New Phytologist

Rapid reduction of arsenate in the medium mediated by plant roots

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Received: *14 May 2007*
Accepted: *20 June 2007*

Summary

• Microbes detoxify arsenate by reduction and efflux of arsenite. Plants have a high capacity to reduce arsenate, but arsenic efflux has not been reported.

• Tomato (*Lycopersicon esculentum*) and rice (*Oryza sativa*) were grown hydroponically and supplied with 10 μM arsenate or arsenite, with or without phosphate, for 1–3 d. The chemical species of As in nutrient solutions, roots and xylem sap were monitored, roles of microbes and root exudates in As transformation were investigated and efflux of As species from tomato roots was determined.

• Arsenite remained stable in the nutrient solution, whereas arsenate was rapidly reduced to arsenite. Microbes and root exudates contributed little to the reduction of external arsenate. Arsenite was the predominant species in roots and xylem sap. Phosphate inhibited arsenate uptake and the appearance of arsenite in the nutrient solution, but the reduction was near complete in 24 h in both –P- and +P-treated tomato. Phosphate had a greater effect in rice than tomato. Efflux of both arsenite and arsenate was observed; the former was inhibited and the latter enhanced by the metabolic inhibitor carbonylcyanide *m*-chlorophenylhydrazone.

• Tomato and rice roots rapidly reduce arsenate to arsenite, some of which is actively effluxed to the medium. The study reveals a new aspect of As metabolism in plants.

Key words: arsenate, arsenate reduction, arsenic speciation, arsenite, efflux, rice (*Oryza sativa*), tomato (*Lycopersicon esculentum*).

New Phytologist (2007) **176**: 590–599

doi: 10.1111/j.1469-8137.2007.02195.x

Introduction

There is widespread contamination of arsenic in the environment caused by anthropogenic activities or from geogenic sources. Arsenic taken up by food crops may present a serious health risk to humans. For example, Meharg & Rahman (2003) reported up to 1.7 μg g^{-1} in rice grain grown in some areas in Bangladesh, where As is building up in the soil because of irrigation with As-contaminated groundwater. Their calculations showed that consumption of rice grain, even at the typical As concentrations found in Bangladesh, could contribute considerably to the total ingestion of As. Smith *et al.* (2006) found that cooked rice and vegetables were a substantial source of As to the Bangladesh population. On the other hand, plants colonizing environments heavily contaminated with As have evolved resistance or detoxification mechanisms to overcome its phytotoxicity (Meharg & Hartley-Whitaker, 2002). At the extreme, some species of fern are able to hyperaccumulate As in the above-ground part to several thousand mg As kg^{-1} dry weight without suffering from toxicity (e.g. Ma *et al.*, 2001; Zhao *et al.*, 2002). For these reasons, the uptake and metabolism of As by plants has attracted much attention in recent years.

Arsenic metabolism has been studied extensively in microbes (see reviews by Rosen, 2002; Silver & Phung, 2005). In both *Escherichia coli* and *Saccharomyces cerevisiae*, arsenate is taken

up into cells by phosphate transporters, whereas arsenite can be taken up directly via the aquaglycerolporin channels. Arsenate is then reduced to arsenite by the bacterial ArsC or yeast Acr2p enzymes. For subsequent detoxification, arsenite is removed from the cytosol by active efflux via the ArsB or ArsAB pump in *E. coli* and Acr3p in the yeast. An arsenite chaperone (ArsD) has recently been identified in *E. coli*, which transfers arsenite to the efflux pump and enhances its extrusion from the cells (Lin *et al.*, 2006).

Similarly, in higher plants, arsenate and arsenite are taken up via phosphate transporters and aquaglycerolporin channels, respectively (see review by Meharg & Hartley-Whitaker, 2002). Arsenate reduction in plants is also likely to be catalysed by arsenate reductase. Based on sequence homology with the yeast arsenate reductase gene *ACR2*, genes encoding arsenate reductase have recently been cloned and characterized from different plant species including *Arabidopsis thaliana* (Dhankher *et al.*, 2006), *Holcus lanatus* (Bleeker *et al.*, 2006), *Pteris vittata* (Ellis *et al.*, 2006) and rice (Duan *et al.*, 2007). Arsenite is then complexed with thiols, such as glutathione and phytochelatins, and sequestered in the vacuoles (Sneller *et al.*, 1999; Pickering *et al.*, 2000; Schmöger *et al.*, 2000; Raab *et al.*, 2005). Reduction of arsenate and complexation of arsenite by thiol peptides are considered to be the main mechanism of As detoxification in plants, although in the As hyperaccumulator *Pteris vittata* most of the arsenite is uncomplexed (Webb *et al.*, 2003; Zhao *et al.*, 2003). A small proportion of As may also be methylated to form compounds such as dimethylarsinic acid (DMA) (Quaghebeur & Rengel, 2003; Raab *et al.*, 2005).

In a preliminary experiment comparing uptake and speciation of arsenate vs arsenite by tomato (*Lycopersicon esculentum*) plants, it was found, unexpectedly, that arsenate added to the nutrient solution was rapidly reduced to arsenite. A series of experiments were conducted to investigate this hitherto unreported phenomenon. Here evidence is shown for arsenate reduction by roots and efflux of arsenite into the external medium, a pathway common in microbes.

