Arsenate impact on the metabolite profile, production, and arsenic loading of xylem sap in cucumbers (Cucumis sativus L.)

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INTRODUCTION
Arsenic uptake and translocation studies on xylem sap focus generally on the concentration and speciation of arsenic in the xylem. Arsenic impact on the xylem sap metabolite profile and its production during short term exposure has not been reported in detail. To investigate this, cucumbers were grown hydroponically and arsenate (AsV) and DMA were used for plant treatment for 24 h. Total arsenic and arsenic speciation in xylem sap was analyzed including a metabolite profiling under AsV stress. Produced xylem sap was quantified and absolute arsenic transported was determined. AsV exposure had a significant impact on the metabolite profile of xylem sap. Four m/z values corresponding to four compounds were up-regulated, one compound down-regulated by AsV exposure. The compound down-regulated was identified to be isoleucine. Furthermore, AsV exposure had a significant influence on sap production, leading to a reduction of up to 96% sap production when plants were exposed to 1000 μg kg−1 AsV. No difference to control plants was observed when plants were exposed to 1000 μg kg−1 DMA. Absolute arsenic amount in xylem sap was the lowest at high AsV exposure. These results show that AsV has a significant impact on the production and metabolite profile of xylem sap. The physiological importance of isoleucine needs further attention.

Keywords: arsenate, dimethylarsinic acid, isoleucine, metabolite profile, principal component analysis, xylem sap
studies have shown that metabolomics is able to uncover the response of plants on metal stress, such as cadmium on Arabidopsis thaliana (Ducruix et al., 2008) and copper, iron, and manganese on Brassica rapa (Jahangir et al., 2008). However, these studies were performed on whole, homogenized plants.

So far, metabolite profiling in xylem sap was done as targeted analysis in comparing the amount of known substances (i.e., targeted metabolomics), such as amino acids, sugars, or carboxylic acids in stressed plants against control plants (Callahan et al., 2009; López-Millán et al., 2009; Larbi et al., 2010). No untargeted metabolomic study on xylem sap under stress, let alone metal(loid) impact (like AsV), on xylem sap was done.

In this work, a study of the impact of AsV and DMA on the production and metabolite profile of xylem sap in Cucumis sativus L. is presented.

EXPERIMENTAL CHEMICALS
Arsenate (AsV, disodium hydrogen heptahydrate) was obtained from BDH Chemicals, Poole, UK. DMA (sodium dimethylarsinic acid) was obtained from Strem Chemicals, UK. Hoagland nutrient solution was made up in double distilled water (DDW). Chemicals were at least of analytical grade [KNO3, Ca(NO3)2, MgSO4, NH4NO3, KH2PO4, FeSO4, Na2MoO4]. For the first week, half-strength, for the rest of the time full-strength nutrient solution was used. Three different plant batches were grown. Plant set B was grown until before developing their two leaf stage, which took approx. 1 week. Reaching that, all stems were cut approx. 1 cm above their root neck, the first drop was wiped away and sap was collected with a micropipette for 1 h. Sap samples were weighed and then either measured right after extraction or stored at −20°C until further analysis. The sap was analyzed by ESI–MS for sucrose in order to test whether the xylem was contaminated with phloem.

QUANTIFICATION AND SPECIATION OF ARSENIC IN SAP
Sap collected from exudation was centrifuged and either diluted with ultrapure water to make up to 500 μL or injected directly into HPLC–ICP-MS (Agilent 1100 HPLC coupled to Agilent 7500c ICP-MS). External calibration was achieved by injection of DMA standard solution ranging from 0.5 to 100 μg kg⁻¹. To detect chloride interferences, m/z values 77 and 82 were also monitored. Indium (50 μg kg⁻¹) was infused continuously post column via a peristaltic pump as internal standard.

MEASUREMENT OF THE METABOLITE PROFILE OF XYLEM SAP

The metabolite profile was determined using plant set A. For separation a reversed-phase column (C18, Eclipse XDB-C18, Agilent, Agilent USA) was used. Hundred microliter of the sample was injected using a 0.1% formic acid in water – 0.1% formic acid in methanol gradient with 1 mL min⁻¹ flow. Starting from 100% water, methanol was added to the eluent after 5 min. From 5 to 25 min the methanol fraction was increased to 100%. The eluent was held at 100% methanol for a further 5 min, followed by re-equilibration. Total chromatographic run time was 40 min. The eluent flow was injected into an ESI–MS (MSD XCT, Agilent USA). The ESI–MS was used in positive/negative switch mode and MS² spectra were recorded when signal intensities were higher than 50,000 counts.

