresponded to that of tritium gas used and to the tritiation method used. Total radioactivity of the product was 8.1 GBq (220 mCi). *N*-(*trans*-2-Decenoyl)-homoserine lactone (5.9 μmol; 1.5 mg) was tritiated in the same manner as the previous compound. The radiochemical purity was higher than 96%; the total radioactivity was 6.3 GBq (170 mCi).

Barley plants were grown axenically as mentioned above. Before application, ethyl acetate was evaporated and [<sup>3</sup>H]C8- and [<sup>3</sup>H]C10-HSL were re-dissolved in ethanol. The plants were grown for 5 days in 9.5 mL mineral media and then sterilely spiked with 0.5 mL AHL media mixture through aseptic silicone septa. The mixture contained non-radioactive and labelled AHL in an amount

that resulted in a final concentration of 10 μM. Total activity of the [<sup>3</sup>H]-labelled AHLs enclosed in the mix was 3.7 MBq (100 μCi). Samples were taken 4, 29, 54, 145, 197 and 315 h after AHL application. Roots were split in halves and one half was washed for 30 s under double distilled water to remove media leftovers, whereas the other half remained unwashed. Afterwards, fresh weights of shoot and root samples were determined immediately. Extraction was done by thoroughly homogenizing plant parts in 1 mL of 25% (v/v) acetonitrile with an Ultra-Turrax (IKA, Staufen, Germany) disperser. The suspension was then processed as mentioned above. A 250-μL aliquot of the extract was added to 5 mL of scintillation liquid Rotiszint eco plus (Carl Roth, Karlsruhe, Germany), mixed and measured for 5 min on an LS 6500 scintillation counter (Beckman, USA).

#### Chiral separation by GC-MS

Samples from plant growth media (5 mL each) were evaporated to dryness and resolved in 200 μL dichloromethane. AHLs were separated on a gas chromatograph (Varian GC 3900 series, Varian Chromatography Systems, Middleburg, the Netherlands) coupled with a mass spectrometer (Varian Saturn 2100T, Varian Chromatography Systems, Walnut Creek, CA, 94598, USA). A 1-μL aliquot of the sample was injected in the splitless mode (2 min) using helium (ultrahigh purity) with a column flow of 1.3 mL min<sup>-1</sup> and pulse mode injection (pulse pressure 15.0 psi, pulse duration 0.25 min). The injection port (CP 1177) contained an unpacked gooseneck injection port liner with a tapered lower section (4-mm internal diameter, deactivated) at 210 °C. The separation capillary column was a fused silica-HYDRODEX β-TBDAC with an inner diameter of 25 μm and 25-m length covered with heptakis-(2,3-di-*O*-acetyl-6-*O*-*t*-butyldimethyl-silyl)-β-cyclodextrin with film thickness of 0.25 μm (Macherey-Nagel, Düren, Germany). The capillary was connected directly from injection port to mass spectrometer via the interface region (280 °C). The oven temperature was programmed to increase from 80 °C at 30 °C min<sup>-1</sup> to 220 °C (21 min). Mass spectrometry conditions were as follows: electron ionization source set to 70 eV, emission current 500 mA at mass spectrometer trap of 190 °C and manifold of 80 °C.

## Results

### Abiotic AHL decrease

Prior to examining AHL kinetics in plants, experiments were carried out to determine AHL losses in media as a

consequence of abiotic processes such as photo-catalyzed oxidation or pH-dependent hydrolysis [23] as well as adsorption to the surface of the glass beads which were used as support for growing plants. Without this information, it would not be entirely clear whether observed changes were indeed contributed by plants only. Abiotic degradation was investigated by installing plantless sterile systems without glass beads under identical experimental conditions. At the end of the respective incubation period, media were checked for their AHL concentration by UPLC. As shown in Table 1, less than 10% of the initial concentration had vanished due to abiotic degradation processes. This amount, however, lies within the allowed relative standard deviation of quantification [24]. Results were different when the same experiment was carried out in the presence of glass beads. While C6- and C8-HSL remained less affected, a reduction in concentration was found for C10-HSL (Table 1). When this compound was incubated with glass beads, approximately 20% higher AHL losses occurred than in experiments without beads, which may be due to adsorption to the glass surfaces. High lipophilicity of C10-HSL (Table 2) might be the reason for this disparity in adsorption. In a further experiment, desorption of AHLs from glass beads was carried out by extraction with organic solvent and it could be proven that this compound actually adsorbs to glass surface (data not shown). Altogether we found that AHL stability was considerably high in our setup. The media used did not contain any possibly interfering organic compounds and exhibited a pH value of  $5.7 \pm 0.1$ , which might support AHL stability according to the findings that they were prone to alkaline hydrolysis, also referred to as chemical lactonolysis [25–27]. After plant harvest, pH was checked again, and it remained unaltered in yam bean media ( $\text{pH } 5.8 \pm 0.3$ ), but decreased to  $\text{pH } 4.1 \pm 0.2$  in barley media. Temperature was