Materials and Methods

Plant culture

Seeds of tomato (*Lycopersicon esculentum* L. cv. Alicante) and rice (*Oryza sativa* L. cv. Oochikara) were surface sterilized by immersing them in 0.5% NaOCl for 15 min. After rinsing, seeds were soaked in deionized water overnight, then transferred to a nylon net floating on 0.5 mM $CaCl_2$ solution in a plastic container. After germination, 45 seedlings were grown in a 30-l container for 2 wk before being used for experiments. Seedlings were cultured hydroponically with a modified 1/5 strength Hoagland nutrient solution for tomato or a 1/2 strength Kimura solution for rice. The compositions of the nutrient solution for tomato were 1.0 mM KNO_3, 1.0 mM $Ca(NO_3)_2$, 0.4 mM $MgSO_4$, 0.1 mM KH_2PO_4, 0.5 μM $MnCl_2$, 3 μM H_3BO_3, 0.1 μM $(NH_4)_6Mo_7O_{24}$, 0.4 μM $ZnSO_4$, 0.2 μM $CuSO_4$, 20 μM NaFe(III)-EDTA, 2 mM MES (pH adjusted to 6.0 with KOH), and for rice 0.091 mM KNO_3, 0.183 mM $Ca(NO_3)_2$, 0.274 mM $MgSO_4$, 0.1 mM KH_2PO_4, 0.183 mM $(NH_4)_2SO_4$, 0.5 μM $MnCl_2$, 3 μM H_3BO_3, 0.1 μM $(NH_4)_6Mo_7O_{24}$, 0.4 μM $ZnSO_4$, 0.2 μM $CuSO_4$, 20 μM NaFe(III)-EDTA, 2 mM MES (pH adjusted to 5.6 with KOH). Nutrient solutions were renewed once every 3 d, and were aerated continuously for the culture of tomato, but not rice because rice roots can secrete oxygen to the external medium. The growth conditions were 16 h photoperiod with a light intensity of 350 μmol m^{-2} s^{-1}, 25 : 20°C day : night temperatures and 70% relative humidity.

Experiment 1

The first experiment was carried out to investigate As speciation in tomato roots, shoots, xylem sap and nutrient solutions, with plants being supplied with either arsenate or arsenite. Tomato plants (15 d old) were transferred to 1-l vessels (two plants per vessel). After 2 d, four treatments were imposed consisting of 10 μM arsenate (Na_2HAsO_4) or arsenite ($NaAsO_2$) with or without 100 μM phosphate. Each treatment was replicated in three vessels. Nutrient solution was aerated continuously. Plants were harvested after As exposure for 1 or 3 d. At harvest, 1 ml nutrient solution was taken from each vessel for analysis of As speciation. Stems were cut at approx. 1 cm above the roots with a sharp blade, and xylem exudates were collected by pipette for 1 h after decapitation. Both nutrient solutions and xylem sap were diluted with a phosphate buffer solution (PBS) containing 2 mM NaH_2PO_4 and 0.2 mM Na_2-EDTA (pH 6.0), which was the eluant solution used for As speciation analysis (see below), and stored on ice before being analysed within 12 h of collection. Addition of EDTA has been shown to preserve As speciation in groundwater samples (Bednar *et al.*, 2002). A preliminary test showed that no changes in As speciation occurred during storage. Plant shoots were rinsed with deionized water, blotted dry and frozen in liquid nitrogen. Plant roots were rinsed briefly in an ice-cold desorption solution containing 1 mM K_2HPO_4, 0.5 mm $Ca(NO_3)_2$ and 5 mm MES (pH 6.0), and immersed in 1 l of the same solution for 10 min to remove apoplastic As. Root samples were blotted dry and frozen in liquid N. Shoots and roots were ground in a mortar and pestle with liquid N. Aliquots (0.2–0.5 g) of the ground materials were extracted with 20 ml PBS for 1 h under sonication. The extracts were filtered through four layers of muslin cloth, followed by filtration through 0.45-μm filters before analysis of As speciation. Total concentrations of As in roots and shoots were also determined.

Experiment 2

Because the nutrient solutions in Expt 1 were not axenic, this experiment was designed to test whether microbes living in

nutrient solution or exudates of tomato roots could mediate transformation of As. The nutrient solution that had been used to grow tomato for 3 d was collected and divided into two portions. A portion was unfiltered and another filtered through a sterilized 0.2-μm filter into a sterilized vial. This filtration procedure was found to remove microorganisms living in the nutrient solution effectively (Zhao *et al.*, 2001). For comparison, the initial nutrient solution that had not been used for growing plants was included. Arsenate or arsenite (10 μM) was added to the three solutions, and incubated in the dark for 1 d inside the same growth cabinet as used for plant experiments. Each treatment was replicated threefold. Arsenic speciation was determined.

Experiment 3

This experiment was set up to investigate the time course of the reduction of arsenate in the nutrient solution. Tomato plants (15 d old) were transferred to 1-l vessels (two plants per vessel) containing the full nutrient solution. Then 2 d later, plants were divided into two groups for the –P and +100 μM P treatments, each having four replicates. Arsenate was added to the nutrient solutions of both treatments at 10 μM. Nutrient solutions were aerated continuously. Aliquots of 0.5-ml nutrient solution were removed from each vessel at 1, 3, 5, 8 and 24 h after the addition of arsenate and diluted with PBS, and the speciation of As was determined.