STATISTICAL ANALYSIS
The variation in xylem sap production, arsenic amount, and the intensities for the selected metabolites was determined by t-test with SigmaPlot 11. Multiple linear regressions were done with Minitab 15.
All chromatograms obtained by RP-HPLC–ESI–MS after sap analysis in positive mode were converted into bins (bin size 1 m/z and 1 min) with ProfileAnalysis 1.0 (Bruker). These bins were imported into PyChem 3.05 g (Jarvis et al., 2006) for PCA. Briefly, chromatograms obtained by the Agilent MSD XCT system were preprocessed by ProfileAnalysis into a matrix in which each column represents a sample and each row 1 m/z/time bin. The specifications were 1 min time and 1 m/z mass per bin. The range was defined from 0 to 35 min and from 80 to 1000 m/z. An average of all intensities in each bin was used. ProfileAnalysis created a matrix with 32,810 bins (rows) and 32 samples (columns) which have a total of 1,049,920 data points. The matrix was exported as ASCII file and used for PCA analysis with the statistical software package PyChem. In PyChem, no data pretreatment was performed. PCA was done on raw spectra by calculating 10 principal components (PC).

**Identification of xylem constituents**

Separation was performed on a C18 reverse-phase column (C18, Eclipse XDB-C18, Agilent, Agilent USA), using a 0.1% formic acid in water – 0.1% formic acid in methanol gradient with 1 mL/min flow. Starting from 100% water, methanol was added to the eluent after 7 min. From 7 to 25 min the methanol fraction was increased to 100%. The eluent was held at 100% methanol for a further 5 min, followed by re-equilibration of the column. Total chromatographic run time was 40 min. Hundred microliter of the sample was used. The eluent flow was injected into a high resolution ESI–MS (LTQ Orbitrap Discovery; Thermo Fisher Scientific) with a resolution of 30,000. Accurate masses of selected m/z values were used for chemical formula generation with X-calibur 2.0 (Thermo Fisher Scientific).

**Results**

**Arsenate impact on the metabolite profile of xylem sap**

Xylem sap collected from plant set A was used for determination of the metabolite profile. One plant of the 12 challenged with 710 μg kg⁻¹ AsV and 3 plants of the 12 challenged with 1400 μg kg⁻¹ AsV did not produce enough sap for analysis, therefore 32 instead of 36 samples were measured with RP-HPLC–ESI–MS. Total ion chromatograms of all 32 sap samples are shown in Figure A1 in appendix. The chromatograms have a good retention time alignment, as can be seen at the peaks of the positive mode chromatograms at ~10.5 and 23 min, respectively. This enables PCA without further peak alignment of the metabolite profile of the xylem sap. PC1 and PC2 combined explain 86% of the variance [PC1 (76% of variation) vs PC2 (10% of variation)], therefore the xylem sap. PC1 and PC2 combined explain 86% of the variance [PC1 (76% of variation) vs PC2 (10% of variation)], therefore the xylem sap. PC1 and PC2 combined explain 86% of the variance [PC1 (76% of variation) vs PC2 (10% of variation)]. This separation of the 710 μg kg⁻¹ samples is also visible in the sap flow results. The three 710 μg kg⁻¹ samples in the group with the 1400 μg kg⁻¹ samples are from one pot where all four samples exuded little xylem sap during extraction which indicates high impact of AsV stress (see Figure A4 in appendix). The other eight 710 μg kg⁻¹ samples were from plants exuding amounts of sap similar to the control plants showing low AsV impact. This however could be the effect of the reduced sap flow and simply a concentration effect. Therefore, individual metabolites need to be studied. This is achieved by analyzing the PCA loading plot. This plot shows which bins (m/z values at respective retention time) are responsible for the clustering in the scores plot; in this case the m/z value at what retention time. The loadings plot (Figure A5 in appendix) reveals that the m/z values of 132, 203, 219, 253, and 312 are significantly different within the 32 samples. For verification purpose all chromatographic peaks resulting from these m/z values in all chromatograms were integrated. The obtained peak areas were used for testing the statistical significance between each condition group. Furthermore, peak areas of a random m/z value which was not identified in the PCA loadings plot were also integrated (m/z 233). This was done to assess if the decreased sap flow, as discussed before, has a major influence. This random value (m/z 233) was chosen because its peak area was within the peak area and retention time range of the other, significantly different peaks. Figure 1 shows box plots of peak areas of all m/z values of all three conditions.