also chosen to be reasonably low so as not to unnecessarily accelerate AHL degradation and to fit the plants' demands at the same time:  $15^\circ\text{C}$  in the barley and  $20^\circ\text{C}$  in the yam bean experiment. As a tropical plant, the legume required higher temperature and 4 days longer growth period than barley to reach a comparable developmental stage. Abiotic AHL decrease was checked under this temperature condition as well (Table 1). It turned out that  $5^\circ\text{C}$  higher temperature caused stronger abiotic degradation, as expected according to earlier reports on temperature-dependent stability of AHLs [23, 28]. Nevertheless, abiotic AHL decrease was altogether small in this experimental setup (10–30%). Increased abiotic losses were documented for C6- and C8-AHL, but not C10-AHL, where the result is more or less identical to the experiment at  $15^\circ\text{C}$ , confirming adsorption rather than degradation of C10-HSL as the main process in our experiment. These trials prove that the abiotic AHL decrease represents a minimal but calculable factor in our setup.

#### AHL depletion in media due to plant contact

Barley (*Hordeum vulgare* L.) and yam bean (*Pachyrhizus erosus* (L.) Urban) media were carefully separated from plants and glass beads, and were used for evaluation of residual AHL concentrations (Fig. 1) by UPLC. The results are shown in Table 3 as a percentage of initial concentration. Firstly, the differences between the two plant species are obvious: higher residual AHL concentrations were measured when the monocot barley was grown in the media as opposed to the legume yam bean. This matches previous data [28] obtained from plants like wheat and clover. A possible influence of pH, which was lowered in barley media but remained more or less stable in legume media as shown before, should thereby be considered. The second and particularly interesting finding is an obvious correlation to the length of the side chain or  $\log P$ , respectively: the higher the number of carbons in the lipophilic moiety, the stronger the plant-induced diminution in AHL concentration is. Thus, the remaining concentrations of C6-HSL were highest in both plants, while C10-HSL seemed to undergo the strongest turnover. An interpolation of plant-dependent AHL depletion calculated by subtracting abiotic degradation and adsorption as well as the abovementioned AHL remains from initial AHL concentrations is shown in Fig. 2. The data indicate that AHLs were actually more prone to increased sequestration by plants the more lipophilic they were.

#### Uptake and translocation

So far, the results yield information about the actual fate of the compounds in these small-scale model rhizospheres.

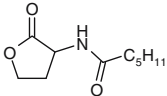
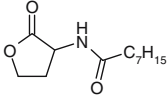
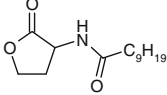
**Table 1** Recoveries of AHLs at different time points

	C6-HSL (%)	C8-HSL (%)	C10-HSL (%)
15 °C without glass beads			
Day 0	100±4.3	100±1.6	100±2.2
Day 8	101±2.3	98±2.9	99±2.3
Day 17	98±7	91±7.5*	92±15
15 °C with glass beads			
Day 0	100±5.1	100±6.1	100±11.2
Day 8	91±12.6	95±4	73±14.6*
Day 17	90±3.7	93±3.4	72±5.9*
20 °C with glass beads			
Day 0	100±0.6	100±2.9	100±4.2
Day 8	87±0.5*	85±5*	76±6.3*
Day 21	85±0.9 *	84±0.9 *	73±7.5*

Stability of the compounds is displayed at the growing conditions but without plant (abiotic degradation)

\* $p < 0.05$  (i.e. a significant difference compared to day 0)

