"Comparative effects of arsenite (As(III)) and arsenate (As(V)) on whole plants and cell lines of the arsenic-resistant halophyte plant species Atriplex atacamensis"

Vromman, Delphine ; Martínez, Juan-Pablo ; Kumar, Mahendra ; Šlejkovec, Zdenka ; Lutts, Stanley

ABSTRACT

Whole plants and hypocotyl-derived calli of the halophyte plant species Atriplex atacamensiswere exposed to 50 µMarsenate (As(V))or 50 µMarsenite (As(III)). At thewhole plant level,As(III) wasmore toxic thanAs(V): it reduced plant growth, stomatal conductance,photosystem II efficiency while As(V) did not. In roots, As accumulated to higher level in response toAs(III) than in response to As(V). Within root tissues, both arsenate and arsenite were identified in response to each treatment suggesting that oxidation of As(III) may occur. More than 40% of As was bound to the cell wall in the roots of As(V)-treated plants while this proportion strongly decreased in As(III)-treated ones. In leaves, total As and the proportion of As bound to the cell wall were similar in response to As(V) and As(III). Non-protein thiol increased to higher extent in response to As(V) than in response to As(III) while ethylene synthesis was increased in As(III)-treated plants only. Polyamine profile was modified in a contrasting way in response to As(V) and As(III). At the callus level, As(V) and As(III) 50 µMdid not reduce growth despite an important As accumulation within tissues. Calli exposed to 50 µMAs did not increase the endogenous non-protein thiol. In contrast to the whole plants, arsenite was not more toxic than arsenate at the cell line level and As(V)-treated calli produced higher amounts of ethylene and malondialdehyde. A very high dose of As(V) (1000 µM) strongly reduced callus growth and lead to non-protein thiols accumulation. It is concluded that As(III) was more toxic than As(...

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RESEARCH ARTICLE

Comparative effects of arsenite (As(III)) and arsenate (As(V)) on whole plants and cell lines of the arsenic-resistant halophyte plant species *Atriplex atacamensis*

Delphine Vromman[1] · Juan-Pablo Martínez[2] · Mahendra Kumar[3] · Zdenka Šlejkovec[4] · Stanley Lutts[1]

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Abstract

Whole plants and hypocotyl-derived calli of the halophyte plant species *Atriplex atacamensis* were exposed to 50 μM arsenate (As(V)) or 50 μM arsenite (As(III)). At the whole plant level, As(III) was more toxic than As(V): it reduced plant growth, stomatal conductance, photosystem II efficiency while As(V) did not. In roots, As accumulated to higher level in response to As(III) than in response to As(V). Within root tissues, both arsenate and arsenite were identified in response to each treatment suggesting that oxidation of As(III) may occur. More than 40% of As was bound to the cell wall in the roots of As(V)-treated plants while this proportion strongly decreased in As(III)-treated ones. In leaves, total As and the proportion of As bound to the cell wall were similar in response to As(V) and As(III). Non-protein thiol increased to higher extent in response to As(V) than in response to As(III) while ethylene synthesis was increased in As(III)-treated plants only. Polyamine profile was modified in a contrasting way in response to As(V) and As(III). At the callus level, As(V) and As(III) 50 μM did not reduce growth despite an important As accumulation within tissues. Calli exposed to 50 μM As did not increase the endogenous non-protein thiol. In contrast to the whole plants, arsenite was not more toxic than arsenate at the cell line level and As(V)-treated calli produced higher amounts of ethylene and malondialdehyde. A very high dose of As(V) (1000 μM) strongly reduced callus growth and lead to non-protein thiols accumulation. It is concluded that As(III) was more toxic than As(V) at the plant level but not at the cellular level and that differential toxicity was not fully explained by speciation of accumulated As. Arsenic resistance in *A. atacamensis* exhibited a cellular component which however did not reflect the behavior of whole plant when exposed to As(V) or As(III).

Keywords Arsenic · *Atriplex atacamensis* · Halophyte · Phytoremediation

Introduction

Arsenic is a toxic metalloid ubiquitously present in the environment and which threaten human health in numerous areas in the world (Dwivedi et al. 2015; Zhao et al. 2010). Arsenic poisoning causes bladder, lung, and skin cancer, induces cardiovascular alteration, and affects thyroid gland and nervous system (Sun et al. 2017; Bohwmick et al. 2018). Arsenic is also highly toxic for plants since it reduces plant growth, impairs mineral nutrition, alters the plant water status, induces oxidative stress, inhibits photosynthesis, and modifies the hormonal content, leading to numerous physiological disorders and ultimately to plant death (Panda et al. 2010; Hettick et al. 2015; Kumar et al. 2015; Farooq et al. 2016a). This element is present in more than 150 minerals and may contaminate the food chain and drinking water (Sharma et al. 2015; Kumar et al. 2015). Ronci et al. (2017) recently provided evidences that even low doses of As in drinking water have a genotoxic impact. Arsenic often has a geogenic origin and is released in the environment through volcanic eruption, erosion of rocks, and low temperature volatilization. Nevertheless, it is also

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✉ Stanley Lutts
Stanley.lutts@uclouvain.be

[1] Groupe de Recherche en Physiologie végétale–Earth and Life Institute-Agronomy (ELI-A), Université catholique de Louvain, 5 (Bte 7.07.13) Place Croix du Sud, 1348 Louvain-la-Neuve, Belgium

[2] Instituto de Investigaciones Agropecuarias, INIA-La Cruz, La Cruz, Region de Valparaiso, Chile

[3] Science Research, Av. Costanera, 4976 Iquique, Chile

[4] Jožef Stefan Institute, Jamova 39, 1000 Ljubljana, Slovenia

released into the environment through anthropic activities such as mining, disposal of industrial effluents, fossil fuel combustion, and use of pesticide and wood preservatives (Mirza et al. 2014; Kumar et al. 2015).

Arsenate (As(V)) and arsenite (As(III)) are the major phytoavailable forms in soil, the former being present in aerobic soils while the latter predominates under anaerobic conditions (Sharma et al. 2015; Hettick et al. 2015; Farooq et al. 2016b). These two As species exhibit contrasting properties in terms of sorption to iron-containing minerals (Vega et al. 2017; Deng et al. 2018). Behaving as a phosphate chemical analog, As(V) is absorbed by phosphate transporters and competes with P for uptake due to similar size and charge. It replaces P in target molecules such as DNA or RNA and inhibits ATP synthesis. It is however commonly assumed that most if not all absorbed As(V) is reduced to As(III) in plant tissues through non-specific reductases (Finnegan and Chen 2012; Triptahti et al. 2012). At natural pH, arsenite is a neutral form (H_3AsO_3) which is more soluble and mobile than As(V). It is transported in the plants through nodulin 26-like intrinsic protein aquaporine channel and exhibits propensity to bind to sulfhydryl groups with consequent detrimental effects on some proteins and their functions (Zhao et al. 2010; Triptahti et al. 2012). However, arsenite may also bind to protecting phytochelatins (PC) which are cysteine-rich small peptides fixing free As(III) on their sulfhydryl groups and forming detoxifying complexes which are then transported and sequestered in vacuoles to avoid cellular damages (Finnegan and Chen 2012; Hettick et al. 2015; Farooq et al. 2016a).

Phytoremediation is now frequently proposed as a promising alternative to expensive physico-chemical techniques for management of As-contaminated large areas (Vithanage et al. 2012; Mirza et al. 2014; Hettick et al. 2015; Franchi et al. 2017; Pittarello et al. 2017; Wang et al. 2017a). *Atriplex atacamenis* is a perennial halophyte shrub species originating from the North part of Chile and which is naturally occurring on As-contaminated areas (Tapia et al. 2013; Vromman et al. 2016a). Mature plants are able to accumulate consistent amounts of As in their shoots (Vromman et al. 2016a) and even seedling remains alive at very high concentration of As(V) (up to 1000 μM) and able to synthesize protecting PC, polyamines (PAs), and glycinebetaine assuming key functions in stress resistance (Vromman et al. 2011). In this species, both As(V) and As(III) were found within plant tissues suggesting that arsenate reduction only partially occurred and a significant proportion of As was bound to the cell wall. At the whole plant level, As accumulation is mainly regulated by root to shoot translocation which is increased by salinity (Vromman et al. 2016b) but decreased in response to phosphate starvation (Vromman et al. 2017).