The plot shows that m/z 132 is down-regulated with increased AsV exposure while m/z 219, 253, 312, and 203 are up-regulated. There is no significant difference between control and challenged plants with m/z 233. All box plots of the m/z values of 132, 219, 253, 312, and 203 show that there is always a statistical significant difference with P < 0.01 between the control and 1400 μg kg⁻¹ samples and with one exception also between the control and the 710 μg kg⁻¹ samples. The exception is at m/z 253, which has no significant difference between control and 710 μg kg⁻¹ samples. There is no statistical difference between both the control and the 710 μg kg⁻¹ samples and control and the 1400 μg kg⁻¹ samples at m/z 233, which shows that the reduced sap flow is not the reason for the statistical significance of the other m/z values. With the peak areas of m/z 132, 219, 253, 312, and 203 and the results for sap flow and total arsenic concentration, multiple linear regressions were done. The m/z values of 132, 312, and 203 have a significant influence on the result, with P < 0.05 (0.022, 0.002, and <0.001, respectively), while for m/z 219 and 253 the P-values were >0.05 (0.791 and 0.726, respectively). Hence, the following equation can be used to calculate the total arsenic concentration in xylem sap from the amount of the three bio-molecules:

\[ c_{\text{model}}(\text{As}_{\text{excluded}}) = 10.502 - (0.0000946 \times \text{m/z} 132) - (0.000410 \times \text{m/z} 312) + (0.00202 \times \text{m/z} 203) \]

Plotting the modeled As concentration of this equation vs the actual measured concentration (see Figure 2A) shows that the resulting curve has a good correlation (R² = 0.71) considering the biological variability.

Furthermore, multiple linear regressions were used to assess possible biomarkers for sap flow, using the peak areas of m/z 132,
219, 253, 312, and 203 and the sap flow. In this case, only \( m/z \) 253 and 203 have a significant influence on the result, or a \( P \)-value ≤0.05 which leads to the following equation (see Figure 2B):

\[
\text{Sap flow} = 3.464 - (0.0000218 \times m/z 253) - (0.0000851 \times m/z 203)
\]

The results show that \( m/z \) 203 and 253 could be used as possible biomarkers for sap flow under arsenic stress.

**Identification of significant \( m/z \) values**

Xylem sap was measured with the LTQ Orbitrap Discovery system to identify the compounds found in experiment A).

For the determination of \( m/z \) 132, xylem sap of control plants was injected since this compound is down-regulated under arsenic stress and is most abundant in xylem sap of control plants. The extracted ion chromatogram shows a distinct peak at 4.5 min (Figure A6 in appendix). The accurate MS for this peak and the fragments of the MS\(^2\) are presented in Figure A7 in appendix and a possible chemical formula is shown in Table A1 in appendix. For the determination of the other 4 \( m/z \) species (203, 219, 253, and 312) xylem sap of plants challenged with \(~1400\) \( \mu \)g kg\(^{-1}\) for 24 h was used (Figure A8 in appendix). All \( m/z \) ranges (species ±0.5 \( m/z \)) resulted in one peak each (chromatograms can be found in the supplement information as well as MS and MS\(^2\) spectra; Figures A8–A12). The accurate MS data for the base peak and proposed chemical formulae are presented in Table 1.
These formulae were then used to search for known natural products in a database\(^1\). Only \(m/z\) 132 was unequivocally identified as isoleucine.

A spiking experiment with isoleucine standard showed that the unknown peak is increasing with added isoleucine (Figure A13 in appendix). Furthermore, the MS\(^2\) of this \(m/z\) value is also matching. The co-elution, matching accurate mass for [M-H]\(^+\) (see Figure A14 for MS and MS\(^2\) of isoleucine) and the matching MS\(^2\) are robust proof that the unknown compound at \(m/z\) 132 is isoleucine, which is down-regulated under As\(^{V}\) stress in cucumbers.