**Table 2** Structure, elemental composition,  $m/z$  and  $\log P$  values of the AHLs used

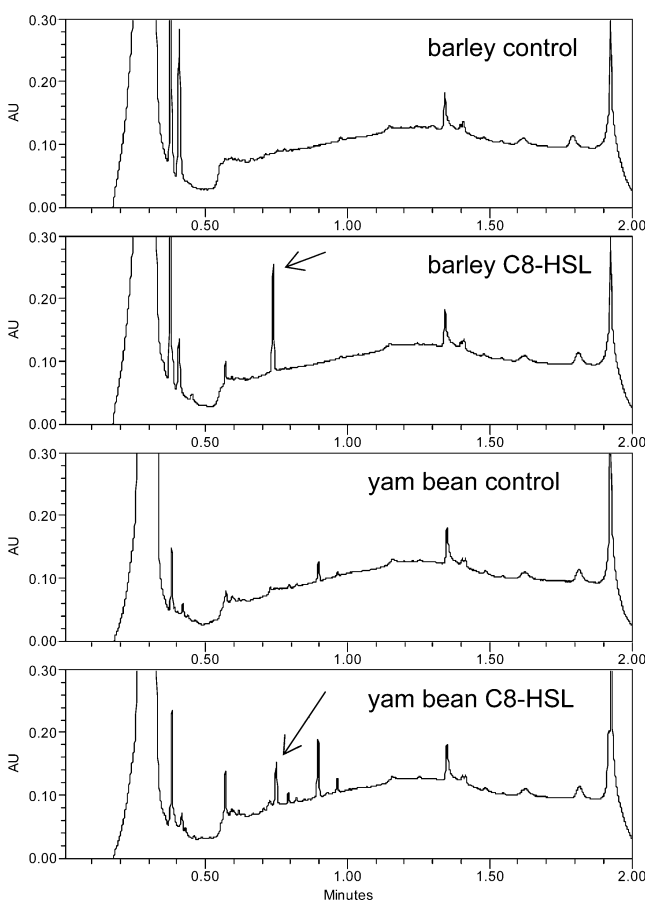
Name	Structure	Elemental composition	[MH] <sup>+</sup>	[MNa] <sup>+</sup>	logP
C6-HSL		C <sub>10</sub> H <sub>17</sub> NO <sub>3</sub>	200.12812	222.11007	1.02
C8-HSL		C <sub>12</sub> H <sub>21</sub> NO <sub>3</sub>	228.15942	250.14137	1.97
C10-HSL		C <sub>14</sub> H <sub>25</sub> NO <sub>3</sub>	256.19072	278.17267	2.96

Plant-borne mechanisms possibly responsible for changes in AHL concentration are manifold, beginning from simple adsorption on root tissue to more destructive processes such as degradation by acylase activity or lactone ring-opening via lactonases and further to active or passive uptake and

even translocation and conversion of the bacterial signals in the whole plant. According to previously published results, AHLs were not accumulated by plantlets of the legume species *Lotus corniculatus* [28]. As expected, UPLC analysis of root and shoot extracts from the legume yam bean did not detect a trace of AHL either. On the contrary, this might probably be due to interference in the elution of the matrix constituents. In barley root extracts, all three AHLs were detected, since these peaks were separated from the matrices. C6- and C8-HSL were also found in the shoot extracts of barley, but the peaks were strongly influenced by matrix effects and coelution of other compounds from the extracts, while C10-HSL was not detected there by UPLC.

A further measurement with FTICR-MS was implemented to confirm the identification of the compounds (Table 4). However, FTICR-MS alone does not yield sufficient information on chemical structure, even though it enables assignment of precise elemental composition with an error less than 2 ppm (relative error of  $2 \times 10^{-4}$  % compared to theoretical mass) to many of the typically more than 1,000 peaks from a single measurement across a sizeable mass range. Hence, combination of exact mass measurement with UPLC results gives unprecedented insight into the nature of the AHLs in the extract [9]. As shown in Fig. 3, all three AHLs detected in barley root extracts by UPLC were also determined by high-resolution mass spectrometry with high reliability and repeatability. In yam bean root extracts (Fig. 4), the mass/charge ratios ( $m/z$ ) of the three AHLs were determined as well. In barley shoots (Fig. 3), two  $m/z$  values corresponding to C6- and C8-HSL were detected, but no trace of C10-HSL. The only compound according to  $m/z$  which could be detected in shoots of yam bean was C6-HSL (Fig. 4).

As mentioned above, FTICR-MS measurements might not yield sufficient information for identification [9].