Experiment 4

This experiment was set up to examine the efflux of As from tomato roots. Tomato plants (17 d old) were exposed to 10 μM arsenate for 24 h, with or without P, as described for Expt 3. Plant roots were rinsed briefly in deionized water, followed by a desorption of apoplastic As in a solution containing 1 mM K_2HPO_4, 0.5 mM $Ca(NO_3)_2$ and 5 mM MES (pH 6.0) for 10 min. Plant roots were then placed in a full nutrient solution with P for 24 h. Aliquots of 0.5-ml nutrient solution were removed from each vessel at 1, 3, 5, 8 and 24 h and diluted with PBS, and As speciation was determined.

Experiment 5

The effects of the metabolic inhibitor carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) and the P-type ATPase inhibitor sodium vanadate were investigated. Tomato plants (17 d old; one plant per vessel) were exposed to 10 μM arsenate for 5 h in a nutrient solution without P. Treatments included control, +200 μM sodium vanadate, +1 μM CCCP (dissolved in ethanol with a final concentration of 0.01% v/v) and +ethanol (0.01% v/v). Each treatment was replicated threefold. Nutrient solutions were aerated continuously. Aliquots of 0.5-ml nutrient solution were removed from each vessel at 5 h after the addition of arsenate and diluted with PBS, and the speciation of As was determined. Additionally, plants in nine vessels of the control treatment were desorbed of the root apoplastic As, as described for Expt 4. Plant roots were then placed in a full nutrient solution with P for 2 h to collect As extruded from the roots. Three treatments were included during the efflux period: control, +200 μM sodium vanadate and +1 μM CCCP. After 2 h, nutrient solutions were taken for the analysis of As species. Plants were harvested for measurement of the fresh weights of roots and shoots.

Experiment 6

Rice plants (20 d old) were used in this experiment. The treatments were the same as those for Expt 1. Plants were exposed to As for 1 d. Roots, shoots and nutrient solutions were sampled for the analysis of As speciation. Expt 2 was also repeated with rice.

Analysis of arsenic speciation

Arsenic speciation in nutrient solutions, xylem sap and plant extracts was determined using HPLC–ICP–MS (Agilent LC1100 series and Agilent ICP–MS 7500ce, Agilent Technologies, Santa Clara, CA, US), similar to the method described by Van den Broeck *et al.* (1998). Arsenic species (arsenite, arsenate, DMA and monomethylarsonic acid) were separated using an anion-exchange As speciation column (Agilent G3154-65001) fitted with a guard column (Agilent G3154-65002). The mobile phase was the PBS solution (2 mM NaH_2PO_4, 0.2 mM Na_2-EDTA pH 6.0), which was pumped through the column isocratically at 1 ml min^{-1}. The outlet of the separation column was connected to a concentric nebulizer and a water-jacketed cyclonic spray chamber of the ICP–MS. Signals at m/z 75 (As) and 35 (Cl) were collected with a dwell time of 500 ms. Possible polyatomic interference of ArCl on m/z 75 was removed by the Agilent Octopole Reaction System operating in the helium gas mode. Peaks were identified by comparison with the retention times of standard compounds. Arsenic species in the samples were quantified by external calibration curves with peak areas. This method did not allow us to quantify arsenite–thiol complexes, as the extraction and separation with an anion-exchange column may have led to dissociation of the complexes (Raab *et al.*, 2004b). However, the method was effective in preserving the oxidation states [As(V) or As(III)] of the As species in the samples, and therefore appropriate for studying the redox reactions between arsenate and arsenite. Analysis of As species was carried out immediately following sample collection or extraction. For each batch of samples, the analysis was completed within 12 h. Samples that were analysed at the beginning of the run were repeated at the end of the run; no changes in As speciation were observed during this period.

Analysis of total arsenic

Ground plant samples (approx. 1 g FW) were digested in 5 ml $HNO_3/HClO_4$ (85/15 v/v). Total As concentrations in the samples were determined by ICP–MS (Agilent 7500ce) operating in helium gas mode to remove possible interference of ArCl on m/z 75. Certified reference materials (seaweed IAEA-140/TM and tomato leaves NIST1573a) and blanks were included for quality assurance. Repeated analysis of the two certified reference materials gave 43.6 ± 1.5 μg g^{-1} As for IAEA-140/TM (certified value 44.3 ± 2.1 μg g^{-1} As) and 0.106 ± 0.0057 μg g^{-1} As for NIST1573a (certified value 0.112 ± 0.004 μg g^{-1} As), respectively. Calibration standards used for As speciation were also analysed for their total As concentrations by ICP–MS.

Statistical analysis

The significance of treatment effects was determined by ANOVA. Where necessary, data were transformed logarithmically to stabilize the variance.

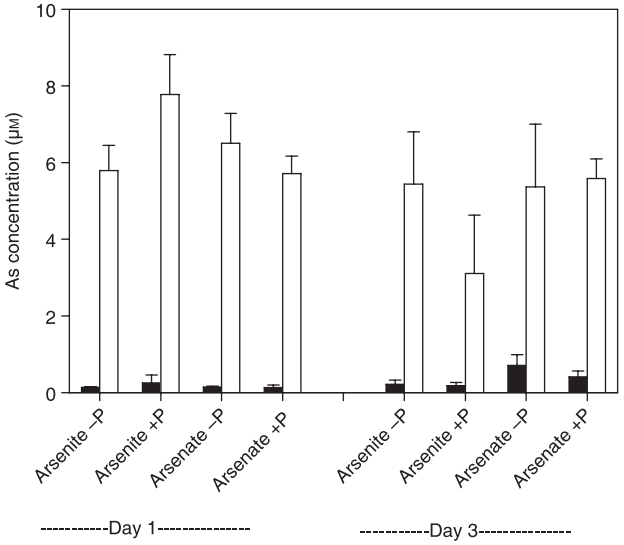

Fig. 1 Concentrations (mean + SE) of arsenate (closed bars) and arsenite (open bars) in the nutrient solutions after tomato (*Lycopersicon esculentum*) growth for 1 or 3 d in Expt 1. Plants were supplied with 10 μM arsenate or arsenite with (+) or without (–) 100 μM phosphate.