**IMPACT OF ARSENIC STRESS ON SAP FLOW**

Plants of set (A) challenged with increasing As\(^{V}\) concentration showed reduced sap flow. This was further investigated with two experiments (plant set B and C) which differ in age (before flowering and during flowering) using the same As\(^{V}\) and DMA concentrations. Xylem sap was collected for 1 h after decapitation of each plant. Sap extracted by exudation was clear and without color. Figure 3 shows sap flows of plant set B and C after challenge with As\(^{V}\) at 100, 500, and 1000 \(\mu\)g kg\(^{-1}\) and DMA at 1000 \(\mu\)g kg\(^{-1}\), respectively compared to sap flow of unchallenged plants.

The plants of set B (challenged before flowering) showed significantly lower sap flow than those of plant set C (challenged

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\(^1\) Dictionary of Natural Products on DVD, ver. 19.1, Taylor & Francis Group, 2010.
during flowering). Unchallenged plants produce the highest sap flow within one set of plants. Plants challenged with AsV show a significant decrease in sap flow with increased arsenic concentration. This accounts both for plants which have reached flowering stage and before reaching flowering stage. High AsV challenge of 1000 μg kg⁻¹ AsV in the nutrient solution leads to 96% reduction of xylem sap production. Plants challenged with DMA show no significant change in sap production.

In order to assess a correlation between inorganic arsenic stress (i.e., inorganic arsenic concentration in nutrient solution) and sap flow, arsenic concentration was determined in all nutrient solutions prior to plant challenge. Figure 4 shows sap flow of cucumber plants subjected to AsV with sap flow of control plants set to 100%. In contrast to the graphs the sap flow is plotted against the measured AsV concentration in the nutrient solution prior to plant treatment. Both slopes show a linear correlation, with $R^2$ of 0.98 for set B and 0.89 for set C. The slope of both graphs is similar and not significantly different, when normalized to control plants. Hence, the combined slope is $-8.8$ with an $R^2$ of 0.89 ($P < 0.001$).

The graph shows that although the absolute sap flow is differing by a factor of 8–10 between the two sets, the relative sap flow reduction is similar in both plant sets. The impact of AsV on the sap flow is independent of the plants developmental stage.

This is the first time that a linear correlation is observed between the arsenic concentration plants are subjected to and the sap flow.

**ARSENIC CONCENTRATION AND SPECIATION IN XYLEM SAP**

Sap samples extracted from plants grown hydroponically and extracted via exudation were injected for arsenic determination and speciation into anion-exchange HPLC–ICP-MS. Chromatograms of xylem sap of plant set B and C can be found in the supplement information and show only the occurrence of DMA and the inorganic arsenic species (Figures A15 and A16 in appendices). Table 2 shows the concentration of those arsenic species found in xylem sap after arsenic challenge with AsV and DMA, respectively.

When plants were challenged with DMA, they transported only DMA in the sap, no other species was detected. When plants were exposed to 100 μg kg⁻¹ AsV, both inorganic arsenic species appeared in the xylem sap of older plants. AsIII, AsV, and MMA were found when plants were exposed to MMA with MMA being the main fraction (Ye et al., 2010).

The main difference between younger plants and plants in the flowering stage is the increased AsV and AsIII concentration in xylem sap of older plants. Younger plants seem to be able to control the xylem loading of AsV. At 500 and 1000 μg kg⁻¹ exposure to AsV only AsIII was detected in the sap, leading to the assumption that all AsV was reduced to AsIII in roots before xylem sap loading.

Arsenic speciation and concentration as well as arsenic amount in plant set A show a main fraction of AsIII with a smaller fraction of AsV. DMAV was detected in traces (see Figure A17 in appendix).

**IMPACT OF ARSENIC STRESS ON ABSOLUTE AMOUNT OF ARSENIC TRANSPORTED IN XYLEM SAP**

In order to calculate the absolute amount of arsenic transported in 1 h the sap flow is multiplied with the total arsenic concentration. The following equation was used:

\[
\text{Arsenic transported [ng h}^{-1}\text{]} = \text{Sap flow [g h}^{-1}\text{]} \times \text{Arsenic concentration [ng g}^{-1}\text{]}
\]

With this formula the actual amount of arsenic transported in the xylem within 1 h is calculated.

