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Evaluation of the influence of arsenic species on the nitrogen metabolism of a model angiosperm: nasturtium, *Tropaeolum majus*

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How the various organic and inorganic arsenic species affect the nitrogen metabolism of a model plant, *Tropaeolum majus*, was studied in order to evaluate the toxicological impact of the various chemical forms of arsenic. For this purpose, the effects on the (a) entire nitrogen pool, (b) protein fraction, and (c) non-protein fraction were distinguished. The arsenic-dependent effects on the nitrogen cycle were assessed by using ^{15}N-labelled KNO_3 as a nutritive substance and optical emission spectroscopy to analyse how ^{15}N is incorporated into the nitrogen cycle. In addition to the ^{15}N-tracer experiments, the uptake and metabolization of the arsenic compounds were examined. The work shows that biochemical indicator systems like ^{15}N-tracer studies are able to characterize the degree of the influence of metabolic processes by arsenic species. For example, the incorporated ^{15}N concentration decreased linearly and independently of the ^{15}N fraction with increasing dimethylarsinate (DMA) concentrations. This behaviour indicates that DMA has prevented the uptake of ^{15}N and hence the formation of amino acids and proteins. Arsenite-treated plants exhibited an elevated concentration of non-protein ^{15}N, which could be an indication either for a stimulated uptake of nitrate or for an interrupted amino acid/protein synthesis. Copyright © 2005 John Wiley & Sons, Ltd.

KEYWORDS: nitrogen metabolism; plants; ^{15}N-tracer experiments; arsenic species; *Tropaeolum majus*

INTRODUCTION

Arsenic occurs in many different compounds, which are known as arsenic species. The main inorganic compounds are arsenious acid (arsenic(III)) and arsenic acid (arsenic(V)), both of which are highly toxic. The extent and type of metabolization in cells depends on the organism group, as well as on the species within it. In terrestrial organisms, including higher plants, the arsenic species are usually restricted to arsenic(III), arsenic(V), monomethylarsonic acid (MMA) and dimethylarsinic acid.[1–3] Only a few bacteria and fungi release volatile, highly toxic arsines.[4] Some other bacteria can use arsenate as a substrate for growth.[5–7] Marine animals, such as mussels, lobsters and crabs, convert arsenic into harmless arsenobetaine and arsenocholine[8] and, therefore, possess an effective detoxification mechanism for ingested toxic inorganic arsenic. Other non-toxic metabolites of arsenic have been found in marine algae, i.e. arsenolipids[9] and arsenosugars.[10–14] Intermediate products of biomethylation[15] include the recently identified trivalent species $MeAs^{III}(OH)_2$ and $Me_2As^{III}(OH)$. Because these species are highly toxic, doubts surround whether the biomethylation of inorganic arsenic really is a method of detoxification. The toxicity of low-molecular-weight methylated arsenicals like MMA and DMA is reflected in the use of their sodium salts as herbicides.[16,17] In prokaryotes, arsenic detoxification is effected by special efflux pumps.[18–20] Studies concerning the mode of action of the herbicide MMA in plant cells revealed an inhibition of the malic enzyme of the C_4-dicarboxylate

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cycle, provoking the loss of the CO_2 source for glucose production.[21]

The pollution level of organisms is often only assessed by the accumulation of the pollutants. By contrast, physiological and biochemical plant reactions to stress caused by the effective intracellular concentration of toxic substances have hardly been investigated.[22]

Nitrogen plays an important role in plant cells, because it is a main constituent of amino acids, peptides, proteins and other biomolecules. Therefore, the nitrogen metabolism, including the uptake and assimilation of nitrate, is essential for the biosynthesis of amino acids and proteins, which are involved in almost all metabolic processes.[23]

Because of nitrogen's crucial role in plant metabolism and the risk of the nitrogen cycle being impaired by toxic substances, the influence of such compounds on the corresponding metabolic pathways needs to be evaluated. Heavy metals have already been found to exert a toxic effect on the nitrogen metabolism of plants.[22]

Nitrate taken up from the soil is reduced to nitrite by the enzyme nitrate reductase in root or leaf cells. The nitrite is then reduced to ammonium by the enzyme nitrite reductase. Ammonium is introduced into amino acids, the precursors of proteins. In order to monitor the fate of a certain nitrogen metabolite or certain nitrogen atoms in the plant metabolism, the nitrogen concerned must be labelled. An established method for determining the nitrogen uptake capacity is the ^{15}N tracer technique, in which the stable, non-radioactive nitrogen isotope ^{15}N is used.[24–28] Determining the incorporation of nitrogen by means of the ^{15}N tracer technique enables information about the reduction of amino acid and protein biosynthesis to be obtained, which is indicative of stress situations in a plant.[29,30]

If arsenic affects the function of proteins (enzyme functions, structure functions, regulator functions) directly or indirectly by metabolites emerging from arsenic stress, then the plant tries to compensate for this functional deficiency by increasing protein synthesis. Additionally, the synthesis of special stress enzymes may occur.[31] In the case of arsenic, the formation of arsenic-complexing peptides, such as metallothioneines or phytochelatines, could be assumed, as is known for Cu^{2+} or Cd^{2+}.[32–34] The turnover of peptides and proteins increases, and because the protein biosynthesis is the last part of the whole nitrogen metabolism, the quantification of nitrogen uptake yields a general parameter for changing protein biosynthesis in plant tissue under arsenic stress.