Results

Arsenate and arsenite used in the hydroponic experiments were checked for purity by HPLC–ICP–MS. The arsenate solution contained only arsenate, whereas the arsenite solution contained approx. 99.4% arsenite and 0.6% arsenate. The stock solutions of both compounds, stored at 4°C, were stable during the entire period of the experiments reported here.

Experiment 1

On average, fresh weights of tomato roots and shoots were 7.1 and 19.9 g per pot, respectively. There was no significant difference in root or shoot fresh weight between treatments. No phytotoxic symptoms were observed.

By days 1 and 3, As concentrations in the nutrient solutions decreased by 23–44 and 42–68%, respectively. Arsenite remained relatively stable in the nutrient solutions of the +arsenite treatments, accounting for > 95% of the solution As on both days 1 and 3 (Fig. 1; Fig. S1 in Supplementary material). By contrast, most (97.7%) of the arsenate added to the nutrient solution was reduced to arsenite by day 1, although this percentage decreased slightly to 91% by day 3 (Fig. 1). The ±phosphate treatments had no significant effect on the percentage distribution of the two As species in the nutrient solution. No methylated As species was found in the solution.

Both As species and ±phosphate treatments had no significant effect on the concentration of total As in roots harvested after 1 or 3 d exposure (Table 1). The extraction with PBS solution recovered 58–72 and 47–60% of the root total As from the day 1 and 3 samples, respectively. In the PBS extracts, As speciation was dominated by arsenite in all treatments (Table 1; Fig. S1), which accounted for 93–99% of the extracted As. The arsenate concentration in the roots was significantly higher when plants were supplied with arsenate than with arsenite. However, even in the arsenate-treated plants, arsenate accounted for only a small percentage (3–7%) of the extracted As. Very small amounts of DMA, accounting for < 0.15% of the extracted As, were detected in some of the root samples after 1 d exposure.

Arsenite was the predominant As species in the xylem sap (Table 1; Fig. S1), representing 85–96 and 82–91% of the total As in the sap samples collected on days 1 and 3, respectively. Arsenate was a minor species of As in the xylem sap (Table 1), accounting for 4–14% of the total As in the sap. DMA was detectable at very low concentrations in some of the sap samples. ANOVA showed that the As and P treatments had significant effects on the arsenite concentration in the xylem sap, but not on the concentrations of both arsenate and DMA. In the absence of P, the concentration of arsenite in the xylem sap was higher with the arsenate than with the arsenite treatment, and the concentration was decreased by the presence of P when arsenate was supplied. Total As concentration in the xylem sap was only 1.5–10% of the initial As concentration in the nutrient solution.

Total As concentrations in the shoots were very low compared with those in the roots, with only 1–3.3% of the total As taken up being distributed to the shoots by day 3 (data not shown). Between 56 and 66% of the PBS-extractable As in the shoots was present as arsenite, with the remainder as arsenate (data not shown).

Table 1 Arsenic speciation in tomato (*Lycopersicon esculentum*) roots and xylem sap after exposure to arsenate or arsenite for 1 or 3 d

Days' As exposure	Treatment	Root arsenate (nmol g^{-1} FW)	Root arsenite (nmol g^{-1} FW)	Root DMA (nmol g^{-1} FW)	Root total As (nmol g^{-1} FW)	Xylem arsenate (μM)	Xylem arsenite (μM)	Xylem DMA (μM)
Day 1	Arsenite – P	2.9 ± 0.7	371 ± 29.6	0.5 ± 0.4	542 ± 41.0	0.01 ± 0.0002	0.15 ± 0.005	0.004 ± 0.002
	Arsenite + P	3.4 ± 0.9	191 ± 79.5	0.1 ± 0.1	333 ± 116	0.04 ± 0.023	0.25 ± 0.052	0.011 ± 0.004
	Arsenate – P	15.5 ± 7.6	237 ± 42.6	0.1 ± 0.1	402 ± 3.1	0.03 ± 0.004	0.83 ± 0.096	0.006 ± 0.005
	Arsenate + P	10.2 ± 3.4	317 ± 6.2	0.1 ± 0.1	454 ± 22.2	0.01 ± 0.002	0.14 ± 0.008	0.005 ± 0.0003
ANOVA†: As species		***	NS	NS	NS	NS	**	NS
P		NS	NS	NS	NS	NS	**	NS
As × P		NS	*	NS	NS	NS	***	NS
Day 3	Arsenite – P	12.0 ± 3.2	352 ± 37.7	ND‡	775 ± 69.8	0.03 ± 0.004	0.41 ± 0.19	0.02 ± 0.013
	Arsenite + P	12.2 ± 1.6	355 ± 33.8	ND	775 ± 41.3	0.02 ± 0.001	0.13 ± 0.016	0.01 ± 0.005
	Arsenate – P	32.9 ± 8.3	427 ± 40.6	ND	762 ± 26.7	0.07 ± 0.031	0.94 ± 0.39	0.02 ± 0.005
	Arsenate + P	13.5 ± 1.2	494 ± 37.4	ND	873 ± 135.0	0.03 ± 0.006	0.33 ± 0.02	0.01 ± 0.004
ANOVA†: As species		*	*		NS	NS	*	NS
P		NS	NS		NS	NS	*	NS
As × P		NS	NS		NS	NS	NS	NS

Values are means ± SE.
DMA, dimethylarsinic acid.
†Significance of ANOVA: NS, not significant, *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.
‡ND, not detectable.