**Figure 5** shows the amount of arsenic in sap samples of plant set B and C. When plants are challenged with 1000 μg kg⁻¹ DMA, the total arsenic amount is highest in both plant sets. In contrast, the amount of arsenic transported is significantly smaller when plants are subjected to 1000 μg kg⁻¹ AsV.

Younger plants (plant set B), the amount of total arsenic in xylem sap is significantly reduced with increasing AsV concentration in the nutrient solution.

Older plants (plant set C) show a different pattern, with the plants challenged with ~500 μg kg⁻¹ AsV having the highest arsenic amount with 46 ± 30 ng h⁻¹. Arsenic amounts of plants challenged with ~100 and ~1000 μg kg⁻¹ are lower, with 5.6 ± 1.4 and 14.0 ± 1.7 ng h⁻¹, respectively. However, there is no statistical significant difference between the arsenic amounts of plants challenged with inorganic arsenic ($P > 0.05$). In comparison to plants challenged with DMA, there is a difference to all AsV contaminated plants ($P < 0.05$).

Younger plants before the flowering stage in this study were able to restrict arsenic loading into the xylem at high AsV concentrations in the nutrient solution. This and the reduced sap flow are leading to a significantly reduced arsenic amount compared to low AsV exposure.

In contrast, older plants are not able to restrict AsV loading into the xylem effectively when treated with increasing AsV concentration. Even the reduced sap flow cannot compensate for the increased arsenic concentration in the sap, leading to statistically similar arsenic amounts at different concentrations. High AsV concentrations in the nutrient solution and low phosphate status will lead to highly efficient uptake of As into the root cells. This resulting high influx may saturate the arsenate reductase, an
Table 2 | Arsenic concentration and speciation of sap samples of plant set B and plant set C after challenge with 1000 μg kg\(^{-1}\) DMA, 100, 500, and 1000 μg kg\(^{-1}\) As\(^{V}\) for 24 h.

| Arsenic species | c(As) in nutrient sol\(^{1}\) (μg/kg) | c(DMA)\(^{2}\) in sap (μg/kg) | c(MMA)\(^{2}\) in sap (μg/kg) | c(As\(^{III}\)) in sap (μg/kg) | c(AsV) in sap (μg/kg) |
|-----------------|-------------------------------------|-----------------------------|----------------------------|--------------------------------|---------------------|
| B DMA           | 117                                 | 973 ± 15.5                  | n.d.                       | n.d.                           | n.d.                |
| B As\(^{V}\)    | 66                                  | n.d.                        | n.d.                       | 3.8 ± 2.4                      | 13.5 ± 5.8          |
| 567             | n.d.                                | n.d.                        | 1.5 ± 0.4                  | n.d.                           | n.d.                |
| 909             | Traces                              | n.d.                        | 9.9 ± 4.6                  | n.d.                           | n.d.                |
| C DMA           | 759                                 | 60.3 ± 3.6                  | n.d.                       | n.d.                           | n.d.                |
| C As\(^{V}\)    | 125                                 | n.d.                        | n.d.                       | 2.7 ± 0.1                      | 1.0 ± 0.1           |
| 554             | n.d.                                | n.d.                        | 13.2 ± 0.6                 | 23.4 ± 8.6                     | n.d.                |
| 759             | Traces                              | n.d.                        | 23.2 ± 4.8                 | 47.7 ± 1.1                     | 23.4 ± 8.6          |

\(^{1}\)Before treatment; \(^{2}\)expressed as μg As/kg.

\(n = 4\). Traces: below LOQ of 1.5 μg/kg (10σ), n.d.: below LOD of 0.5 μg/kg (3σ). Selected HPLC–ICP-MS chromatograms are shown in Figures A15 and A16 in appendices in the supplement.

between these redox species. While As\(^{III}\) is efficiently complexed (Raab et al., 2005; Bluemlein et al., 2008) with phytochelatins, this is resulting in higher As\(^{V}\) loading into the xylem.

Plants treated with high concentrations of DMA do neither show a decreased arsenic amount nor a decreased sap flow. This lack of response indicates the non-existence of toxicity or the absence of a specific defense response by the plant.