Until now, *A. atacamensis* was only tested in the presence of As(V). In its native environment (desert of Atacama, one of the most dry area in the world), *A. atacamensis* has to cope with an extremely arid environment characterized by mean annual rainfall lower than 5 mm $year^{-1}$ (Queirolo et al. 2000). The plant is thus found along few rivers present in this area and close to the river bed. *A. atacamensis* may be exposed to transient flooding conditions inducing an anaerobic environment leading to As(V) reduction and As(III) accumulation. No data, however, are available regarding the physiological behavior of this species exposed to arsenite.

Plants are complex organisms and their responses to ion toxicities vary depending on the phenological stage and morphological development of the whole plant system (Lefèvre et al. 2009). This is especially the case for As non-hyperaccumulating plant species where most As is retained in the roots with only a small fraction accumulating in the shoots. Nevertheless, some unicellular organisms such as several microalgae are able to resist the adverse effects of As (Wang et al. 2017b, 2017c) suggesting that morphological complexity is not an absolute pre-request for As resistance. As far as higher plants are concerned, in vitro callus culture of proliferating cell lines offers a convenient tool to study the behavior of plant cell lines exposed to ion toxicities independently of the plant morphological complexity (Lutts et al. 1996). Lefèvre et al. (2010a) demonstrated that the cotyledon-derived calli issued from plants belonging to two populations of *Zygophyllum fabago* exhibited similar levels of Cd and Zn resistance than the whole plants. Similarly, a water stress-resistant cell line of *Atriplex halimus* selected in the presence of polyethylene glycol displayed a higher ability to cope with Cd-induced oxidative stress (Lefèvre et al. 2010b). To the best of our knowledge, no data are available regarding the cellular basis of As resistance in *A. atacamensis*.

The present work was therefore undertaken in order (1) to compare the response of *A. atacamensis* to As(V) and As(III) in terms of plant nutrition and synthesis of protecting compounds (non-protein thiols and polyamines) and (2) to analyze the behavior of hypocotyl-derived cell lines exposed to those toxic forms of arsenic.

Materials and methods

Plant material and growth conditions

Seeds of *A. atacamensis* were collected in the north part of Chile (Quillagua 442015 E, 7609153 N). This site exhibited an As concentration of 220 ± 20 mg As kg^{-1} soil DW and plants were located on the bank of Rio Loa containing 2.5 mg As L^{-1} in water as detailed by Vromman et al. (2016b). Enclosing bracts were gently removed and seeds were sterilized using 5% NaClO (*w*/*v*) (20 min at room temperature). Seeds were then germinated on a peat/loam substrate (1:1) and the obtained seedlings (5 weeks old) were then transferred in a growth chamber (12 h daylight photoperiod

with a minimal PPFD of 180 μmol m^{-2} s^{-1} at 26 °C during the day and 22 °C during the night under a relative humidity of 70%). Plants were distributed among 10 tanks containing 50 L of nutrient solution (323 μM of $NaH_2PO_4 \cdot 2H_2O$; 1.43 mM of NH_4NO_3; 1.64 mM of $MgSO_4 \cdot 7H_2O$; 512 μM of K_2SO_4; 11.4 μM of $MnSO_4 \cdot H_2O$; 750 μM of $CaCl_2 \cdot 2H_2O$; 57.8 μM of H_3BO_3; 14 μM of $Na_2MoO_4 \cdot 2H_2O$; 0.96 μM of $ZnSO_4 \cdot 7H_2O$; 42.7 μM of Fe-EDTA; 0.4 μM of $CuSO_4 \cdot 5H_2O$). Plants were fixed in plugged holes on polystyrene plates floating at the top of nutrient solution (25 plants per tank). Treatments were applied after 2 weeks of acclimation and consist in 0 (Control), 50 μM of As(V), added in the form of $Na_2HAsO_4 \cdot 7H_2O$, or 50 μM As(III) added in the form of As_2O_3. Redox potential was maintained below 100 meV, ensuring the stability of the redox state of added As species during the time course of the experiment. Solutions were renewed every weeks and tanks were randomly rearranged in the growth chamber.

For calli obtention, sterile seeds were germinated on Whatman no. 1 filter paper moistened with deionized water (12 h photoperiod; PPFD 110 μmol m^{-2} s^{-1}). Hypocotyls were aseptically excised as recommended by Lefèvre et al. (2010b) and placed in Petri dishes on a Linsmaier and Skoog (1965) medium supplemented with 1 mg L^{-1} of naphthaleneacetic acid, 1 mg L^{-1} benzylaminopurine, 0.5 mg L^{-1} 2,4-dichlorophenoxyacetic acid, and 30 g L^{-1} of sucrose. Medium was solidified by 2.5 g L^{-1} gelrite (phytagel; Sigma), pH was set at 5.7, and medium was autoclaved at 120 °C and 150 kPa during 20 min. After 6 weeks, the obtained calli were transferred to medium corresponding to the four following treatments: control, 50 or 1000 μM of arsenate ($Na_2HAsO_4 \cdot 7H_2O$) or 50 μM of arsenite (25 μM of As_2O_3) (all calli exposed to 1000 μM arsenite rapidly died). Calli were exposed to these treatments during 2 months. Relative growth rate of individual callus was estimated according to RGR = (ln Wf −ln Wi) / Δt where Wf and Wi are final and initial fresh weights, respectively, and Δt is the elapsed time in day between the two measurements.

Ion quantification and As speciation

For total S, P, and major cations quantification in roots, leaves, and calli, 75 mg of dry material was digested in 35% HNO_3 and evaporated to dryness on a sand bath at 80 °C. Minerals were incubated with a mix of HCl 37%/HNO_3 68% (3:1) slowly evaporated and dissolved in distilled water. Mineral concentration was assessed through inductively coupled plasma-optical emission spectroscopy (Varian, type MPX).

Arsenic speciation was performed for roots and leaves only using high performance liquid chromatography-hydride generation-atomic fluorescence spectrometry (HPLC-HG-AFS) (Šlejkovec and Van Elteren 1999). Freeze-dried samples were ground in methanol/water (1:1) and incubated overnight. Centrifugation was performed for 10 min at 1000×*g* and extraction was repeated twice. Pooled extracts were dried (rotary evaporator at 45 °C, c.a. 20 min), resuspended in 4 mL of MilliQ water, and stored at 4 °C until analysis. Anionic arsenic species (As(III), As(V)) were separated on Hamilton PRP-X100 anion using a mobile phase (15 mM KH_2PO_4, pH 6.1). Non-volatile arsenic compounds were converted into corresponding volatile hydrides using an on-line hydride generation step (1.5% $NaBH_4$ in 0.1% NaOH, 3 mL min^{-1}). Hydrides were detected in an Excalibur atomic fluorescence spectrometer. A similar setup in flow-injection mode (column omitted, FI-UV-HGAFS) with UV-decomposition prior to HG (3.1 m long, 0.5 mm i.d FEP Teflon tubing coiled around an 8-W Camag UV lamp, 254 nm with a flow of 2% $K_2S_2O_8$ in 2% NaOH) was used for the determination of total arsenic.

Discrimination between the elements bound to cell walls and those present in the symplasm soluble fraction was performed according to Chen et al. (2013). The tissues powder was washed three times with 40 mL of 75% ethanol for 20 min. The homogenates were then centrifuged for 10 min at 1000×*g*, and the pellets were sequentially washed in three steps, with 40 mL of each (1) ice-cold acetone (1:7, *w*/*v*), (2) methanol–chloroform mixture (1:1, *v*/v), and (3) methanol. The final pellet was composed of crude cell walls and was analyzed by ICP for ions quantification as previously described.

Stomatal conductance, chlorophyll content, and fluorescence-related parameters

The leaf stomatal conductance (g_s) was measured on unfolded leaves located at the upper part of the plant using an AP4 system (Delta-T Devices, Cambridge, UK) at 11:00 a.m. Photosystem II efficiency (Φ_{PSII}) and non-photochemical-quenching (NPQ) were measured on leaves of six plants per treatment using a Fluorescence Monitoring System II (Hansatech instruments, Norfolk, England) as detailed in Vandoorne et al. (2012). Osmotic potential (Ψs) was quantified on extracted tissue sap with a vapor pressure osmometer (Wescor 5500) as previously described (Lefèvre et al. 2010a).