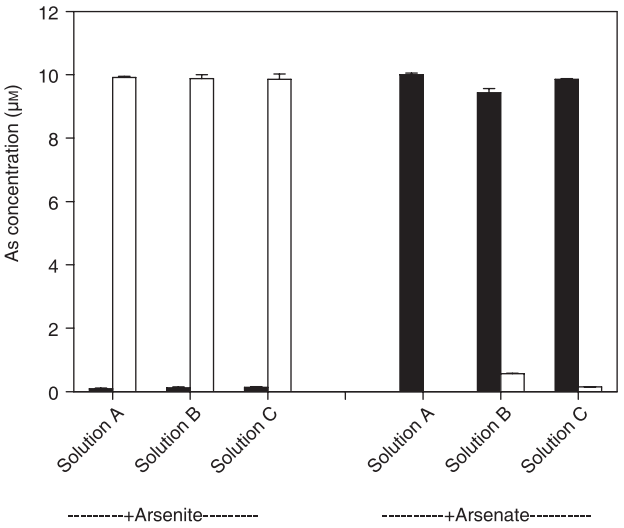

Fig. 2 Transformation of arsenate (closed bars) and arsenite (open bars) added to three nutrient solutions without plants (Expt 2): solution A, nutrient solution not used for growing plants; solution B, nutrient solution after being used for growing tomato (*Lycopersicon esculentum*) plants for 3 d; solution C, solution B after being filtered through sterilized 0.2-μm filter. Data are means + SE.

Experiment 2

Arsenite or arsenate (10 μM) was added to three types of nutrient solution: initial solution that had not been used to grow plants (solution A); solution that had been used to grow tomato plants for 3 d (solution B); and solution B filtered through a sterilized 0.2-μm filter (solution C). After 24 h incubation, arsenite remained stable in all three solutions, accounting for 99% of the initial concentration (Fig. 2). No reduction was observed when arsenate was added to solution A. A small percentage of arsenate was reduced to arsenite in solutions B (5.7%) and C (1.5%) (Fig. 2), which was much smaller than the extent of reduction observed in the nutrient solution bathing growing tomato roots (cf. Figs 1, 2).

Experiment 3

The time course of the reduction of arsenate in nutrient solution, in the presence of tomato roots, was followed over a 24-h period (Fig. 3). The concentration of arsenate decreased, with a concomitant increase of arsenite concentration in the nutrient solution. This reduction was very rapid in the absence of P, and by 8 h 97% of the As remaining in the nutrient solution was present as arsenite. Reduction of solution arsenate was slower in the presence of P; 25% of the As in the solution was arsenite by 8 h. Nevertheless, the extent of arsenate reduction was almost identical in the ±P treatments after 24 h. The net decrease in the concentration of solution As reflects uptake by plants; this was also similar in the ±P treatments by 24 h. During the first 3 h following arsenate addition, arsenate was being depleted faster than the production of arsenite,

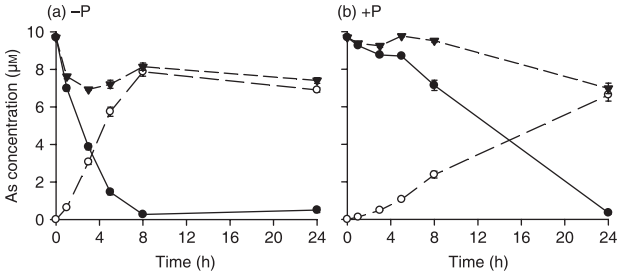

Fig. 3 Changes in arsenate (closed circle) and arsenite (open circle) concentrations in the nutrient solution (with (+) or without (–) 100 μM phosphate) with tomato (*Lycopersicon esculentum*) plants during a 24-h time course after arsenate addition (Expt 3). Total (triangle) is the sum of arsenate and arsenite in the solution. Data are means ± SE.

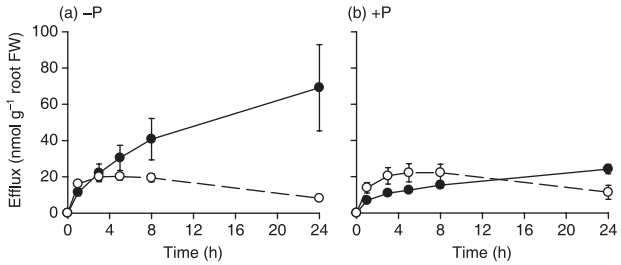

Fig. 4 Efflux of arsenate (closed circle) and arsenite (open circle) after tomato (*Lycopersicon esculentum*) plants had been supplied with 10 μM arsenate with (+) or without (–) 100 μM phosphate for 24 h (Expt 4). Results are expressed as the amount of arsenate or arsenite in the nutrition normalized by root FW. Data are means ± SE.

particularly in the –P treatment. By contrast, more arsenite occurred in the nutrient solution than the depletion of arsenate between 3 and 8 h. This pattern suggests a lag between arsenate uptake by roots and the occurrence of arsenite in the nutrient solution. Total uptake of As by roots was similar between the ±P treatments after 24 h exposure (data not shown).