**DISCUSSION**

This metabolite study is the first available which is focused on the impact of arsenic stress on the organic composition of xylem sap. So far, only iron-deficient plants were used for targeted metabolite analysis. The concentration of organic anions in xylem sap was monitored in iron-deficient *Lycopersicon esculentum* L. (tomato), which resulted in a significant change of the organic anion composition (López-Millán et al., 2009). This effect was also monitored in xylem sap of iron-deficient *Beta vulgaris* (sugar beet; Larbi et al., 2010).

In this study, five m/z values were identified as being up- or down-regulated under arsenate stress, corresponding to five peaks in the LC-MS chromatograms and therefore to five compounds. Four of the compounds were up-regulated, one down-regulated. This up- or down-regulation was not due to the reduced sap flow induced by arsenate stress as shown by the integration of a random m/z value (m/z 233) which was unaffected by As\(^{V}\) treatment. Three of these m/z values can be used as potential biomarkers for either total arsenic concentration or sap flow.

Identification of the down-regulated compound was achieved for isoleucine (m/z 132). The data from the metabolite profiling study and the identification part show that under arsenic stress isoleucine is down-regulated in the xylem sap of cucumbers.

So far, isoleucine has not been found to be involved in the plant response toward arsenic. In *Arabidopsis thaliana*, isoleucine is part of the γ-glutamyl-cycle along with all other 20 α-amino acids. This cycle is thought to be used by plants to transport amino acid inter- and intra cellular since the γ-glutamyl-enzyme is part of the cell membrane. Interestingly, this cycle is based on the transformation of glutathione, which is also the initial biothiol in the phytochelatin synthesis (leading to As\(^{III}\), PC complexes).

**FIGURE 5** | Arsenic amount of sap samples of decapitated cucumbers in experiment B (Figure 5A) and C (Figure 5B) after challenge of plants with no arsenic, DMA at 1000 μg kg\(^{-1}\) and As\(^{V}\) at 100, 500, and 1000 μg kg\(^{-1}\) for 24 h. Sap was collected for 1 h \(n = 4\). Statistical similarity in one graph is indicated by letter above bar.
Furthermore, the genes encoding the enzymes that convert the amino acid (e.g., isoleucine) and glutathione into a 5-L-glutamyl-L-amino acid and L-cysteineglucine are closely related to the PC encoding genes (Ohkama-Ohtsu et al., 2009; Destro et al., 2011). It might be that isoleucine is involved in the glutathione production and is therefore consumed under arsenic stress.

Furthermore, isoleucine is a substrate for a plant stress hormone. In Arabidopsis thaliana, (-)-jasmonoyl-L-isoleucine is synthesized from jasmonic acid and L-isoleucine when the plant is attacked by an herbivore, wounded by mechanical means or infected by a pathogen (Svoboda and Boland, 2010; Sato et al., 2011). It might be that AsV contamination is also causing stress in the roots of cucumbers leading to an increased formation of (-)-jasmonoyl-L-isoleucine and a depletion of L-isoleucine in the roots and subsequently in the sap.

Furthermore, isoleucine along with valine and leucine was decreased in rat plasma and red blood cells when rats were fed with AsV (Kilroe-Smith and McLoughlin, 1979). Isoleucine-containing enzymes were also identified as playing a role in the arsenic methylation in humans, which are up-regulated under arsenic stress (Wood et al., 2006). However, no function for isoleucine in xylem sap under arsenic stress has so far been described in the literature.

Arsenic concentration and speciation in xylem sap was found to be consistent with other studies. Plants challenged with DMA did transport only DMA in the xylem. Plants challenged with AsV transported both inorganic arsenic species in the xylem, which is consistent with previous studies (Pickering et al., 2000; Mihucz et al., 2005; Xu et al., 2007). There are probably several factors governing the loading of AsV into the xylem, including the AsV reduction capacity of the roots. In this study, xylem sap of plants in the flowering state contains both inorganic arsenic species. The in vivo reduction of AsV is thought to be done by the enzyme ACR2, which has shown in vitro AsV reducing capacity (Bleeker et al., 2006; Ellis et al., 2006). Here, plants which have reached flowering stage had a significantly higher concentration of AsV in the xylem than younger plants. This leads to the assumption that

- arsenate reductase was not able to quantitatively reduce AsV to AsIII in plants at the flowering stage with high xylem sap flow
- the overall arsenic uptake is significantly higher in older plants.