For leaf chlorophyll a, b and carotenoids (xantophylls + β-carotene) concentration, leaf tissue (c.a. 100 mg FW) was homogenized with sand in 10 mL chilled acetone (80%) in the dark. The homogenate was centrifuged at 5000×*g* for 10 min at 4 °C. Absorbance of the supernatant was recorded directly at 663, 647, and 470 nm for chlorophyll a, chlorophyll b, and carotenoids, and content was calculated as recommended by Lichtenthaler (1987) and expressed as milligram per gram FW.

Ethylene, polyamines, and soluble sugars measurements

The ethylene production was estimated through an on-line photoacoustic ethylene detector ETD-300 (Sensor Sense)

(Cristescu et al. 2002). *A. atacamensis* seedlings were placed in glasses box of 400 mL on filter paper Whatman no. 1 moistened with the corresponding nutrient solution. The box and Petri dishes containing *A. atacamensis* seedlings or calli was covered with a plexiglass plate with an inlet and outlet for gas flow, and tightly closed. The measurements were conducted in stop-and-flow mode with each cuvette being alternatively flushed during 20 min with a flow of 3 L h^{-1}. The flow from each cuvette was directed into a photoacoustic cell where ethylene was quantified. The obtained results were analyzed with the use of Valve Controller software and expressed as nanoliters per hour per gram of plant FW. Measurements were performed with three experimental repetitions.

PAs were extracted and quantified according to Lefèvre et al. (2001). Tissues were ground in a cold pestle and mortar and extracted twice in 500 μL of cold $HClO_4$ 4% (*v*/*v*) containing 5 mg L^{-1} 1,7-diaminoheptane as internal standard with subsequent centrifugation at 13,000×*g* (4 °C, 20 min). The free polyamines present in the supernatant were derivatized as previously described (Lefèvre et al. 2001). Extracts were dried under a nitrogen flow, dissolved in 1 mL methanol, and filtered at 0.45 μm. For each sample, 5 μL was injected on a Nucleodur C18 Pyramid column (125 × 4.6 mm internal diameter; 5 μm particle size) maintained at 40 °C. Analysis were performed using high performance liquid chromatography coupled with a fluorescence detector (HPLC-FLD) on a Shimadzu HPLC system, equipped with a solvent delivery unit LC-20AT, a SIL-HTc autosampler, and a RF-20A Fluorescence Detector (Shimadzu, 's-Hertogenbosch) with the excitation wavelength at 340 nm and the emission wavelength at 510 nm. The mobile phase was prepared with water (eluent A) and acetonitrile (eluent B) and used at a flow of 1.0 mL min^{-1}. The gradient program was as follows: 40% B to 91% B (20 min), 91% B to 100% B (2 min), 100% B (8 min), 100% B to 40% B (1 min), and column equilibration at 40% B during 4 min. A 6-point calibration curve was used for PAs quantification and prepared with custom-made external standard solutions and internal standard (1,7-diaminoheptane) at concentrations ranging from 3.125 to 100 μM. A check standard solution was regularly used to confirm the calibration of the system.

Soluble sugars were extracted according to Yemm and Willis (1954). Frozen samples were ground in 7 mL of 70% ethanol (*v*/*v*) and incubated for 5 min on ice before centrifugation at 8000×*g* for 10 min at 4 °C. The supernatants were evaporated in a thermomixer under nitrogen flux (45 min at 50 °C); dry residues were resuspended in 200 μL of HPLC water and were filtered through 0.45 μm microfilters (Minisart® RC4, Sartorius, Goettingen, Germany). Aliquot (20 μL) was injected into a HPLC system (Prominence System, Shimadzu, The Netherlands) equipped with a Rezex RCM-Monosaccharide Ca^{2+} (8%) column (300 × 7.8 mm) and a guard column (Phenomenex Carbo-Ca 4*3 mm ID). Elution was performed at 80 °C at a flow rate of 0.6 mL min^{-1} using pure water over 40 min. Detection of individual sugars was performed with a Shimadzu RID-10A refractive index detector. Fructose, glucose, and sucrose were quantified using five-point calibration curves with custom-made external standard solutions, ranging from 1 to 50 mM and every ten injections, a check standard solution was used to confirm the calibration of the system.

Non-protein thiols, hydrogen peroxide, malondialdehyde, and anthocyanins measurements

The total non-protein thiol (NP-SH) concentration was determined according to De Vos et al. (1992). For each sample, 200 mg FW was ground in liquid nitrogen. Two milliliters of 5% (*w*/*v*) sulfosalicylic acid was added to 6.3 mM diethylenetriaminepentaacetic acid (pH < 1). After centrifugation at 10,000×*g* for 10 min at 4 °C, the supernatants were used for the determination of thiols using Ellman's reagent. Three hundred microliters of supernatant was mixed with 630 μL of 0.5 M KH_2PO_4 and 25 μL of 10 mM 5,5-dithiobis 2-nitrobenzoic acid (final pH 7.0). The absorbance at 412 nm was recorded after 2 min, and the NP-SH concentration was determined using an extinction coefficient of 13,600 M^{-1} cm^{-1}.

Hydrogen peroxide was quantified spectrophotometrically at 508 nm using titanium reagent in phosphate buffer K_2HPO_4/KH_2PO_4 100 mM at pH 7.8 according to Arasimowicz et al. (2009). Calibration curve was 0, 3.125, 6.25, 12.5, 25, and 50 nM of H_2O_2.

The level of lipid peroxidation was estimated as the amount malondialdehyde (MDA) quantified according to Heath and Packer (1968). Samples ground in a pre-chilled mortar were homogenized in 5 mL of trichloroacetic acid 5% (*w*/*v*) and glycerol 1.25%. The homogenates were centrifuged at 4 °C, 8000×*g* for 10 min and filtered through Whatman no. 1 filter paper. A 2-mL aliquot of thiobarbituric acid (0.67% (*w*/*v*)) was added to 2 mL of supernatant: the mixture was heated at 100 °C for 30 min and then quickly cooled on ice. Samples were centrifuged at 8000×*g* for 1 min and the absorbance was measured at 532 nm. The non-specific absorption at 600 nm was subtracted. The concentration of MDA was calculated using an extinction coefficient of 155 mM^{-1} cm^{-1}.

For anthocyanins extraction from leaves, approximately 200 mg FW was mixed with 2 mL of methanol/water/acetic acid (80:19.5:0.5, *v*/*v*/*v*). This mixture was vortexed for 50 s and shaken for 20 min at 4 °C. After centrifugation at 12,000×*g* for 10 min at 4 °C, the supernatant was collected. Two hundred microliters of supernatant was added to 800 μL of potassium chloride buffer pH 1 (0.025 M) on the one hand and to 800 μL of sodium acetate buffer pH 4.5 (0.4 M) on the other hand (André et al. 2007). Total anthocyanins were measured using the pH differential method absorbance at 510 and

700 nm in pH 1.0 and 4.5 buffers according to Benvenuti et al. (2004) with a molar extinction coefficient of 26,900 corresponding to cyanidin-3-glucoside.

Statistical analysis

All analyses were performed on at least six biological replicates. Technical triplicates were also performed for each sample to check the accuracy of the technical procedures. Normality of the data was verified using Shapiro-Wilk tests and data were transformed when required. For each organ (root, stem, leaves, and calli), ANOVA 1 were performed at a significance level of $P < 0.05$, $P < 0.01$, or $P < 0.001$ using SAS Enterprise Guide 6.1 (SAS 9.4 system for windows) considering the As treatment, as main factor. Post-hoc analyses were performed using Student-Newman-Keuls test at a 5% level. Results are presented as means ± standard errors.

Results

Whole plant

All plants exposed to As(V) remained alive until the end of the treatment while 7% died when exposed to As(III). Arsenate had no impact on the root, stem, and leaf fresh weight while As(III) significantly reduced fresh weight of all organs after 2 weeks of treatment (Fig. 1a). The tested treatments had no impact on the leaf Ψs (mean value of − 2.37 MPa) or on the water content of the different organs (detailed data not shown). Arsenite strongly reduced g_s while As(V) had no effect on stomatal conductance (Fig. 1b). Similarly, Φ_{PSII} and NPQ values remained unaffected in As(V)-treated plants while As(III) significantly impacted them (Fig. 1c, d). Arsenite increased Chl b but had no impact on Chl a and significantly decreased the carotenoid concentration (Fig. 1e). Fructose concentration was not affected by the treatment while glucose and sucrose both increased in response to As(III), only (Fig. 1f).