**Fig. 1** Detection of C8-HSL in mineral growth media. Example UPLC runs. The *top* two chromatograms show media from barley plants, the *bottom* two below samples from yam bean media

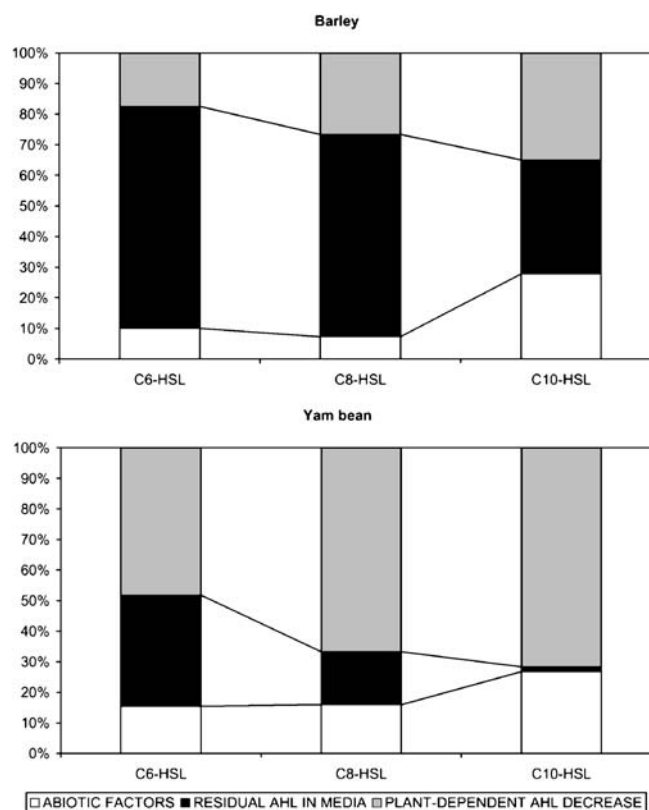
**Table 3** AHL recoveries in media after plant growth

	C6-HSL (%)	C8-HSL (%)	C10-HSL (%)
Barley day 0	100±0.64	100±4.32	100±11.15
Barley day 17	72.51±3.12	66.06±5.10	37.24±6.14
Yam bean day 0	100±3.43	100±9.56	100±11.51
Yam bean day 21	36.38±3.67	17.38±2.80	1.63±0.17

Data shown as percentage of initially measured concentration. Abiotic factors are included in this calculation

However, the reliability of the AHL identification in the tissue extracts connected to UPLC analysis could be confirmed by several facts. Firstly, the  $m/z$  values of the AHLs were measured as their protonated form  $[MH]^+$  and as their sodium adduct  $[MNa]^+$ . Both forms were calculated and compared with the measured  $m/z$  in the mass spectra of the extracts. For example, the measured  $m/z$  of 250.14161 in barley root extract (Fig. 4) corresponds to the elemental composition of  $C_{12}H_{21}NO_3Na$  (250.14137) with an error of 0.96 ppm, so that this could most probably be the sodium adduct of C8-HSL. The masses measured in plant extracts with errors compared to putative AHLs are summarized in Table 3. Additionally, isotope peaks of possible  $[MH]^+$  and  $[MNa]^+$   $m/z$  were also detectable by increasing the scan numbers of the FTICR-MS measurement (data not shown). This, in combination with UPLC results, confirms the identity of the investigated AHLs.

**Fig. 2** Contribution of plants to depletion of AHL in media. Data shown as percentage from initial concentration (10  $\mu$ M or 100%). Values for abiotic AHL diminution have been considered and incorporated



## Uptake kinetics

For further investigation of short-term AHL uptake, a mixture of non-radioactive and tritium-labelled AHLs ( $[^3H]$  AHL) was added to growth media. Barley was chosen for the experiments, since it was shown in previous experiments that yam bean caused considerably higher AHL decrease in media, which would have resulted in the detection of AHL derivatives or fragments instead of intact AHL. Radioactivity in plant tissue of barley as well as in media was then investigated by scintillation measurements. Roots of plants treated with  $[^3H]$ AHLs were split in halves and one half was washed immediately under flowing distilled water to check the impact of possibly loosely adhering AHLs in media leftover. The upper four curves in Fig. 5a show that a part of the AHLs was actually not bound to the surface but removable by water. Average amounts of non-removable radioactivity were approximately 65% for C8-HSL and 67% for C10-HSL. Uptake curves of both AHLs in roots in correlation to plant fresh weight seemed to follow a saturation function. Increasing counts of radioactivity were thereby compensated by a steady increase in plant mass when data were expressed as becquerels per milligram fresh weight. Shoot translocation curves shown below (Fig. 5a) appeared to share a similar correlation, while fluctuations of the values were much higher than in root samples.