During sap extraction a new arsenic stress effect was observed. With increasing inorganic arsenic stress (i.e., increasing inorganic arsenic concentration in the nutrient solution) a decreased sap flow was monitored. This was first mentioned in a recent publication where castor beans challenged with 3.75 and 7.5 mg kg⁻¹ AsV did not exudate xylem sap (Ye et al., 2010). In another recent hydroponic study this effect was studied more closely, revealing that xylem sap exudation of cucumbers was significantly reduced at high arsenic levels (7.5 mg kg⁻¹) in the nutrient solution (Czech et al., 2011). Furthermore, leaf transpiration and biomass production were significantly inhibited.

Here, this effect was analyzed in more detail. Decreased sap flow with increased inorganic arsenic stress is consistent for all plant experiments shown in this study. Reduced sap production was not monitored with high concentrations of dimethylated arsenic species. The correlation between sap flow vs. AsV concentration in nutrient solution is linear for younger plants, which have not yet reached flowering stage and older plants which have reached flowering stage. This might be another reason for AsV toxicity because reduced sap flow could lead to less nutrients and water intake and therefore to lower biomass production. However, it is yet unclear what mechanisms are involved in lower sap production.

The decreased sap flow leads furthermore to a decreased arsenic amount within the plant. Even with increased total arsenic concentration in the xylem, the decreased sap flow is the significant factor. It is interesting that the highest sap amount monitored in plant set A was at the lowest concentration in the nutrient solution. There might be a threshold concentration which triggers the reduction of sap flow.

The decrease in arsenic amount under AsV stress compared to methylated arsenic could be due to two reasons:

- the plants have a mechanism to shut down sap production in order to avoid arsenic contamination in the xylem and throughout the plant, or
- the concentration of AsV is already causing toxicity symptoms leading to a decrease in cell functionality.

Usually, the total arsenic concentration in leaves and fruits is higher in plants grown on highly contaminated soil or nutrient solution (Abedin et al., 2002). However, it seems as if translocation factors from roots to shoots are higher when plants are grown on less contaminated soil or nutrient solution as presented later (Caetano et al., 2008). They have investigated the metal retention in belowground and aboveground biomass of salt marsh plants compared to the metal composition in the surrounding marsh. For arsenic they found that plants grown on soil with the highest As concentration had also the highest As concentration in the belowground biomass. Interestingly, those plants had a significantly lower As concentration in the above ground biomass than plants grown on soil with lower As concentration. This means that a large reduction of available arsenic in the soil has only a small effect on the arsenic concentration in the above ground biomass. In another study it was revealed that of 13 different plants grown on 3 different soils with different arsenic concentrations in the surface water, the plants grown on the least contaminated soil had the highest arsenic translocation factor from root to shoot (Zandsalimi et al., 2011). The same effect was observed in a recent study where the highest translocation factors were observed in plants grown on the lowest bio-available arsenic fraction in three different soils (Baig et al., 2011).

These results suggest the hypothesis that there is a concentration threshold at which plants react with their defense mechanisms (phytchelatin synthesis/xylem sap reduction). However, these results raise the question whether concentration data obtained by this xylem sap sampling technique is valid for inter-comparison (control – challenged plants). The significant reduction of sap production could have a significant influence on the arsenic loading.

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FIGURE A1 | RP-HPLC-ESI-MS total ion chromatograms of all 32 sap samples from plant set A.

FIGURE A2 | PCA score plot of PC1 vs PC2 of the HPLC-ESI-MS obtained from plant set A. Gray numbers: Group A, black numbers: group B according to cluster analysis. Letters and numbers are coded as: 10–3: 10 = arsenate concentration in nutrient solution (1000 μg kg⁻¹); 3 = pot number, there were four plants in one pot.
**Figure A3** | Dendrogram of the data points in PCA score plot of plant set A using two clusters, calculated by using the Euclidean distance with complete linkage.