Roots of *A. atacamensis* exposed to As(III) accumulated higher total As concentration than those exposed to As(V) (Table 1). More than 60% of accumulated As in the roots was in the non-extractable fraction in arsenate-treated plants while this percentage was reduced to 30% for As(III)-treated plants. As far as the extractable fraction is concerned, similar endogenous proportion of As(V) and As(III) accumulated within the tissues, although the concentration of both As species was by far higher for arsenite than for arsenate-treated plants. The total As concentration was lower in the leaves than in the roots but appeared similar in response to both As treatments. Once again, the proportion of non-extractable As was higher for As(V)-treated plants than for plants exposed to As(III). Both endogenous As species were recorded in the extractable fraction. Almost 40% of accumulated As was bound to cell walls in As(V)-treated plants while only 16% of As was bound to the cell wall in the roots of As(III)-treated ones. In the leaves, almost half of the accumulated As was bound to the cell walls for plants exposed to As(V) or As(III).

Both As(III) and As(V) reduced the root K concentration while only As(III) decreased it in the leaves (Fig. 2a). Magnesium and S remained unaffected by arsenic treatments, but both As(V) and As(III) decreased P concentration in the roots (Fig. 2).

Non-protein thiols increased in the roots of As-treated plants and the recorded increase was higher for As(V) than for As(III) (Fig. 3a). As far as the leaves were concerned, NP-SH increased in response to As(V) but decreased in response to As(III). The H_2O_2 concentrations decreased in roots of As(V)-treated plants comparatively to control, but increased in As(III)-treated ones (Fig. 3b). Arsenite induced an increase in anthocyanins but As(V) had no effect on this parameter (Fig. 3c). The MDA concentration was similar in controls and in As(V)-treated plants (mean values of 29.7 nmol g^{-1} FW for roots and 56.2 nmol g^{-1} FW in leaves). Exposure to As(III) increased the MDA concentration in roots (57.6 nmol g^{-1} FW) and leaves (110 nmol g^{-1} FW).

Putrescine and tyramine concentrations were higher in the roots than in the shoots. Arsenite increased the concentration of these compounds in both organs while As(V) increased Put concentration in the roots only (Fig. 4). Arsenate had no significant impact on Spd concentrations while As(III) increased Spd content in the roots. The concentration of Spm remained unaffected by the treatments. As a consequence, the Put/(Spd + Spm) ratio increased in the roots of arsenate-treated plants while it decreased in the roots of As(III)-treated ones. Arsenate did not increase ethylene synthesis by the leaves, while As(III) increased it by more than 37% (Fig. 4f).

Callus culture

The relative growth rate of calli exposed to 50 μM As(V) or 50 μM As(III) remained similar to controls while 1000 μM As(V) strongly decreased callus growth (Fig. 5a). Arsenic content was the highest in response to 1000 μM As(V) (Fig. 5b), and in this case, only 18% of the accumulated As was bound to the cell wall. Calli exposed to 50 μM As(V) and 50 μM As(III) accumulated the same concentrations of As and similar proportion of As was bound to the cell walls in the two cases (39%). As(V) and As(III) 50 μM induced a moderate increase in S concentration comparatively to controls (Fig. 6a) while the highest dose of As(V) (1000 μM) induced more than 100% increase in S content. In contrast, P and Ca concentrations remained unaffected by the considered treatments.

The NP-SH concentration increased in the calli exposed to 1000 μM As(V), only (Fig. 7). The MDA content was the

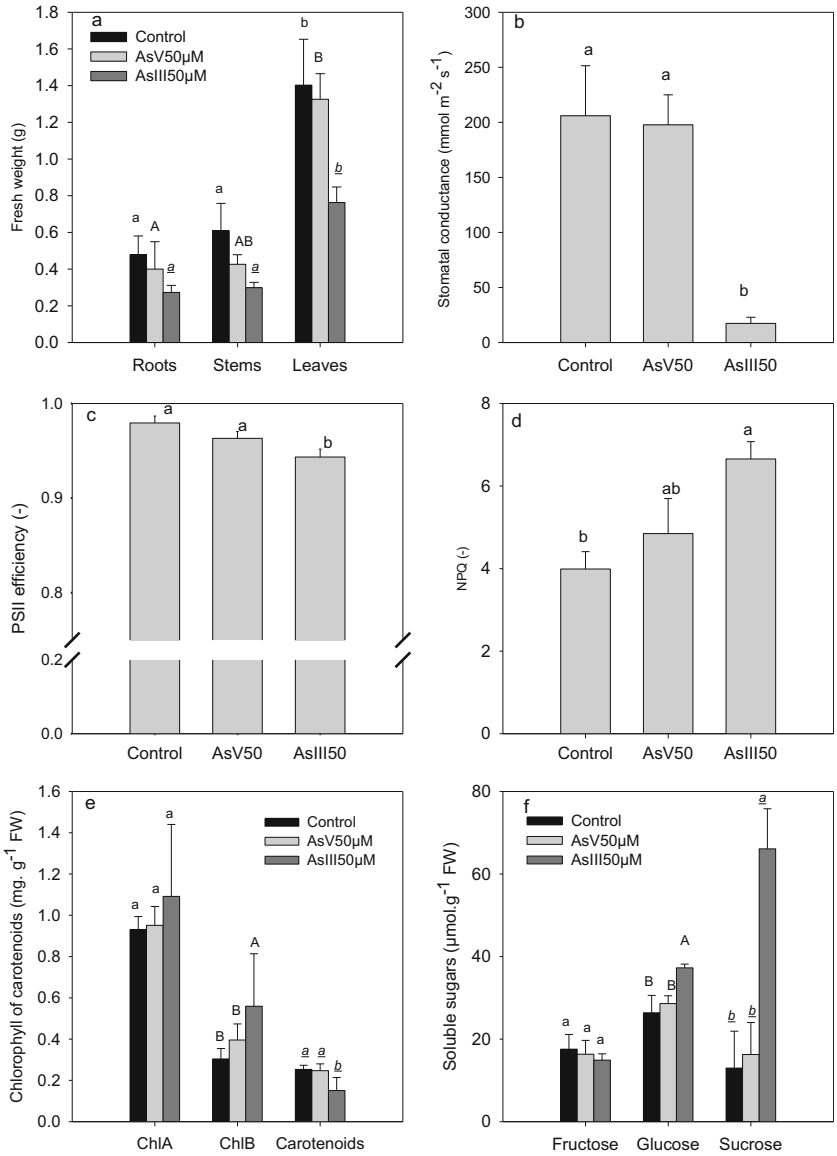

Fig. 1 Root, stem, and leaves fresh weight (**a**), stomatal conductance (g_s; **b**), PSII efficiency (Φ_{PSII}; **c**), non-photochemical quenching (NPQ; **d**); chlorophyll and carotenoid concentration (**e**), and leaf sugar concentration(**f**) in *Atriplex atacamensis* exposed during 2 weeks to nutrient solution containing 50 μM As(V) or 50 μM As(III). Each value is the mean of six replicates and vertical bars are S.E. For a given organ (**a**) or compound (**e** and **f**), values exhibiting different letters are significantly different at $P < 0.05$ according to Student-Newman-Keuls test at 5% level

highest for calli exposed to 1000 μM As(V). A low dose of As(V) (50 μM) also increased MDA comparatively to controls while a similar dose of As(III) had no impact. Treatments had no impact on H_2O_2 concentration in calli (detailed data not shown).