**Table 4** Target analysis of shoot and root extracts from barley and yam bean

	Barley shoot	Barley root	Yam bean shoot	Yam bean root
Retention of C6-HSL	n.d.	det	n.d.	n.d.
MH <sup>+</sup> of putative C6-HSL	200.12791 (−0.9)	200.12796 (−0.7)	200.12793 (−0.9)	200.12799 (−0.6)
MNa <sup>+</sup> of putative C6-HSL	222.10979 (−1.2)	222.10982 (−1.1)	222.10983 (−1.0)	222.10988 (−0.8)
Retention of C8-HSL	n.d.	det	n.d.	n.d.
MH <sup>+</sup> of putative C8-HSL	–	228.15926 (−0.7)	–	228.15927 (−0.6)
MNa <sup>+</sup> of putative C8-HSL	250.14107 (−1.1)	250.14113 (−0.9)	–	250.14116 (−0.7)
Retention of C10-HSL	n.d.	det.	n.d.	n.d.
MH <sup>+</sup> of putative C10-HSL	–	256.19052 (−0.8)	–	256.19046 (−1.0)
MNa <sup>+</sup> of putative C10-HSL	–	278.17236 (−1.1)	–	–

In UPLC chromatograms, AHL peaks were detectable (*det.*) or not detectable (*n.d.*) compared to retention times and spectra of standards. In FTICR-MS spectra, *m/z* values of AHLs were detected as a protonated form or as sodium adduct with error (shown in ppm, in brackets) compared to theoretical *m/z*

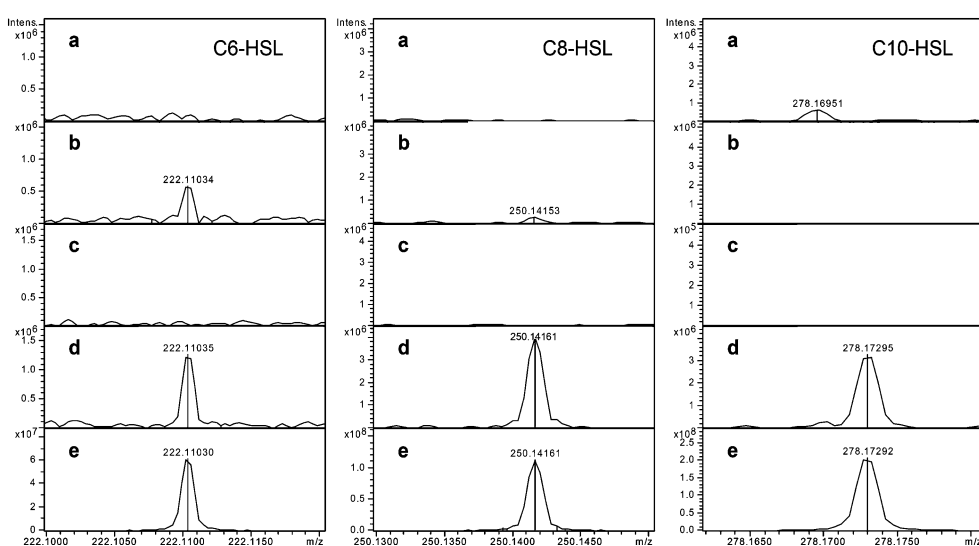
In contrast to previous results, C10-HSL was detected in shoots of barley. Counts per milligram fresh weight were small, less than 160 Bq mg<sup>−1</sup> for C10-HSL and in total, approximately 40 kBq of tritium-labelled C10-HSL were counted in the whole shoot at the end of the experiment, which then represented roughly 1% of the initially applied radioactivity or a 0.1 μM concentration. Since no trace of C10-HSL was detectable in shoot extracts by the other measurements, it must not be overseen that, besides high sensitivity of the scintillation method, radioactive derivatives and fragments of tritium-labelled AHLs could have been detected as well, though not forgetting the possibility that a small amount of the compound may have entered the shoot.