**Figure A4** | Xylem sap flow of cucumbers subjected to different arsenic treatment: No arsenic (C-x); 5–3: 677 μg/kg; 5–4: 666 μg/kg; 5–5: 791 μg/kg; 10–3: 1405 μg/kg; 10–4: 1407 μg/kg; 1405 μg/kg, (n = 4).
FIGURE A5 | PCA loadings plot of weighting 1 vs weighting 2 of the LC-MS runs of respected tR and m/z obtained from plant set C. PC1: 76%, PC2: 10%. Numbers given are m/z (three digits) followed by retention time in seconds (three digits).
FIGURE A6 | RP-HPLC-ESI-MS (Orbitrap) base peak chromatograms of xylem sap of plants not challenged with arsenate. (A) the whole mass range (m/z 100–2000) and (B) the mass range m/z 131.50–132.50.
FIGURE A7 | Accurate mass MS and MS2 of m/z 132 at 4.46 min.
FIGURE A8 | RP-HPLC-ESI-MS (Orbitrap) base peak chromatograms of xylem sap of plants challenged with 1000 μg/kg arsenate with (A) the whole mass range (m/z 100–2000), (B) the mass range m/z 202.50–203.50, (C) the mass range m/z 218.50–219.50, (D) the mass range m/z 252.50–253.50, and (E) the mass range m/z 311.50–312.50.
FIGURE A9 | Accurate mass MS and MS2 of m/z 203 at 3.35 min.
FIGURE A10 | Accurate mass MS and MS2 of m/z 219 at 2.38 min.
FIGURE A11 | Accurate mass MS and MS2 of m/z 253 at 11.59 min.
FIGURE A12 | Accurate mass MS and MS2 of m/z 312 at 4.63 min.
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FIGURE A13 | RP-HPLC–ESI-MS (Orbitrap) base peak chromatograms of (A) isoleucine showing the m/z range 132.10–132.11, (B) an unspiked xylem sap sample, showing the m/z range 132.10–132.11, and (C) a xylem sap sample spiked with isoleucine, showing the m/z range 132.10–132.11.
FIGURE A14 | Accurate mass MS and MS2 for isoleucine.

FIGURE A15 | Anion-exchange HPLC–ICP-MS chromatograms of xylem sap samples of plants challenged with 1000 μg/kg DMAV, 100, 500, and 1000 μg/kg AsV for 24 h. Samples of 500 and 1000 μg/kg AsV had to be diluted to make up enough sample for injection. All chromatograms have an offset for clarity (Plant set B).
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FIGURE A16 | HPLC–ICP-MS chromatograms of xylem sap samples of plants challenged with DMA 1000 μg/kg, As(V) 100, 500, and 1000 μg/kg, respectively for 24 h. Chromatograms are shifted for clarity (Plant set C).

FIGURE A17 | Arsenic concentration and speciation in xylem sap of plants grown for 28 ± 3 days (plant set A) and challenged with no arsenic and As(V) at 710 and 1400 μg/kg for 24 h. Sap extracted for 1 h. Concentration of blank xylem sap was below LOD of 0.5 μg/kg (3σ of noise; n = 12).
### Table A1 | Chemical formulae calculated with m/z 132.

| Index | Formula   | RDB | Δ ppm |
|-------|-----------|-----|-------|
| 1     | C₆H₁₄O₂N  | 0.5 | −0.872|

### Table A2 | Chemical formulae calculated with m/z 203.

| Index | Formula       | RDB | Δ ppm |
|-------|---------------|-----|-------|
| 1     | C₇H₁₇O₂N₅     | 2.0 | 3.267 |
| 2     | C₉H₁₉O₃N₂     | 1.5 | −3.342|

### Table A3 | Chemical formulae calculated with m/z 219.

| Index | Formula       | RDB | Δ ppm |
|-------|---------------|-----|-------|
| 1     | C₁₂H₉N₉       | 8.0 | −2.113|
| 2     | C₈H₁₅O₃N₂     | 2.5 | −2.136|
| 3     | C₆H₁₃O₄N₅     | 3.0 | 3.992 |

### Table A4 | Chemical formulae calculated with m/z 253.

| Index | Formula       | RDB | Δ ppm |
|-------|---------------|-----|-------|
| 1     | C₁₂H₁₇O₄N₂    | 5.5 | −1.871|
| 2     | C₁₀H₁₅O₃N₅    | 6.0 | 3.434 |

### Table A5 | Chemical formulae calculated with m/z 312.

| Index | Formula       | RDB | Δ ppm |
|-------|---------------|-----|-------|
| 1     | C₁₀H₁₆O₄N₈    | 7.0 | −1.642|
| 2     | C₁₁H₂₂O₈N     | 1.5 | −1.658|
| 3     | C₉H₂₀O₈N₄     | 2.0 | 2.643 |