The nitrogen cycle as a reductive process in plants is dependent on the supply of reduction equivalents ($NADH + H^+$, $NADPH + H^+$, ferredoxine) originating from photosynthesis or from dissimilatory pathways.[35] By quantifying nitrogen uptake and incorporation in reduced nitrogen compounds such as amino acids and proteins, a broad spectrum of effects caused by the toxic arsenic species can be covered, delivering information about ecotoxicological potentials.

The experimental separation of the nitrogen fractions of a plant provides more detailed information about the location of interference. The nitrogen-containing compounds in a plant can be separated into a protein pool and a non-protein pool. The former comprises proteins, such as enzymes and regulator proteins, that are soluble in aqueous media, as well as insoluble proteins like membrane or other structural proteins and storage proteins, whereas the non-protein pool mainly contains free amino acids accompanied by other nitrogen compounds, like amines, amides, pyrimidines and purines in considerably smaller concentrations.

Measuring nitrogen incorporation in the protein fraction ought to provide information about the degree of protein synthesis, whereas nitrogen incorporation in the non-protein fraction demonstrates the turnover of amino acids.

The aim of the investigations described was to correlate the accumulation and metabolization behaviour of arsenic in plants with the physiological effect of arsenic stress on the plant nitrogen metabolism. *Tropaeolum majus* (nasturtium) was chosen for these experiments as a model angiosperm. The arsenic species used were selected from the point of: (1) highest toxicity, i.e. arsenite; (2) lower toxicity and steric hindrance, i.e. dimethylarsinate (DMA); and (3) aspects of present applications as feed additives, i.e. phenylarsonic acids, like roxarsone (Rox).

EXPERIMENTAL

Plant cultivation, arsenic and ^{15}N-tracer application

Plant cultivation and arsenic exposure took place in a phytochamber (Bioline Pflanzenwuchsschrank Typ VB 1514, Vötsch Industrietechnik GmbH Balingen, Germany) under controlled light (60 W m^{-2}), temperature (day: 21 °C; night: 15 °C) and humidity conditions (day: 60%; night: 75%). The day/night cycle was fixed to 13 h and 11 h respectively.

Nasturtium seeds (*T. majus*) were obtained from Walz Samen GmbH Stuttgart, Germany. After sowing in pots with soil (800 g dry weight (d.w.) per pot), the seedlings were watered for 2 weeks. Aqueous solutions of the separately used arsenic species arsenite (arsenic(III)), DMA, Rox and *p*-aminophenylarsonic acid (*p*-arsanilic acid, pAA) were then added at regular intervals for 3 weeks. Arsenic(III) was applied in four concentrations ([As] = 7.5, 15, 30, 60 mg l^{-1}), DMA in three concentrations ([As] = 7.5, 15, 30 mg l^{-1}) and Rox and pAA in concentrations of [As] = 100 mg l^{-1} each. For reproducibility, five pots each containing five plants were used for each concentration.

To study the nitrogen-uptake capacity ^{15}N-labelled KNO_3 (20 mg nitrogen (10 at.% ^{15}N)/20 ml (as KNO_3) solution per 400 g dry soil) was added as tracer to the irrigation water 2 days before the plants were harvested.

Sample preparation for the determination of arsenic species and total arsenic concentrations in plants

After exposure to arsenic, the plants were harvested and washed first with tap water and finally with deionized water.

The plants were divided into stems, leaves and leaf stalks and ground homogeneously with mortar and pestle under liquid nitrogen. Weighed quantities of 0.5–2 g were placed into 11 ml extraction cells for pressurized liquid extraction (PLE) using bidistilled water (ASE 200 device, Dionex, Sunnyvale, USA). Parallel to this, the dry weight of the appropriate fresh samples was determined by drying at 90 °C.

The PLE parameters selected after optimization were as follows: pressure, 100 bar; temperature, 120 °C; static time, 10 min; purging, 60% of cell volume; number of cycles, one; purge time, 180 s. The extract obtained was filled up to 50 ml and then analysed within 24 h to determine its arsenic species content, as well as after stabilization by acidification with 32% HCl (500 µl acid on 4.5 ml sample) to determine its total arsenic content.

To gauge the total arsenic content without prior extraction, the plant samples underwent microwave digestion (Multiwave, Anton Paar, Perkin Elmer). After drying at 90 °C, the plant samples were ground to a fine powder in an ultracentrifugal mill (ZM1, Retsch, Germany) and weighed in portions of approximately 500 mg into digestion containers. Extraction residues of the PLE were dried and weighed without further cutting into the digestion containers. 0.5 ml 30% H_2O_2 (Merck) and 5 ml 65% HNO_3 p.a. (Merck) were added to each sample. Microwave digestion was conducted in a four-stage programme (80 min; maximum pressure: 75 bar; maximum power: 1000 W). The digested samples were transferred to 50 ml graduated flasks and filled up with deionized H_2O (Millipore). Total arsenic was determined with hydride generation atomic absorption spectrometry (HG-AAS) and ICP-atomic emission spectrometry (ICP-AES).

Sample preparation for the determination of arsenic species and total arsenic concentrations in the water-soluble fraction of soil used for plant cultivation

Immediately after the plants had been harvested, the soil in the pots was homogenized by agitation. Fresh soil samples were divided into 10 g portions and shaken in 50 ml distilled water for 2 h. Similar to the plant samples, the dry weight was determined by drying three representative mixed samples at 105 °C. The extracts were analysed in terms of their arsenic species composition with ion chromatography and mass spectrometry (IC–ICP-MS) detection, as well as of their total arsenic concentrations with HG-AAS and ICP-AES.