Figure 5b shows the change in radioactivity in the mineral media over several time points in the growth phase. AHL depletion caused by plants appears to be a more or less steady uptake process. Regression coefficients for a linear correlation are  $R^2=0.9$  for C8-HSL and  $R^2=0.85$  for C10-HSL and therefore fit better than other applied trend lines. However,

mathematical characterisation of AHL decrease influenced by plants remains a difficult task, since several factors contribute to the process. Adsorption must be considered, especially for C10-HSL. According to previous results (Table 1) it can be concluded that adsorption processes should occur rather rapidly in the first days of the experiment, while plant-dependent processes begin to unfold their activities slowly and are dependent on a constant increase in plant and especially root mass, resulting in a roughly linear correlation.

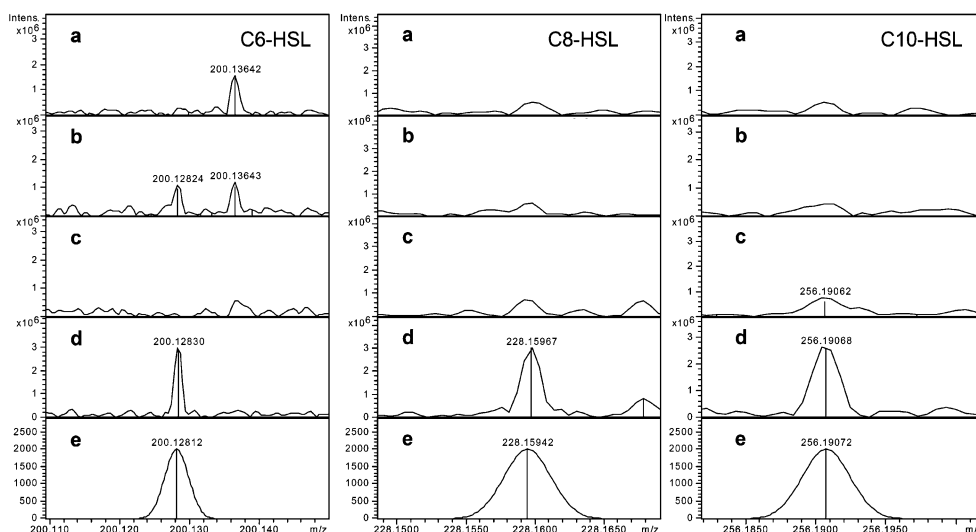
Basically, Fig. 5b again demonstrates the stronger depletion of C10-HSL in media compared to the less lipophilic C8-HSL. Calculating AHL leftover values for time point 400 h (nearly 17 days) shows that these lie in a range approximately 20% higher for both AHLs as shown in Fig. 2, indicating that this fraction consists of radioactively labelled derivatives like homoserines and probably so far unidentified fragments of AHL in the media, which were not detected by UPLC and FTICR-MS, which were used to detect only intact AHL molecules.

**Fig. 3** Identification of AHLs in barley plant extracts: *a* controls shoot, *b* AHL-treated shoots, *c* controls root, *d* AHL-treated roots, *e* standard



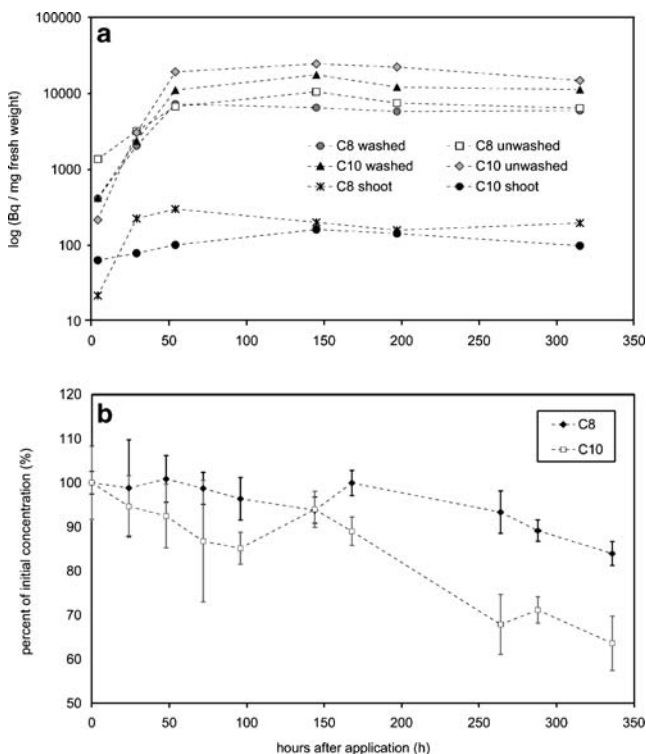


**Fig. 4** Identification of AHLs in extracts from yam bean: *a* controls shoot, *b* AHL-treated shoots, *c* controls root, *d* AHL-treated roots, *e* theoretical  $[MH]^+$  mass