As(V) and As(III) applied at 50 μM increased the Put concentration to a similar extent (Fig. 8) but As(V) at 1000 μM significantly decrease the Put content. The considered treatments had no impact on Tyr or Spm content. Arsenate at 50 μM increased the Spd concentration while it slightly

Table 1 Total arsenic, As non-extractable, As(III) extractable, As(V) extractable (in μg g^{-1} DW), and estimation of As in the cell wall (%) in roots and in leaves of *Atriplex atacamensis* seedlings exposed for 2 weeks to 0 (control), 50 μM arsenate (AsV), or 50 μM arsenite (AsIII). Each value is the mean of three replicates ± standard errors

Arsenic species in μg g^{-1} DW						% of As in the cell wall
Organs	Treatment	As Total	As non-extractable	As(III) extractable	As(V) extractable	
Root	AsV-50 μM	521 ± 37 a	321 ± 35 a	83 ± 36 b	117 ± 15 b	40 ± 11%
	AsIII-50 μM	663 ± 43 b	202 ± 31 b	238 ± 23 a	226 ± 28 a	16 ± 4%
Leaves	AsV-50 μM	18 ± 4 a	8 ± 4 a	4 ± 1 a	6 ± 2 a	47 ± 5%
	AsIII-50 μM	23 ± 4 a	3 ± 1 a	9 ± 3 a	11 ± 2 a	46 ± 7%

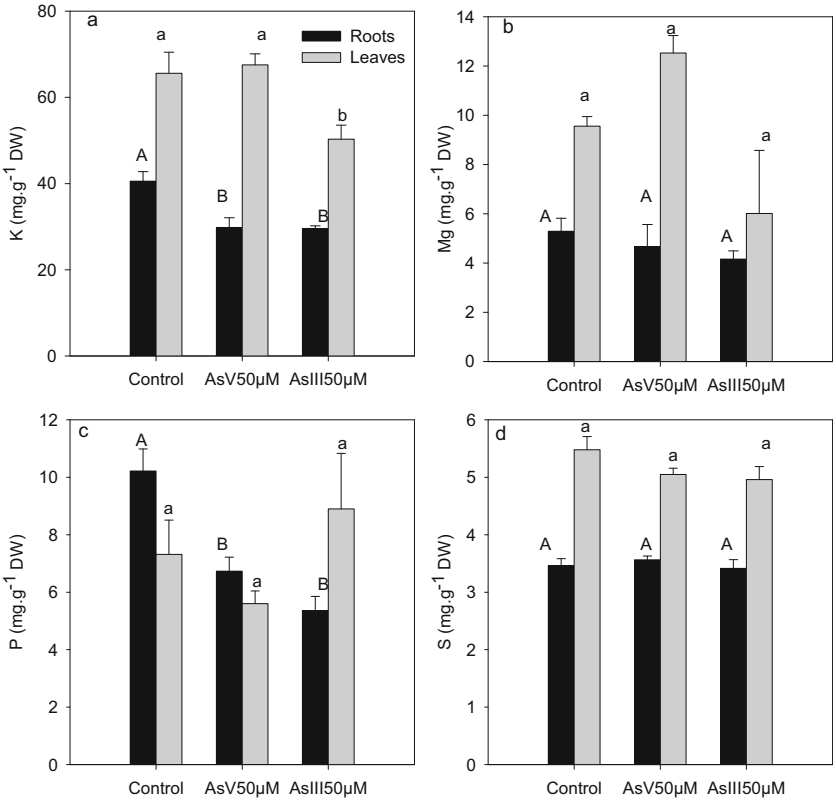

Fig. 2 Potassium (**a**), magnesium (**b**), phosphorous (**c**), and sulfur (**d**) concentration in leaves and roots in *Atriplex atacamensis* exposed during 2 weeks to nutrient solution containing 50 μM As(V) or 50 μM As(III). Each value is the mean of six replicates and vertical bars are S.E. For a given organ, values exhibiting different letters are significantly different at $P < 0.05$ according to Student-Newman-Keuls test at 5% level

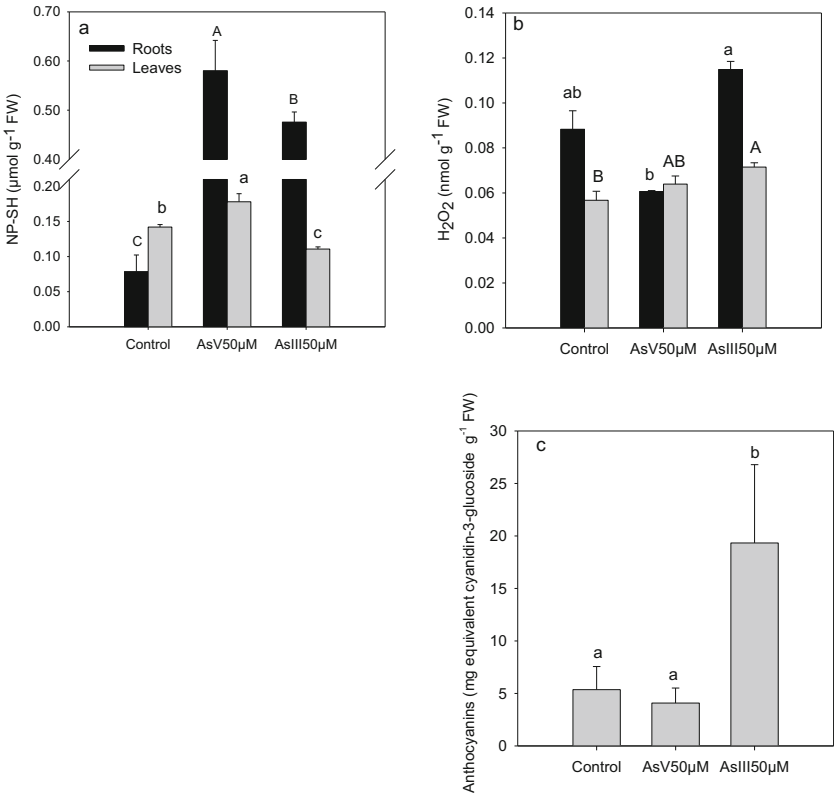

Fig. 3 Root and leaves non-protein thiols (**a**), root and leaves hydrogen peroxide (**b**), stem and leaves anthocyanins (**c**) in *Atriplex atacamensis* exposed during 2 weeks to nutrient solution containing 50 μM As(V) or 50 μM As(III). Each value is the mean of six replicates and vertical bars are S.E. For a given organ (**a** and **b**), values exhibiting different letters are significantly different at $P < 0.05$ according to Student-Newman-Keuls test at 5% level

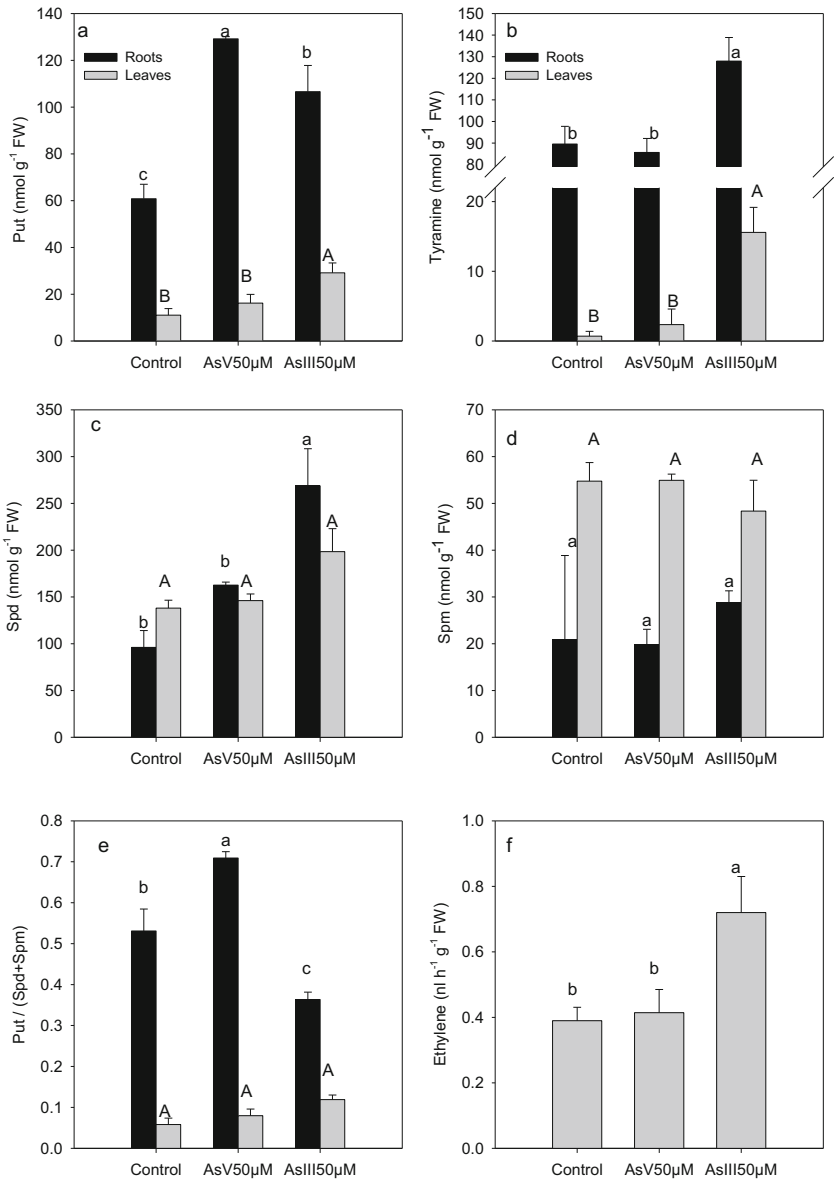

Fig. 4 Root and leaf putrescine (Put; **a**), tyramine (**b**), spermidine (Spd; **c**), spermine (Spm, **d**), putrescine/(spermidine + spermine) ratio (**e**), and leaf ethylene synthesis (**f**) in *Atriplex atacamensis* exposed during 2 weeks to nutrient solution containing 50 μM As(V) or 50 μM As(III). Each value is the mean of six replicates and vertical bars are S.E. For a given organ, values exhibiting different letters are significantly different at $P < 0.05$ according to Student-Newman-Keuls test at 5% level