Determination of arsenic species with IC–ICP-MS

The arsenic species were determined using the method developed by Londesborough *et al.*[36] The separation of different arsenic species was performed by IC with an IonPac AS7 separation column in combination with an IonPac AG7 pre-column (both Dionex). The injection volume was 200 µl. A gradient program with two eluents (eluent A: 0.4 mM HNO_3; eluent B: 50 mM HNO_3) was used. Coupling took place with a quadrupole mass spectrometer (ELAN 5000, PE Sciex) for element-specific detection at $m/z = 75$ using a cross-flow nebulizer.

Determination of total arsenic concentrations with HG-AAS and ICP-AES

When almost solely inorganic arsenic compounds were present in the samples, HG-AAS could be used for total arsenic determination without having to consider the problems arising in connection with organic compounds due to incomplete hydride formation.[37,38] A combination of an FIAS 400 for HG in the flow injection mode and an atomic absorption spectrometer (ZL 4100, Perkin Elmer) with an electrothermal atomizer was used. The hydrides were enriched in a graphite furnace (permanently modified with palladium). The absorption intensity was measured at a wavelength of 193.7 nm. Arsenic hydride was produced by nascent hydrogen (pre-reduction of arsenic(V) with potassium iodide and ascorbic acid). The nascent hydrogen was formed by acidifying $NaBH_4$ with HCl.

When organic arsenic compounds were expected in the samples, ICP-AES (Spectroflame, Spectro-A.I. Kleve, Germany) was employed to determine the total arsenic concentrations. In this technique, the limit of detection for arsenic was 60 µg l^{-1}. Even if ICP-AES has worse detection limits than HG-AAS, the former method has the great advantage of the constant sensitivity of arsenic detection irrespective of the element's original chemical form. The liquid sample was introduced into the ICP through a pneumatic cross-flow nebulizer. The intensity of the emission line of arsenic at $\lambda = 189.042$ nm was measured.

Sample preparation for ^{15}N analysis and determination of total nitrogen

The samples were prepared for ^{15}N tracer analysis using the micro-Kjeldahl technique.[39] 0.1–0.5 g freshly harvested leaves were digested with 2 ml nitrogen-free sulfuric acid (H_2SO_4 conc.) by heating until the solutions were clear. The resulting $(^{15}NH_4)_2SO_4$ solutions were distilled using a modified micro-Kjeldahl apparatus by adding 10 ml 32% NaOH. The amount of nitrogen in the sample was determined by titration against a 0.01 M H_2SO_4 standard solution. The samples were then dried in a water bath and the pure ammonium salts were collected.

As an alternative to the Kjeldahl technique, an elemental analyser (vario EL, Elementaranalysensysteme GmbH Hanau, Germany) was used as the sample preparation system for ^{15}N analysis. Plant samples were dried at 60 °C, ground in a ball mill (MM 2000, Retsch, Germany), and weighed into small tin crucibles in 9–10 mg portions. In the elemental analyser, the prepared samples were exposed to a highly oxygenated atmosphere in which they completely combusted. For ^{15}N analysis, part of the nitrogen gas flow produced was diverted to the emission spectrometer as explained below. To determine the total nitrogen, the combustion gases were detected by a thermal conductivity detector.

Nitrogen fractionation

To separate the total plant nitrogen pool into protein and non-protein fractions, freshly harvested leaf material was pulverized homogeneously with mortar and pestle under liquid nitrogen. Afterwards, 0.7–0.9 g of this powder was weighed into 50 ml centrifuge tubes. After adding 10 ml of 10% trichloroacetic acid, the sample was homogenized by an ultra-turrax with a dispersing tool S25N/18G (IKA Werke GmbH & Co. KG, Staufen, Germany) for 3 min. Sample pieces sticking to the ultra-turrax were rinsed back to the sample by 5 ml of 10% trichloroacetic acid. The precipitation of proteins was carried out overnight for at least 18 h at 4 °C. After centrifugation (10 000 rpm, 15 min, 2 °C), the supernatant representing the non-protein nitrogen fraction was decanted into a Kjeldahl flask and boiled in accordance with the previous section. The pellet was washed by resuspension in 5 ml 0.5% trichloroacetic acid and subsequent centrifugation (10 000 rpm, 5 min, 2 °C) in order to remove residues of the non-protein fraction. The resulting supernatant was discarded and the pellet was dissolved in a small amount of distilled water and transferred into a Kjeldahl flask for digestion. The ammonium sulfate solutions finally obtained from both fractions (protein and non-protein) were subjected to spectrometric $^{15}N/^{14}N$ analysis as described below.

Analysis of the ^{15}N isotope by emission spectrometry

Optical emission spectrometry is a very useful tool for the analysis of ^{15}N because of the distinct emission wavelengths of the 'isotope shifts': $^{14}N_2$, 297.68 nm; $^{14}N^{15}N$, 298.29 nm; $^{15}N_2$, 298.6 nm. The measurement of the ^{15}N abundance (^{15}N at.%) of the nitrogen fractions of plants was carried out by emission spectrometry on a ^{15}N analyser system (NOI-7, Fischer Analyseninstrumente, Leipzig, Germany). Nitrogen was measured as gaseous dinitrogen excited to light emission in a high-frequency electric field under reduced pressure. The required molecular nitrogen was produced from the liquid sample (aqueous solution of ammonium sulfate) by reaction with sodium hypobromite.