Experiment 4

Tomato roots were first exposed to arsenate with or without P in the nutrient solution for 24 h. Efflux of As from roots to the nutrient solution (containing P) was then monitored during the following 24 h (Fig. 4). More arsenite than arsenate was effluxed after 1 h in the –P-treated plants, and for 1–8 h in the +P-treated plants, suggesting that the efflux of arsenite was initially faster than that of arsenate. At other, subsequent time points, arsenate efflux exceeded that of arsenite. The pattern of arsenite efflux was unaffected by the ±P treatments imposed during the initial As-loading period. By contrast, more efflux of arsenate occurred from the –P-treated plants than from the +P-treated plants, probably because more arsenate was taken up in the –P treatment during the loading period. At the end of the 24 h efflux period, 16.4 and 7.4% of the root As was excreted into the nutrient solution with the –P- and +P-treated plants, respectively.

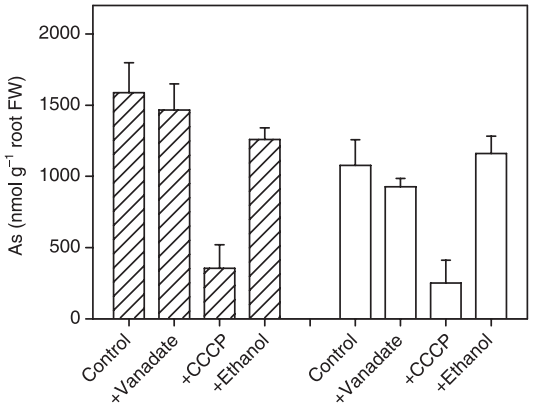

Fig. 5 Effects of sodium vanadate (200 μM) and CCCP (1 μM) on the decrease of arsenate (hatched bars) and appearance of arsenite (open bars) in the nutrition solution (–P) after tomato (*Lycopersicon esculentum*) plants were supplied with 10 μM arsenate for 5 h (Expt 5). Results are normalized by root FW. Data are means ± SE.

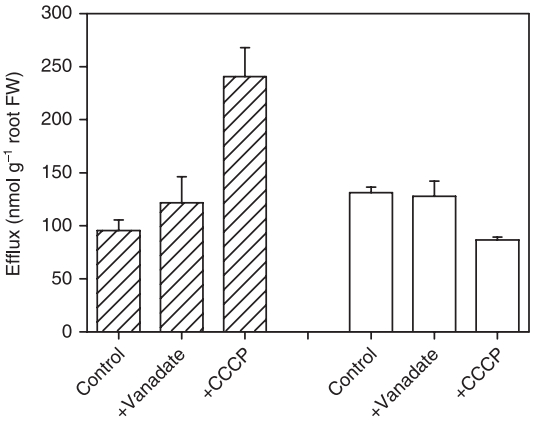

Fig. 6 Effects of sodium vanadate (200 μM) and CCCP (1 μM) on the efflux of arsenate (hatched bars) and arsenite (open bars) from tomato (*Lycopersicon esculentum*) roots to the nutrition solution (Expt 5). Plants were supplied with 10 μM arsenate for 5 h before being used in the efflux experiment. Results are normalized by root FW. Data are means ± SE.

Experiment 5

Similarly to Expt 3, 62% of the As in the nutrient solution had been reduced to arsenite after 5 h incubation with tomato roots in the control treatment (without P). The decrease in arsenate, reflecting uptake by plants, and the appearance of arsenite in the nutrient solution, were expressed on the basis of root FW (Fig. 5). The treatments with vanadate or ethanol (0.01%) did not significantly affect the decrease in arsenate or the appearance of arsenite. By contrast, CCCP significantly ($P < 0.01$) inhibited both the decrease in arsenate (by 72%) and the appearance of arsenite (by 78%), compared with the +ethanol treatment.

In the efflux experiment (Fig. 6), substantially more arsenate and arsenite were effluxed (44–64% of the As uptake) than observed in Expt 4. This was probably because the As-loading period was only 5 h in Expt 5 compared with 24 h in Expt 4. The vanadate treatment had no significant effect on the efflux of either arsenate or arsenite. By contrast, CCCP significantly ($P < 0.01$) enhanced the efflux of arsenate (by 2.5-fold), but significantly decreased the efflux of arsenite (by 34%) (Fig. 6).

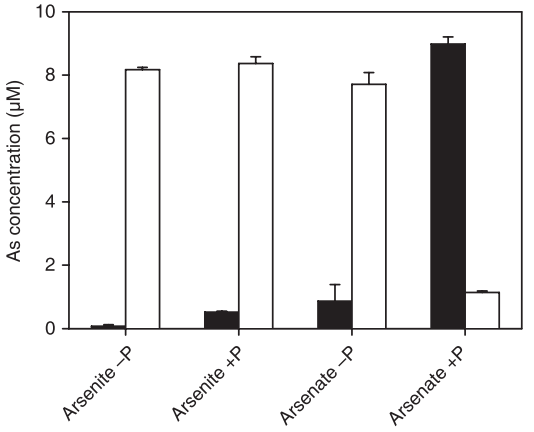

Fig. 7 Concentrations (mean ± SE) of arsenate (closed bars) and arsenite (open bars) in the nutrient solutions after rice (*Oryza sativa*) growth for 1 d in Expt 6. Plants were supplied with 10 μM arsenate or arsenite with (+) or without (–) 100 μM phosphate.