decreased it at the highest dose (1000 μM). The Put/(Spm + Spd) ratio was decreased in the case of exposure to As(V) 1000 μM, only. Ethylene synthesis was strongly increased by As(V) 50 μM and slightly decreased in response to As(III) 50 μM. The highest dose of As(V) had no impact on ethylene synthesis comparatively to controls.

Discussion

Several species belonging to the genus *Atriplex* have been identified in South America and were reported to be present in harsh environmental conditions (Brignone et al. 2016). Considering their ability to cope with ion toxicities, those species were frequently reported as promising candidates for phytoremediation of heavy metal-contaminated areas (Lutts et al. 2004; Lefèvre et al. 2010b; Eissa 2017). As far as the metalloid arsenic is concerned, *A. atacamensis* displays a fascinating capacity to survive and grow under very high doses of As (Vromman et al. 2011, 2016a, 2017). The present work however demonstrates that *A. atacamensis* response to this pollutant is highly dependent of its speciation in the environment.

Arsenite was indeed more toxic than equivalent concentration of arsenate since As(III) compromised plant survival and plant growth, decreased PSII efficiency, reduced K nutrition, and increased ethylene synthesis, H_2O_2 concentration, and lipid peroxidation index while those effects were not observed in As(V)-treated plants. Arsenite also increased sugar concentration in *A. atacamensis* which might be explained by a decreased growth with only marginal decrease in photosynthesis or by a direct impact of As on enzyme activities involved in carbohydrate metabolism and leading to soluble sugar

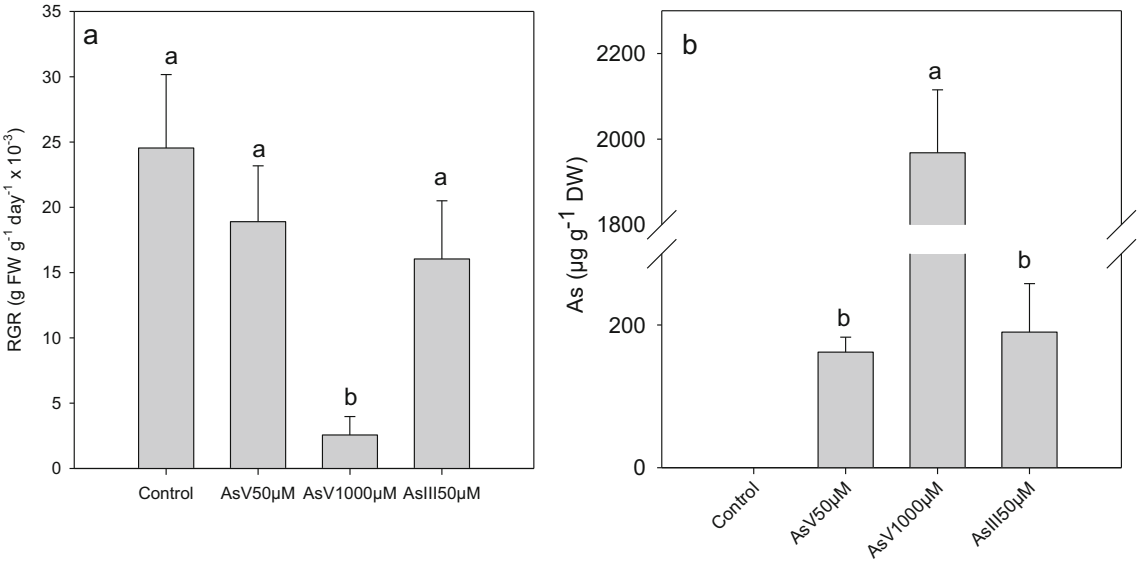

Fig. 5 Relative growth rate (RGR; **a**) and total arsenic concentration (**b**) in hypocotyl-derived calli of *Atriplex atacamensis* cultivated during 2 months on LS medium containing no As (control), As(V) 50 μM, As(V) 1000 μM, and As(III) 50 μM. Each value is the mean of 12 replicates and vertical bars are S.E. Vertical bars exhibiting different letters are significantly different at $P < 0.05$ according to Student-Newman-Keuls test at 5% level

accumulation (Panda et al. 2010). It might be argued that As accumulation was higher in plants exposed to As(III) than in those exposed to As(V) but this is true for roots only, and the difference was not significant for aerial parts. Arsenic had no impact on Ψs values which may be related to the fact that the xerohalophyte *A. atacamensis* in its native habitat is living

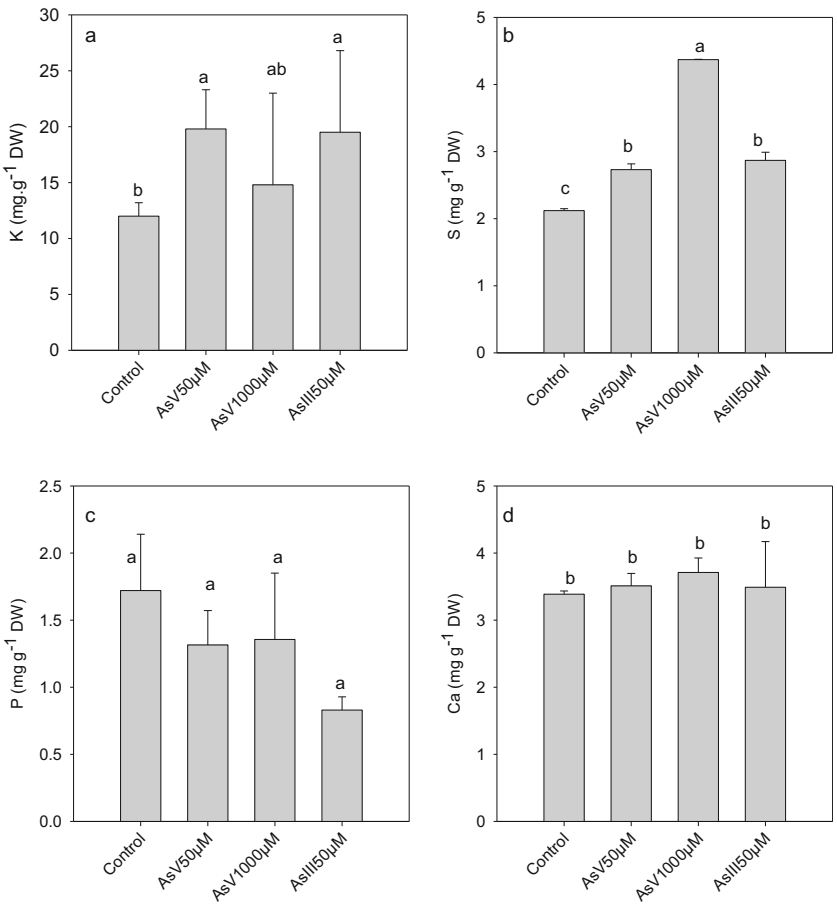

Fig. 6 Potassium (**a**), sulfur (**b**), phosphorous (**c**), and calcium (**d**) concentration in hypocotyl-derived calli of *Atriplex atacamensis* cultivated during 2 months on LS medium containing no As (control), As(V) 50 μM, As(V) 1000 μM, and As(III) 50 μM. Each value is the mean of 12 replicates and vertical bars are S.E. Vertical bars exhibiting different letters are significantly different at $P < 0.05$ according to Student-Newman-Keuls test at 5% level