10 μg of nitrogen as $(^{15}NH_4)_2SO_4$ dissolved in 30 μl distilled water was used. After measuring the ^{15}N abundance, the ^{15}N excess abundance (^{15}N at.% exc.) was calculated by subtracting the natural ^{15}N abundance of 0.366 at.%.

RESULTS AND DISCUSSION

Influence of arsenic contamination on the total nitrogen content of leaves

Comparing the total nitrogen concentrations of plants exposed to different arsenic species does not allow the detection of effects of arsenic stress (Table 1). The metabolic activity can be ascertained only by means of the sensitive ^{15}N-tracer, since the large entire nitrogen pool is not changed significantly.

Table 1. Comparison of nitrogen concentrations in leaves of arsenic(III)- and DMA-exposed plants (*T. majus*) determined by digestion and titration in the Kjeldahl procedure and by combustion in an elemental analyser

As species applied	[As] applied (mg l^{-1})	[N] in leaves (g kg^{-1} d.w.)	
		Elemental analyser	Kjeldahl
As(III)	0	34	34
	7.5	28	27
	15	39	34
	30	26	29
	60	20	22
DMA	7.5	30	26
	15	31	28
	30	28	26

Effect of arsenic(III), DMA and pAA on the metabolic activity in different nitrogen fractions of leaves

To study how the uptake of arsenic species affects the biological activity of the nitrogen metabolization, a distinction was made between the protein and non-protein fractions in the nitrogen pool of *T. majus*, as explained in the Introduction. ^{15}N incorporation in different nitrogen fractions of DMA-exposed plants is compared in Fig. 1a with control plants without arsenic application.

At the lowest DMA application ([As] = 7.5 mg l^{-1}) the nitrogen balance is not affected, since both separated nitrogen fractions and the total nitrogen have the same ^{15}N concentration as in controls. However, higher DMA amounts applied yield a nearly linear concentration-dependent depression of ^{15}N incorporation in all the nitrogen fractions considered (cf. linear regression based on the relative ^{15}N incorporation at the concentration levels 15, 30 and 60 mg l^{-1} in Fig. 1a). This indicates that the arsenic-affected plants are subjected to a general inhibition of both the uptake of nitrogen and protein synthesis. In contrast to DMA, the arsenite application series behaves quite differently (Fig. 1b). The lowest arsenic concentration applied (7.5 mg l^{-1}) implies a decrease in nitrogen uptake, whereas the higher arsenite quantities cause an increase of the turnover of the nitrogen metabolism, especially in the non-protein fraction. The activation of the nitrogen metabolism is regarded as a symptom of the alarm phase of stress response in plants with general metabolic stimulation appearing.[40]

The metabolic activity in the protein and non-protein fractions differs sharply. The increasing ^{15}N incorporation into the entire nitrogen pool of the plant is attributable solely to the non-protein fraction. The proteins do not contribute to this enhancement, since their ^{15}N incorporation does not exceed those values of the control plants. The linkage of

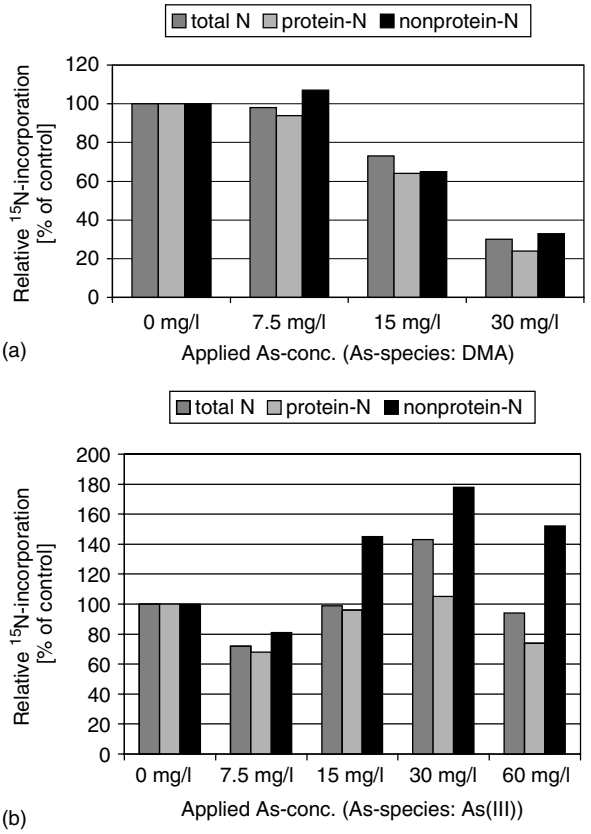

Figure 1. Relative ^{15}N incorporation in different nitrogen fractions of arsenic-exposed plants relating to control plants (*T. majus*). (a) DMA exposure. Linear regression from $x = 7.5,\ 15,\ 30\ mg\ l^{-1}$, where $x = [As]$: for non-protein, $y = -36.82x + 142.15$, $R^2 = 0.994$; for protein: $y = -34.96x + 130.81$, $R^2 = 0.994$; for total nitrogen, $y = -34.15x + 135.28$, $R^2 = 0.978$. (b) As(III) exposure.

the large amount of amino acids formed into proteins is restricted. According to Bernstam and Nriagu,[31] arsenic(III) and As(V) both inhibit protein synthesis.

The remarkable result of an initially inhibited nitrogen metabolism followed by increasing ^{15}N incorporation is similar to the behaviour of *Silene vulgaris*.[41]

In this study, dimethylarsinic acid exerted a more negative effect on nitrogen incorporation than inorganic arsenite. This was confirmed by other investigations, in which dimethylarsinic acid was found to be more phytotoxic than inorganic arsenic.[42,43] A direct interaction of arsenic with the amino acid biosynthesis is supposed by Edmonds.[44] In this study, the possibility of a replacement of ammonium by an arsenic species at the step of the transfer of ammonium into a 2-oxo acid normally resulting in an amino acid is discussed.