### Selective uptake of stereoisomers

GC-MS was employed to check potential AHL stereo-selectivity of plants by separation of the optical isomers on a chiral capillary (Fig. 6). The comparison of D/L ratios shown in Table 5 clearly shows that plants favoured L-



**Fig. 5**  $[^3H]$ -Labelled AHLs in media and roots and shoots of barley. Data points were obtained at several time points (hours) after application of  $[^3H]$ AHL. **a** shows the uptake of  $[^3H]$ AHLs in roots (washed and unwashed) and shoots in relation to plant fresh weight. **b** demonstrates the changes in  $[^3H]$ AHL radioactivity in mineral media during growth of barley. Values are shown as percentage of initially applied radioactivity

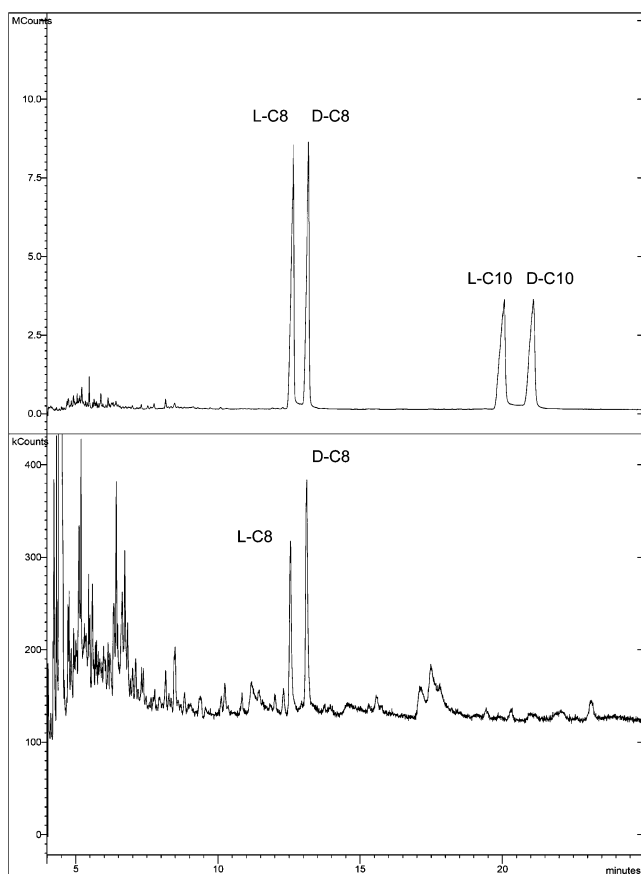
instead of D-isomers. Obvious differences between species were also reflected in selective uptake patterns. Concerning barley, a rather homogeneous increase in D/L ratio of around 11% on average as compared to  $T=0$  was documented for all three AHLs. This means that D-AHLs have been slightly discriminated during plant growth either by more rapid uptake or turnover of L-isomers, since their lipophilic features do not differ.

Yam bean *Pachyrhizus* also selected the L-form, but not all AHLs in approximately equal measure as barley does. Instead, the legume most strongly discriminated D-isomers of C6-HSL (D/L ratio of circa 2.7 after plant growth), but those from C8- and C10-HSL to a lesser extent, as shown in Table 3. C6-HSL represents the least lipophilic compound of the three (Table 2), so that indirect uptake into plant tissue by diffusion would seem more probable; however, since D-C6-HSL was clearly segregated by yam bean, some active uptake mechanism must be involved, which remains to be elucidated.

### Discussion

In this experimental setup, less than 10% of the AHLs underwent abiotic degradation processes like photo-catalyzed oxidation or alkaline hydrolysis. Adsorption, in this experimental setup to glass beads, represented an additional abiotic process which might influence AHL concentration in the media. It turned out that actually the longest side chain AHL used here, C10-HSL, was clearly more subject to adsorption processes than the other two compounds. However, we found that these abiotic processes contributed only in a minor way to the decline of AHLs in the plant growth solution of the axenic setup used in our experiments.