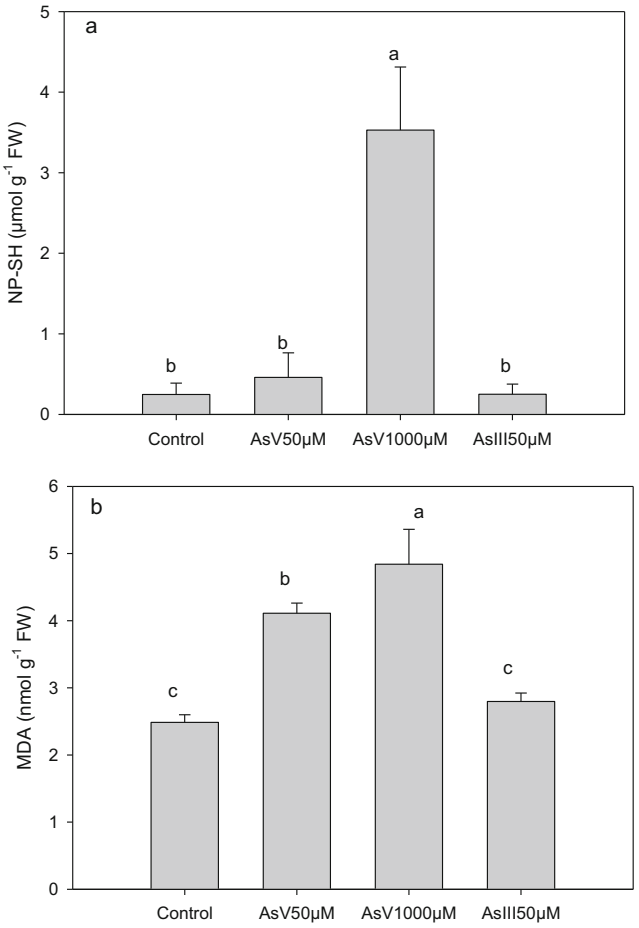

Fig. 7 Non-protein thiols (**a**) and malondialdehyde (MDA, **b**) concentration in hypocotyl-derived calli of *Atriplex atacamensis* cultivated during 2 months on LS medium containing no As (control), As(V) 50 μM, As(V) 1000 μM, and As(III) 50 μM. Each value is the mean of 12 replicates and vertical bars are S.E. Vertical bars exhibiting different letters are significantly different at $P < 0.05$ according to Student-Newman-Keuls test at 5% level

under permanent stress conditions and is thus able to constitutively synthesize high amounts of osmoprotectants such as proline (Vromman et al. 2016a) or glycinebetaine (Vromman et al. 2011).

Within plant cells, As(III) may bind to sulfhydrl groups and consequently alter numerous enzyme activities (Finnegan and Chen 2012; Kumar et al. 2015). Accordingly, endogenous As(III) was reported to be more toxic than As(V) in several plant species (Triptahti et al. 2012; Farooq et al. 2016a). It is however frequently assumed that absorbed As(V) is quickly reduced to As(III) in plant tissue (Panda et al. 2010; Tripathi et al. 2007). Numerous unspecific reductases were identified in several plant species and reduction of As(V) to As(III) by glutathione may also occur non-enzymatically (Delmondieu et al. 1994). Our data suggest that the situation is probably more complex in *A. atacamensis* since, in plants exposed to As(III), an important proportion of accumulated arsenic was in the As(V) form and this suggests that the inverse process of As(III) oxidation may also occur. The two forms were also reported in plants exposed to As(V) suggesting that arsenate reduction was not complete. Although some plant species growing on As-contaminated sites may contain various organic compounds such as methylarsonate or dimethylarsinate (Ruiz-Chancho et al. 2008), these compounds were not identified in our sample suggesting that As methylation did not occur in *A. atacamensis*.

Beside As speciation, As compartmentation is also an efficient strategy to reduce As toxicity. In roots of As(V)-treated plants, 40% of accumulated As was bound to the cell wall fraction. Since this sequestered As is part of the non-extractible fraction, we do not have information regarding As speciation of bound arsenic. This binding process is only marginal in roots of plants exposed to As(III). The situation appeared somewhat different in leaves where a similar proportion of As was bound to the cell wall. Farooq et al. (2016b) also recently reported that leaf cell walls accumulated up to 50% of absorbed As in *Brassica napus* exposed to 50 μM As(III) while Mohd et al. (2017) reported As precipitation within cell walls of *Piriformospora indica*. By using surface-enhanced Raman scattering approaches, Tian et al. (2012) provided clear evidences that As(V) could specifically bind to the cell wall components, including polysaccharides and flavin derivatives, C-N, CH_2, amino, thiol, and hydroxyl groups. Similarly, Dixit and Singh (2016) applied a Fourier transforms infrared (FTIR) spectroscopy method to analyze arsenite binding in the cell wall and concluded that amine, alkenes, and C-N functional groups are involved.

The extractable fraction should contain the unbound As or As fixed on small chelators, such as PC. It is considered that only As(III) is able to bind to –SH group of cysteine (Triptahti et al. 2012). In our plant material, non-protein thiol synthesis was however higher in response to As(V) than to As(III) and As(V) reduction in the tissue was not complete. A similar observation was reported by Wang et al. (2017b) in *Dunaliella salina* where As(V) also triggered higher PC accumulation than As(III), although only As(III) is supposed to bind to PC.

Beside PC, the non-protein thiol fraction contains glutathione which assumes a crucial role in the management of oxidative stress (Noctor et al. 2012) and an improved antioxidative status could explain that H_2O_2 and MDA concentration were lower in As(V)- than in As(III)-treated plants. An increased content of anthocyanin in As(III)-treated plants could also be a consequence of a higher oxidative stress since reactive oxygen species are frequently required as key signals to trigger anthocyanin biosynthesis (Cheynier et al. 2013). Anthocyanin may behave as antioxidant in plant tissues (Benvenuti et al. 2004).

Polyamines are small aliphatic amines behaving as polycations at cellular pH. They assume a wide range of crucial functions in plant growth and development, from seed germination to flowering processes, and are also directly involved in plant responses to abiotic and biotic stresses (Liu et al. 2015). Polyamines contribute to stabilization of biological membranes