In contrast to the *p*-aminophenylarsonic acid exposure discussed here, plants treated with Rox suffered impaired growth and extensive leaf necrosis, preventing comparison

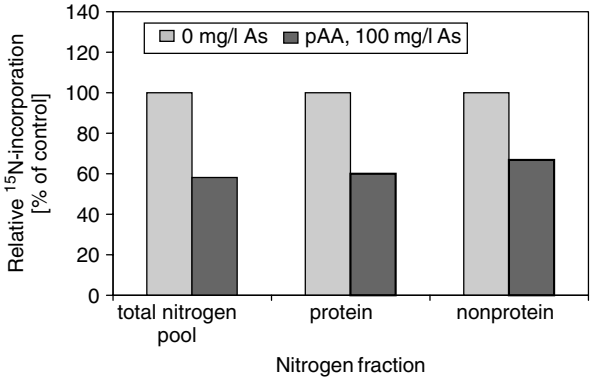

Figure 2. Relative ^{15}N incorporation in different nitrogen fractions of *T. majus* treated with *p*-aminophenylarsonic acid relating to control plants.

with control plants regarding their ^{15}N uptake. Plants exposed to the former phenylarsonic acid showed no growth impairment. As can be seen from the results of ^{15}N-tracer experiments (Fig. 2), the nitrogen uptake of these plants was inhibited, equally affecting both separated nitrogen fractions and, hence, the total nitrogen pool. The degree of inhibition amounted to about 40% of nitrogen incorporation in controls.

Statistical validation of the ^{15}N incorporation values

For emission spectrometric ^{15}N analysis, the relative error estimated by six parallel measurements for each sample was less than 1%. The reproducibility of the sample preparation procedures was examined both by parallel measurements of separately prepared samples (Table 2) and by comparing two different sample preparation techniques (Table 3).

Table 2. Reproducibility of ^{15}N incorporation in leaves of arsenic-exposed and control plants (*T. majus)* after separation of total nitrogen in protein and non-protein fractions. In each case, three parallel samples were digested individually by the micro-Kjeldahl technique

		^{15}N-incorporation (^{15}N at.% exc.)	
Samples, parallels		Non-protein	Protein
As control	1	5.47	2.77
	2	6.00	2.76
	3	6.29	2.82
Mean value plus/minus standard deviation		5.92 ± 0.40	2.78 ± 0.03
As(III) exposure	1	4.89	2.84
	2	4.87	2.59
	3	5.06	2.64
Mean value plus/minus standard deviation		4.94 ± 0.1	2.69 ± 0.1

Table 3. Comparison of the amounts of incorporated ^{15}N using two different sample preparation procedures for leaves of *T. majus*

As species applied	[As] applied (mg l^{-1})	^{15}N incorporation (^{15}N at.% exc.)		
		Elemental analyser (EA)	Micro-Kjeldahl	EA/Micro-Kjeldahl ratio
Without	0	3.08	3.02	1.02
As(III)	7.5	2.68	2.4	1.12
	15	3.23	3.06	1.06
	30	4.90	5.17	0.95
	60	3.24	3.38	0.96
DMA	15	1.71	1.73	0.99
	30	2.04	1.95	1.05

After the digestion of three parallel samples by means of the Kjeldahl technique, the relative standard deviation of ^{15}N incorporation in protein- and non-protein fractions amounted of 1 to 7% (Table 2). From these results it can be concluded that the sample preparation chosen provides good reproducibility. Moreover, the ^{15}N data obtained by Kjeldahl sample digestion and by the combustion of samples in an elemental analyser are in good agreement (Table 3).

Arsenic accumulation in *T. majus* after the application of arsenic(III) and DMA

The accumulation of arsenic in leaves and stems of *T. majus* grown under the continuous application of arsenic species (Figure 3) is quantified in Fig. 4. Comparing the ordinates of the two diagrams reveals the greater accumulation of the methylated arsenic compound than inorganic arsenic in above-ground parts of plants. The application of arsenic(III) (Fig. 4a) only caused low arsenic accumulation. The application of DMA (Fig. 4b) led to a linear dependence of the arsenic concentration accumulated in leaves and stems on the arsenic concentration applied (see linear regression

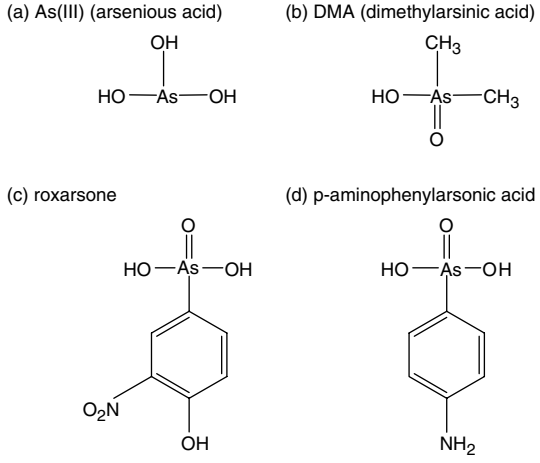

Figure 3. Arsenic compounds applied to *T. majus*.