Experiment 6

This experiment was conducted to investigate whether the rapid transformation of As species observed in tomato also occurs in a different plant species, rice. Average fresh weight of rice roots (8.1 g per pot) was similar to that of tomato in Expt 1, but shoot fresh weight (11.9 g per pot) was smaller. Similarly to the experiments with tomato, arsenite added to the rice nutrient solution remained largely stable after 1 d (Fig. 7). By contrast, in the arsenate treatment without P, most of the As in the solution (90%) had been reduced to arsenite. When P was present in the nutrient solution, only 11% of the solution As was reduced to arsenite. Phosphate had no significant effect on the concentration of total As in roots from the arsenite treatment, but decreased root As concentration by fivefold in the arsenate treatment (Table 2). PBS extracted 45–69% of the root total As, similarly to those with tomato roots in Expt 1. In the PBS extracts of rice roots, 97 and 92% of the As was arsenite in the +arsenite and +arsenate treatments, respectively (Table 2). The ±P treatments had significant effects on the concentrations of both arsenate and arsenite, but not on their relative proportions in the extracts. DMA was not detected in rice roots after 1 d exposure to either As species.

Expt 2 was repeated with rice nutrient solutions, and identical results were obtained (data not shown).

Table 2 Arsenic speciation in rice (*Oryza sativa*) roots after exposure to arsenate or arsenite for 1 d

Treatment	Root arsenate (nmol g^{-1} FW)	Root arsenite (nmol g^{-1} FW)	Root total As (nmol g^{-1} FW)
Arsenite – P	3.3 ± 0.6	98.3 ± 13.7	168.3 ± 6.6
Arsenite + P	2.4 ± 0.3	93.5 ± 10.9	193.9 ± 35.9
Arsenate – P	10.8 ± 2.5	131.8 ± 13.9	266.3 ± 18.2
Arsenate + P	2.1 ± 0.8	23.0 ± 6.5	53.6 ± 3.6
ANOVA† As species	NS	*	NS
P	***	**	**
As × P	**	*	***

Values are means ± SE.
†Significance of ANOVA: NS, not significant; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

Discussion

The most interesting finding from the present study is that arsenate added to the nutrient solution was rapidly reduced to arsenite (Figs 1, 3, 7). More than 95% of arsenate was reduced to arsenite within 1 d in the experiments with tomato, and 92% in the case of the –P-treated rice. To our knowledge, this observation has not been reported before. The rapid reduction of arsenate in the nutrient solution was not mediated by microbes or root exudates, as both contributed to arsenate reduction only to a very limited extent (Fig. 2). Thus roots must be responsible for most of the reduction of arsenate in the external medium. This is supported by the fact that most of the As in both tomato and rice roots was in the form of arsenite, regardless of whether arsenate or arsenite was initially supplied to the plants. Arsenite was also the main form of As transported in the xylem sap of tomato. Some of the arsenite in tomato and rice roots is likely to be complexed with thiols, as has been shown in sunflower, *Holcus lanatus* and *Pteris vittata* by Raab *et al.* (2004a, 2005).

Most plants appear to have high levels of endogenous arsenate reductase activity (Dhankher *et al.*, 2006), which explains the predominance of the arsenite species in the roots of tomato and rice observed in this study. However, unlike microbes, efflux of arsenite or arsenate from plant roots to the external medium has not been reported before. Raab *et al.* (2007) noted that roots of sunflower did not excrete As back into its rooting environment. However, their observation was based on the mass balance of total As in the root and shoot tissues over a time-course of 32 d following arsenate exposure. In the present study, we monitored changes of As species in the nutrient solution directly during the period of As exposure, and also after As exposure with root apoplastic As having been removed first. The results shown in Fig. 3 suggest a rapid uptake of arsenate, which was inhibited by phosphate, followed by a rapid reduction of arsenate and efflux of arsenite, and a short lag between uptake and efflux. Figs 4, 6 show clear evidence for an efflux of both arsenite and arsenate from tomato roots, with arsenite efflux being faster than arsenate efflux initially under normal nutrient conditions. Efflux of arsenite and arsenate measured after a 5-h loading (Fig. 6) were much larger than those measured after a 24-h loading (Fig. 4). This difference can be explained by the fact that, by 24 h, most of the arsenate in the roots and the nutrient solution had been reduced to arsenite, leaving little arsenate inside the root cells for reduction and efflux. Furthermore, some arsenite could have been complexed by thiols and sequestered in the vacuoles. Efflux of arsenite measured after 24 h exposure may therefore underestimate the rate of efflux during arsenate exposure. In support of this explanation, Raab *et al.* (2005) found that the amount of As not complexed by thiols fell from 90% of total As in sunflower roots after 1 h exposure, to 43% after 4 d exposure.