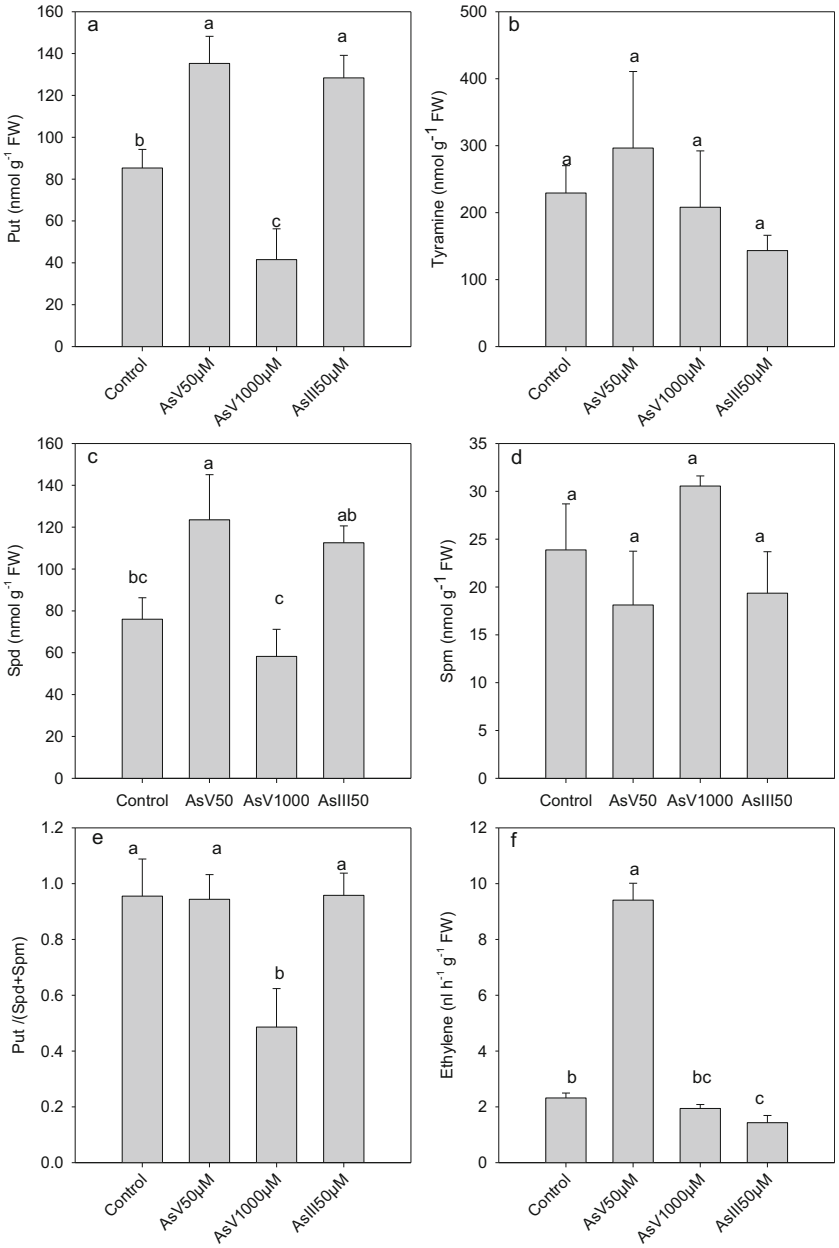

Fig. 8 Putrescine (Put; **a**), tyramine (**b**), spermidine (Spd; **c**), spermine (Spm, **d**), putrescine/(spermidine + spermine) ratio (**e**), and ethylene synthesis (**f**) in hypocotyl-derived calli of *Atriplex atacamensis* cultivated during 2 months on LS medium containing no As (control), As(V) 50 μM, As(V) 1000 μM, and As(III) 50 μM. Each value is the mean of 12 replicates and vertical bars are S.E. Vertical bars exhibiting different letters are significantly different at $P < 0.05$ according to Student-Newman-Keuls test at 5% level

and numerous cellular structures, as well as scavenging of reactive oxygen species (ROS). In *A. atacamenis* exposed to a very high dose of As(V) (1000 μM), polyamine (Spm and Spd) increased while the diamine Put decreased (Vromman et al. 2011). Such a high dose of exogenous As may afford some valuable information regarding the "potential" of *A. atacamensis* in terms of As resistance but is not realistic regarding As dose commonly encountered in the field, even in highly polluted areas (Vithanage et al. 2012; Mirza et al. 2014; Wang et al. 2017a). In the present study performed at a more realistic (although still high) dose, polyamine accumulation appeared different since Put accumulated in roots of plants exposed to As(V) and to As(III) while Spd accumulated only in roots of As(III)-treated plants. Interaction between amine groups and arsenic is an interesting aspect which is still poorly documented in a biological context. Silica-polyamine composite may be used to remove arsenic in acid mine drainage (Hughes et al. 2006) and retains both As(III) and As(V) (Kailasam et al. 2009). Some synthetic amines were recently produced to specifically retain As(V) only (Al Hamouz and Akintola 2017). Hence, it is not excluded that endogenous polyamine may act in a direct sequestration of free As, ensuring a protection of the cell against As-induced damages. Beside this putative binding function, polyamine acting as "hormone-like" molecule may trigger defense processes in stressed plants but Put is usually considered as a toxic compound comparatively to Spd and Spm which are directly involved in cellular structure protection (Liu et al. 2015). It is noteworthy that

the Put/(Spd + Spm) ratio remained unaffected in leaves of *A. atacamensis* while it was the highest in roots of As(V)-treated plants and the lowest in roots of As(III)-treated ones which might be regarded as an unsuccessful attempt of adaptation. In plant species, ethylene is sharing with Spd and Spm the same precursor (S-adenosylmethionine). Oversynthesis of ethylene in As(III)-treated plants however did not appear to limit Spd and Spm synthesis in these plants. Ethylene itself, acting as a senescing hormone may induce numerous physiological disorders associated with premature aging and cell death (Koyama 2014). Ethylene may also lead to K deficiencies (Schachtman 2015) and it has to be mentioned that As(III)-induced leaf ethylene synthesis correlates with K deficiencies in *A. atacamensis*. Beside ethylene, tyramine was obviously increased in both leaves and roots of *A. atacamensis* when exposed to As(III), only. Tyramine is an aromatic amine and its function in plant metabolism is still poorly documented. However, tyramine was recently found to specifically increase in shoots of the halophyte plant species *Inula chrithmoides* when concomitantly exposed to NaCl and heavy metal (Ghabriche et al. 2017).

Whatever As speciation in the nutrient solution, As mainly accumulated in the roots and only a small part was translocated to the shoot. It has to be noticed that stomatal conductance was drastically reduced by As(III) and that transpiration thus is expected to be reduced. Despite a lower stomatal conductance recorded for As(III)-treated plants only, As accumulation in the leaves was similar in both plants exposed to As(III) and plants exposed to As(V), suggesting that As loading might have been more efficient in plants exposed to arsenite than in those exposed to arsenate. Environmental factors influencing As resistance in *A. atacamensis* may act directly on As translocation from the root to the shoot: phosphorous deficiency decreased it (Vromman et al. 2017) while salinity increased As(V) translocation (Vromman et al. 2016a). The impact of salinity on As(III) behavior in planta still needs to be clarified.

In vitro cultivated cell line provides a convenient tool to study the impact of ion toxicities independently of the plant morphological complexity (Lutts et al. 1996; Lefèvre et al. 2010a, b). In the present work, two doses of As(V) were applied: the first one (50 μM) was similar to the dose of As(III) and correspond to the doses tested at the whole plant system in the present study. The second dose (1000 μM) corresponds to the dose previously defined as the maximal dose supported by *A. atacamensis* (Vromman et al. 2011). In contrast to the whole plant system, As(III) 50 μM was not more toxic at the cell level than As(V) 50 μM. Arsenic accumulation was similar in the two cases and RGR values were even lower and MDA higher in response to As(V) than in response to As(III). Arsenite is thought to enter in the plant via the nodulin26-like intrinsic protein aquaporin channel (Kumar et al. 2015). However, there is no transpiration stream in undifferentiated calli and water movement inside the structure remains limited or is even absent in a saturated closed vial. Once entered in the cell, there is no, or only few plasmodesmata for cell to cell movement (Lutts et al. 1996). This may explain why the As accumulation in response to 50 μM As(V) or 50 μM As(III) was lower for callus than for roots. Nevertheless, the As in calli was ten times higher than in leaves and did not drastically compromise growth since RGR of As(V)- or As(III)-treated calli were similar to control, thus confirming that As resistance in *A. atacamensis* presents a cellular component. Another difference between dark-grown cell lines and whole plants is that the former does not perform photosynthesis which is known to be extremely sensitive to As toxicity since chloroplasts constitute major site of reactive oxygen species production (Panda et al. 2010, Finnegan and Chen 2012; Farooq et al. 2016a). Similarly, stomatal and mesophyll conductances drastically influence whole plant photosynthesis but should not be considered in proliferating undifferentiated cell lines.

Despite such a high level of As accumulation, NP-SH did not significantly increase in those calli, suggesting that, in contrast to whole plant, they did not assume crucial functions in cellular resistance. Callus growth was reduced only in response to 1000 μM As(V) which induced a very important As accumulation. NP-SH was increased in those calli only, and should be regarded as an unsuccessful attempt to cope with As accumulation. Arsenic speciation was not analyzed on callus but As(III) and As(V) 50 μM induced a similar proportion of binding to the cell wall. This proportion drastically decreased in response to As(V) 1000 μM, which could be related to the fact that in those calli with a reduced growth, the cell wall compartment should be completely saturated and that a consistent part of As accumulated in the symplasm or in intercellular spaces which represent an important volume in calli (Lutts et al. 1996). The fact that ethylene synthesis was higher in response to As(V) 50 μM than in response to 1000 μM As(V) remains puzzling. In some halophyte plant species, ethylene was reported to assume positive function in response to ion toxicity and is not simply considered as a senescing hormone (Gharbi et al. 2017). On the other hand, high doses of plant growth regulators used in the in vitro medium may modify hormonal metabolism of tissues maintained in closed vials.

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