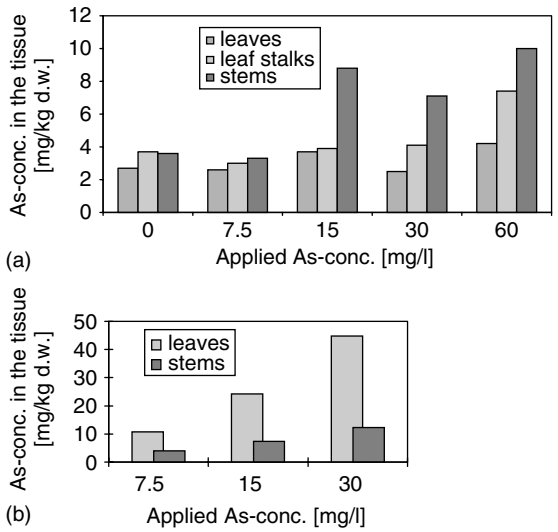

Figure 4. Arsenic accumulation in above-ground organs of *T. majus* exposed to different arsenic species: (a) arsenic(III) application; (b) DMA application. Linear regression: for leaves, $y = 17.05x - 7.5$, $R^2 = 0.987$; for stems, $y = 4.15x - 0.43$; $R^2 = 0.986$. Mean values from two parallel samples each. Sample preparation: PLE; arsenic analysis: HG-ETA-AAS and IC-ICP-MS.

of Fig. 4b). The leaves contained more arsenic than the corresponding stems. The distribution of dimethylarsinic acid within plant organisms depends on the type of plant. *S. vulgaris,* investigated by Schmidt *et al.*,[45] stored the methylated arsenic compound mainly in leaves, like *T. majus*, whereas in rice plants this compound was found to dominate in stems.[46]

Arsenic species composition in *T. majus* after the application of arsenic(III) and DMA

In addition to the dominating inorganic species arsenite and arsenate, some unidentified arsenic compounds in concentrations near the detection limit of IC–ICP-MS (0.6 µg l^{-1}) occurred in arsenic(III)-treated plants. The

Table 4. Comparison of arsenic concentrations in plant compartments depending on digestion or extraction techniques

Plant organ	As species applied	[As] (mg kg^{-1} d.w.) Extracted with PLE	In the extraction residue[a]	Extracted + extraction residue	In the total digest[a]	[As] extracted with PLE as a percentage of total conc. (%)
Leaves	As(III)	0.59	0.13	0.72	0.8	82
		2.77	0.18	2.95	2.1	94
		2.70	0.20	2.90	3.1	93
		3.36	0.22	3.57	3.1	94
Leaf stalks	As(III)	7.36	0.81	8.17	7.3	90
Stems	As(III)	2.19	0.32	2.52	2.3	87
Leaves	DMA	11	—	11	10	110
		24	—	24	22	109
		45	—	45	41	110

[a] Microwave digestion.

prevalence of inorganic arsenic in terrestrial plants has been emphasized in many recent publications.[1–3,41,47,48] In all the organs considered, the concentration of arsenite exceeded the concentration of arsenate, as demonstrated in the concentration ratios of both arsenic species [As(III)]/[As(V)] in leaves: 2.3 ± 0.4; in stems: 7 ± 3). The correlation between prevailing arsenite and living tissues is discussed elsewhere.[41]

In the case of DMA applications, the arsenic species applied dominated in the plants and was accompanied by lower concentrations of arsenite ($5 \pm 3\%$ in leaves, $20 \pm 5\%$ in stems—as percentage of total extractable arsenic), arsenate ($1 \pm 1\%$ in leaves, $7 \pm 9\%$ in stems) and unidentified compounds (<1.5% of total extractable arsenic) in some samples. In other plants, dimethylarsinic acid was not converted either.[49] However, *S. vulgaris* demethylated and reduced a large part of the ingested methyl compound.[45]

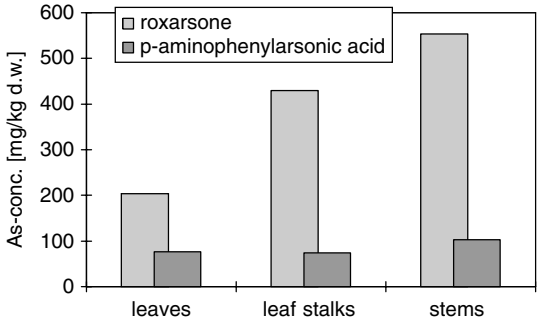

Figure 5. Distribution of arsenic in *T. majus* after application of Rox and *p*-aminophenylarsonic acid. Sample preparation: PLE plus microwave digestion of extraction residue; arsenic analysis: IC-ICP-MS and ICP-AES. The arsenic concentrations shown here are the sums of concentrations extracted with PLE and concentrations remained in the extraction residue. Arsenic concentrations in control plants are already listed in Fig. 4a.

Extraction efficiency for arsenic from plant tissue

A high extraction efficiency ranging between 82 and 100% was achieved by means of an optimized extraction procedure using PLE[50] (Table 4). A comparison of the arsenic concentrations obtained by a total digestion of plant material with the sum of arsenic concentrations, extracted by PLE and remaining in the extraction residue, exhibits good agreement.

Accumulation and metabolization of phenylarsonic acids in *T. majus*

No precise data exist on the plant uptake of phenylarsonic acid derivatives such as Rox and *p*-aminophenylarsonic acid (Fig. 3c and d), even though these compounds are of practical importance because of their use as antiprotozoic drugs for livestock.