Another basic question we focussed on was whether such distant related plant species as barley and yam bean



**Fig. 6** Separation of enantiomers by GC-MS. The *upper* chromatogram shows two standards (C8- and C10-HSL); the *bottom* chromatogram shows real samples from yam bean treated with C8-HSL after 21 days. The first of the two respective isomer peaks represents L-AHL

would exhibit significant differences in their AHL uptake and turnover. Since C6-HSL half-life has already been reported to be dependent on plant species [28], we hypothesized that differences may also be the case for barley and yam bean. Furthermore we intended to demonstrate that AHL depletion also depends on chemical features of the applied signal compounds, like in this case lipophilicity ( $\log P$ ) according to side chain length. AHLs from media samples were analysed by UPLC and FTICR-MS and it was found that AHL sequestration actually depended on both plant species and on lipophilicity of the compound. This gives rise to speculations that lipophilicity may represent a

**Table 5** D/L ratios of AHLs in media before and after plant growth

	C6-HSL	C8-HSL	C10-HSL
Barley D/L (day 0)	0.92	0.99	0.99
Barley D/L (day 17)	1.04	1.08	1.11
Yam bean D/L (day 0)	0.97	1.02	1.07
Yam bean D/L (day 21)	2.70	1.25	1.65

significant factor in plant–AHL interactions, where signal compounds may encounter lipophilic sites.

A considerable difference in AHL depletion during growth of the plants was observed. The legume yam bean caused clearly more AHL diminution in the growth media. It is most likely that the significantly higher AHL depletion observed in media of the legume is due to enzymatic activities, since these results match the previously described ones by Delalande et al. [28], which have reported on degradation of AHL signal molecules by lactonase or acylase in the legume species *Lotus corniculatus*. However, there is evidence that activity of lactonases may represent an important factor in yam bean, since the comparison of relative signal intensities of AHL and HS signal gained by FT-ICR/MS measured in a continuous trial indicated that the ratio of AHL to HS was always  $>1$  in barley media, but  $<1$  in media after yam bean growth. The impact of acylase leading to cleavage of the acyl side chain could not be examined, on the one hand, due to the low  $m/z$  of the lactone entity which was below the FT-ICR/MS detection limit. On the other hand, fatty acids represent ubiquitous contaminants as well as naturally occurring compounds in plants. Besides enzymatic influence, it must also be considered that at least some proportion of the AHLs may be taken up intactly into plant tissue or stay adsorbed on surfaces.

AHL uptake and translocation into the plant was investigated by off-line UPLC and alternatively with tritium-labelled compounds. To confirm the identity of the compounds, a further measurement with FTICR-MS was implemented. Evaluation of the data actually revealed completely different uptake and translocation patterns of the two plants. In the monocot barley, all AHLs seemed to be taken up and transported into the whole plant, except for C10-HSL in the shoot. Yam bean merely transported C6-HSL into the shoot, while the two other compounds were restricted to root tissue. However, it remains to be elucidated whether they actually enter the root or stay adsorbed on surfaces. Interestingly, a C10-HSL signal was detected in shoots of barley using tritium-labelled AHL, while neither UPLC nor FTICR-MS measurements detected the presence of this molecule in barley shoots. As the scintillation method offers high sensitivity, it must be considered that not only unmodified structures (as measured with UPLC and FTICR-MS) of [ $^3\text{H}$ ]AHLs can be taken up, but also radioactive fragments which may, for instance, result from the abovementioned enzymatic metabolism of the plant.

Another point in these trials was that all three signal compounds tested in the previous experiments were commercially available AHL preparations in racemic state, with optical D- and L-isomers of AHLs in equal amounts. L-Isomers of AHLs had previously been reported to possess main biological activity, while D-isomers, in comparison, seemed to elicit only negligible activity [29, 30]. Despite identical

chemical composition of optical isomers, living organisms such as plants might exhibit competency in discriminating for one special isomer. It is known that L-isomers of AHLs help facilitating quorum sensing in bacteria, since no D-AHL-producing organism has yet been found [30]. Similarly, no information was available on selective uptake of these bacterial signals by plants. GC-MS was used to investigate whether plants are capable of chiral discrimination of AHL signal molecules. The results indicate that both plants exhibit the potential to select for L-isomers of AHLs to different extents. This finding gives rise to speculations that active processes, for example by involvement of membrane proteins, might be responsible for the discrimination of D-isomers in media, or that isomerase enzymes could interfere with D/L-AHL. Our further investigations will concentrate on the identification of processes involved in stereoselectivity and on the characterization of AHL metabolites and their enzymatic formation in media and plant tissue.

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