Similar to the studies on *E. coli* (Dey *et al.*, 1994) and on Chinese hamster cells (Wang *et al.*, 1996), vanadate was not an effective inhibitor of arsenite efflux from tomato roots (Fig. 6), suggesting that the efflux is not mediated by a P-type ATPase. CCCP is a protonophore and an uncoupler of oxidative phosphorylation, and causes a dissipation of the proton motive force across the membranes. CCCP significantly inhibited arsenite efflux from tomato roots (Fig. 6), indicating that arsenite efflux is an energy-dependent active process. This is consistent with findings from studies on microorganisms and animal cells (Wang *et al.*, 1996; Meng *et al.*, 2004). Meng *et al.* (2004) propose that the arsenite efflux pump ArsB in *E. coli* is an antiport that mediates the exchange of arsenite (or antimonite) with proton. In *E. coli* there is a second mechanism of arsenite efflux mediated by the ArsAB complex, with ArsA being an ATPase that provides the energy for the efflux (Rosen, 2002; Silver & Phung, 2005). The mechanism of arsenite efflux by the yeast transporter Acr3p is possibly similar to that of ArsB, relying on the proton motive force for energy (Wysocki *et al.*, 1997). No arsenite efflux transporters have yet been reported in plants. However, the physiological data presented in this study strongly suggest that such transporters exist. There is an intriguing possibility that some arsenate might be reduced in the root apoplast or on the outer surface of plasma membranes. This is only speculation, and requires further research to prove or disprove it. Examples of reductases that reside on the plasma membranes of plant roots and can catalyse reduction of substrates outside the membranes include ferric-chelate reductase FRO2 and some nitrate reductase (Robinson *et al.*, 1999; Berczi & Moller, 2000). Given the synchronized effect of P and CCCP on the dynamics of arsenate and arsenite in the nutrient solution (Figs 3, 5), the model of arsenate uptake, followed by reduction and arsenite efflux, is much more plausible than a surface reduction.

Interestingly, efflux of arsenate from tomato roots appears to be a passive process, with CCCP markedly enhancing the efflux (Fig. 6). Because uptake of arsenate across plasma membranes to cytoplasm is an active process up an electrochemical gradient, diminishing the proton motive force by CCCP would be expected to decrease arsenate uptake (Fig. 5) and to

increase the leakage of arsenate back to the external medium (Fig. 6), especially when roots were placed in a solution initially without arsenate. The effect of ±P during the loading period on the subsequent efflux of arsenate (Fig. 4) is consistent with the above interpretation for a passive leakage of arsenate.

The ±phosphate treatments had no significant effect on the total amount of As accumulated by tomato roots after 1 or 3 d exposure (Table 1). This finding would be difficult to explain without the knowledge of a rapid reduction of external arsenate to arsenite. The presence of phosphate did inhibit arsenate uptake by tomato roots, leading to a slower depletion of arsenate in the solution (Fig. 3). However, within 24 h most of the external arsenate had been reduced to arsenite even in the +P treatment, and the subsequent uptake of arsenite was likely to be via aquaglyceroporins that are not inhibited by phosphate (Meharg & Jardine, 2003). Phosphate had a much more pronounced influence on arsenate uptake and reduction of the external arsenate by rice than by tomato (Tables 1, 2). The difference may reflect a different selectivity for phosphate/arsenate by the phosphate transporters in rice and tomato. In the absence of phosphate, rice roots also reduced external arsenate rapidly, suggesting that the root-mediated reduction of external arsenate is common in different plant species.

It remains to be tested whether the rapid reduction of external arsenate mediated by roots is a part of the detoxification mechanism, as is the case in microbes (Rosen, 2002; Silver & Phung, 2005). Short-term (20-min) influxes of arsenate and arsenite in rice roots were similar (Abedin *et al.*, 2002; Chen *et al.*, 2005). Therefore reduction of external arsenate to arsenite would not decrease As uptake by rice. In aerobic soils, arsenite is oxidized rapidly to arsenate, either chemically by reactions with manganese oxide (Oscarson *et al.*, 1981), or by arsenite-oxidizing microbes (Macur *et al.*, 2004). Thus soil, plant roots and microbes are likely to be engaged constantly in the reduction–oxidation cycle of arsenate–arsenite.

The results from the present study also have an important implication for the interpretation of some previous data comparing uptake, assimilation and toxicity of arsenate vs arsenite in hydroponic culture (Marin *et al.*, 1992; Carbonell-Barrachina *et al.*, 1998; Burló *et al.*, 1999; Raab *et al.*, 2005). Because arsenate in the nutrient solution can be rapidly reduced to arsenite by roots, treatments with arsenate would become a mixture of arsenate and arsenite, or even be dominated by arsenite, over exposure periods of hours to days. Contrary to general expectation, arsenite added to the aerated nutrient solution remained rather stable up to at least 3 d in our experiments. This may be because of a lack of arsenite-oxidizing bacteria in the medium, and that transition metals, which are catalysts of arsenite oxidation, were complexed with EDTA in the nutrient solution. Alternatively, roots may maintain the dominance of arsenite in the solution by reducing arsenate. This study highlights the importance of monitoring As speciation in the experimental media in studies of As metabolism in plants.

Acknowledgements

We thank the Chinese Academy of Sciences for a one-year fellowship to Xiaoyan Xu, Dr Tony Miller for advice on the metabolic inhibitors, and Dr Jian Feng Ma for providing rice seeds. Rothamsted Research receives grant-aided support from the Biotechnology and Biological Sciences Research Council of the UK.

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Supplementary Material

The following supplementary material is available for this article online:

Fig. S1 Examples of HPLC–ICP–MS chromatograms of (a) standards; nutrient solutions (b) before and (c) after tomato (*Lycopersicon esculentum*) growth; (d) tomato root extracts; (e) xylem sap.

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