As can be seen from the experiments, large amounts of these aromatic arsonic acids present in the soil were transferred to plants (Fig. 5). Plants treated with Rox accumulated more arsenic than plants treated with *p*-aminophenylarsonic acid. This behaviour correlates with the higher concentrations of Rox found in the water-soluble fraction of soil (see below).

In the case of Rox application, a graduated increase in arsenic content was registered in the following order: leaf < leaf stalk < stem.

The IC–ICP-MS chromatograms of extracts from different plant parts shown in Fig. 6 indicate the metabolization of ingested phenylarsonic acids. Hence, it appears that the phenylarsonic acids predominating in the water-soluble soil fraction (see below) after their strong accumulation in plants were metabolized to some degree. Low amounts of arsenite and arsenate occurred in all samples. In order to obtain arsenite from *p*-aminophenylarsonic acid or Rox, the arsonic acid group must be cleaved from the phenyl group and reduced. However, the majority of the phenylarsonic acids applied remain in the initial state in plants, as is evident from the unchanged main peak in Fig. 6.

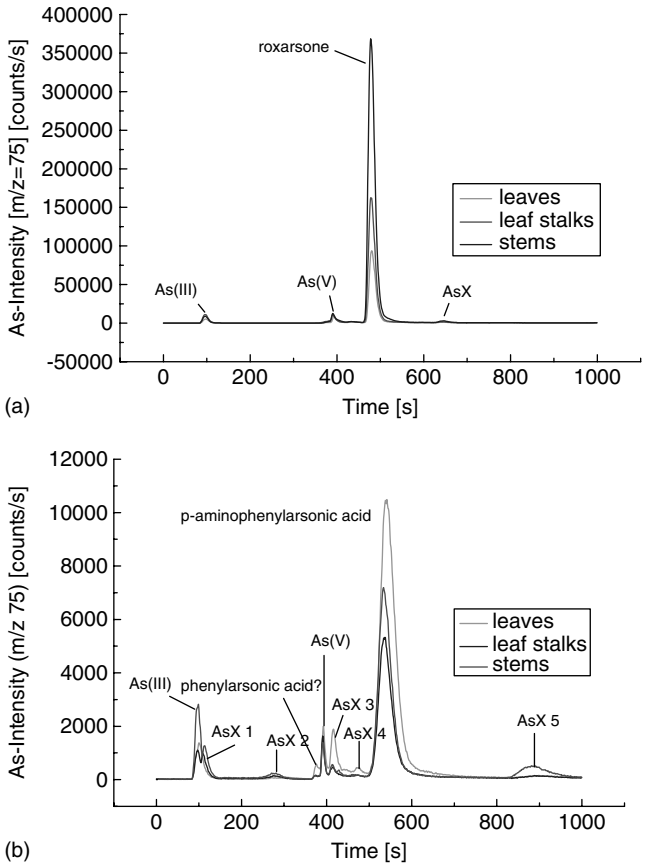

Figure 6. Chromatograms (IC–ICP-MS) of arsenic species from different organs of *T. majus*. Sample preparation: PLE; peak intensities are normalized to a net weight of 1 g wet weight. (a) After application of Rox, (b) after application of *p*-aminophenylarsonic acid.

Extractability of the accumulated phenylarsonic acids from plant tissues

Comparison of arsenic amounts extracted with PLE and amounts remaining in the residue of PLE shows that the extractability of arsenic from plants treated with *p*-aminophenylarsonic acid is considerably lower than from plants treated with dimethylarsinic acid, arsenite (Table 4) or Rox (Table 5).

As far as the form of non-extractable arsenic is concerned, it can presently only be assumed that arsenic ought to be fixed on non-extractable components of the plant matrix.

Arsenic species in soils of different arsenic-species applications

Arsenic species analysis in water eluates of soil showed that in the case of all arsenic(III) applications, arsenate dominated at the end of arsenic exposure (Fig. 7a). This is a general problem arising from arsenic(III) applications using soils with oxidizing conditions.[41,49] Nevertheless, a new portion of arsenite was added to the soil in each application step during plant cultivation, ensuring continuous arsenic(III) exposure in addition to a growing arsenic(V) pool.

In the case of DMA applications, this arsenic species was found to be the main component in water eluates of soil (Fig. 7b). Small amounts of arsenic(V) and an unidentified

Table 5. Extractability (PLE) of arsenic species from plants treated with phenylarsonic acids[a]

As species applied	Extractability from different plant parts (%)		
	Leaves	Leaf stalks	Stems
Rox	94	89	70
p-Aminophenylarsonic acid	73	46	33

[a] Sample preparation: PLE of fresh plant material plus microwave digestion of extraction residues; arsenic analysis: IC–ICP-MS and ICP-AES.

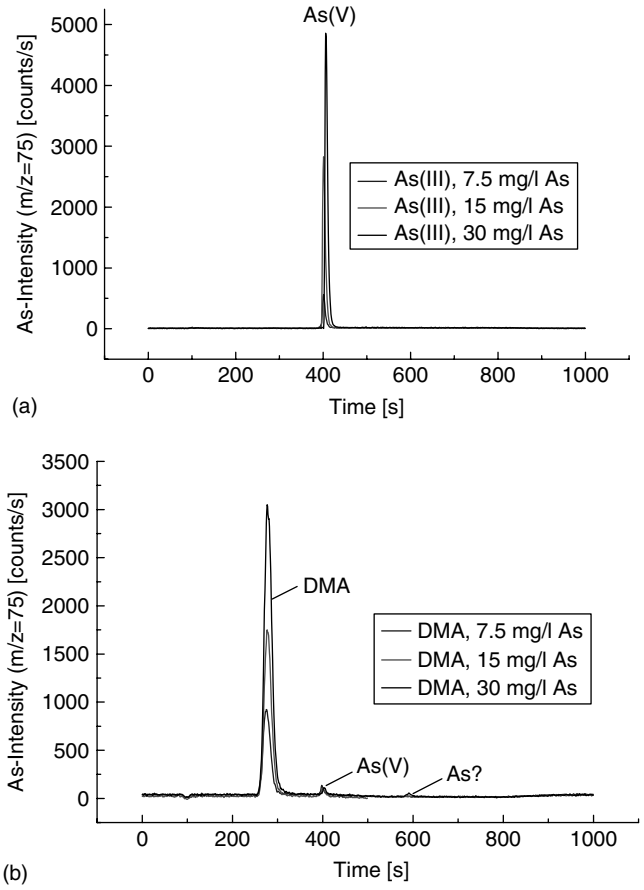

Figure 7. Chromatograms of arsenic species from water eluates of DMA- and arsenic(III)-treated soil (application of three and four different arsenic-concentrations respectively). Arsenic species analysis by IC–ICP-MS; the extracts were diluted (1 : 100) prior to measurement. (a) Arsenic(III) application; (b) DMA application.

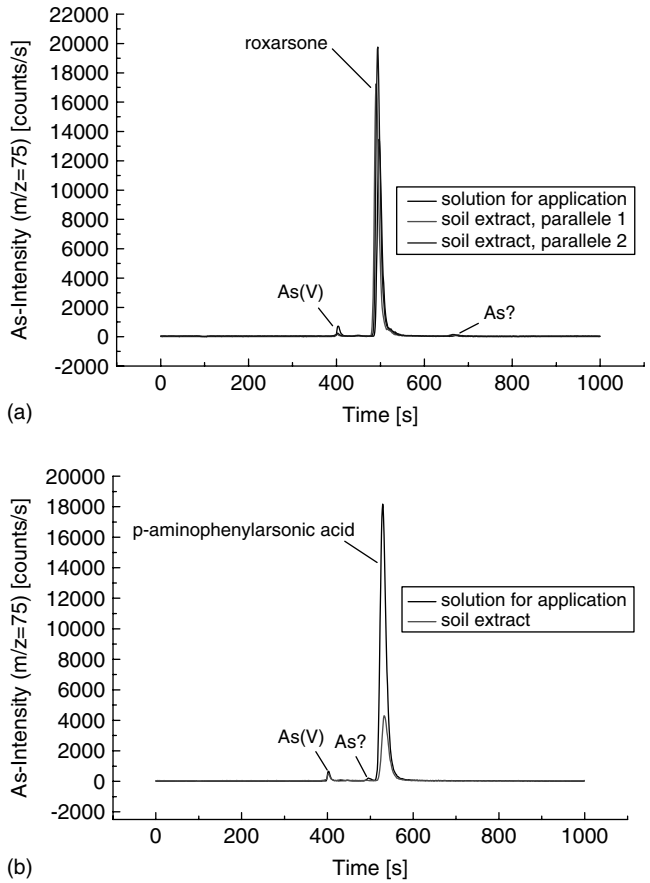

Figure 8. Chromatograms (IC–ICP-MS) of water eluates of soils treated by aqueous solutions of Rox and *p*-aminophenylarsonic acid as well as of the corresponding solutions: (a) Rox: (b) *p*-aminophenylarsonic acid.

compound also appeared.

Arsenic species analysed in the water-soluble soil fraction after the application of Rox and *p*-aminophenylarsonic acid are shown in the IC–ICP-MS chromatograms (Fig. 8). Comparison with the chromatograms of aqueous solutions of the two phenylarsonic acids applied to soil during plant cultivation proves the stability of the compounds concerned in the soil environment. Despite similar treatment, the concentration of aminophenylarsonic acid in the water-soluble soil fraction is much lower than that of Rox. This may be due to the stronger adsorption of aminophenylarsonic acid on soil components.

CONCLUSION

It was demonstrated that different arsenic species have different influences on the nitrogen-metabolic activity, as expressed by the degree of formation of amino acids (non-protein fraction) and their transformation to proteins (protein fraction) by means of the incorporation of ^{15}N into the amino acid and protein pool. The sensitive analysis of the stable nitrogen isotope ^{15}N allows the investigation of the uptake of nitrogen by plants, as well as of the synthesis of amino acids and proteins and their dependence on the arsenic species and their concentrations. Concrete differences were found in plants irrigated with DMA and arsenite solutions. Comparison with the control plants, the incorporated ^{15}N concentration decreased linearly and independently of the ^{15}N fraction with increasing DMA concentrations. This behaviour indicates that DMA prevented the uptake of ^{15}N and, hence, the formation of amino acids and proteins. An elevated concentration of non-protein ^{15}N was determined in plants irrigated with arsenite solutions, which can be attributed either to a stimulated uptake of nitrate or to an interrupted amino acid/protein synthesis. An argument to favour the stimulation of nitrate uptake ought to be finding in the similarity of the ^{15}N incorporation in the protein fraction of the control plants and of the arsenite treated plants. If the amino acid synthesis is the rate-determining step then an accumulation of ^{15}N in the non-protein fraction can occur. An analysis of the amino acid and protein status of the plants investigated is planned for the future.

The knowledge about arsenic-influenced physiological processes is very important to regulate the arsenic uptake by plants in order to perform an effective phytoremediation.

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