JOURNAL OF ENVIRONMENTAL SCIENCES 49 (2016) 150–161

Available online at www.sciencedirect.com

ScienceDirect

www.elsevier.com/locate/jes

JES JOURNAL OF ENVIRONMENTAL SCIENCES

www.jesc.ac.cn

The importance of glutathione and phytochelatins on the selenite and arsenate detoxification in *Arabidopsis thaliana*

Fatai Adigun Aborode[1,*], *Andrea Raab*[1], *Matthias Voigt*[1], *Leticia Malta Costa*[1,2], *Eva M. Krupp*[1], *Joerg Feldmann*[1,*]

1. *Trace Element Speciation Laboratory, Department of Chemistry, University of Aberdeen, Aberdeen AB24 3UE, Scotland, UK*
2. *Departamento de Química, Universidade Federal de Minas Gerais, Belo Horizonte, MG CEP: 31270-901, Brazil*

ARTICLE INFO

Article history:
Received 26 April 2016
Revised 8 August 2016
Accepted 13 August 2016
Available online 18 September 2016

Keywords:
Toxicity
Phytochelatins
Glutathione
Selenopeptides
ICP–MS
ESI–MS
Arabidopsis thaliana

ABSTRACT

We investigated the role of glutathione (GSH) and phytochelatins (PCs) on the detoxification of selenite using *Arabidopsis thaliana*. The wild-type (WT) of *Arabidopsis thaliana* and its mutants (glutathione deficient Cad 2–1 and phytochelatins deficient Cad 1–3) were separately exposed to varying concentrations of selenite and arsenate and jointly to both toxicants to determine their sensitivities. The results of the study revealed that, the mutants were about 20-fold more sensitive to arsenate than the WT, an indication that the GSH and PCs affect arsenate detoxification. On the contrary, the WT and both mutants showed a similar level of sensitivity to selenite, an indication that the GSH and PCs do not significantly affect selenite detoxification. However, the WT is about 8 times more sensitive to selenite than to arsenate, and the mutants were more resistant to selenite than arsenate by a factor of 2. This could not be explained by the accumulation of both elements in roots and shoots in exposure experiments. The co-exposure of the WT indicates a synergistic effect with regards to toxicity since selenite did not induce PCs but arsenic and selenium compete in their PC binding as revealed by speciation analysis of the root extracts using HPLC–ICP–MS/ESI–MS. In the absence of PCs an antagonistic effect has been detected which might suggest indirectly that the formation of Se glutathione complex prevent the formation of detrimental selenopeptides. This study, therefore, revealed that PC and GSH have only a subordinate role in the detoxification of selenite.

Introduction

Selenium is known for its roles in biological systems because of its antioxidant and pro-oxidant properties (Mezes and Balogh, 2009). Apart from its role as a growth elicitor and abiotic stress tolerance enhancer, (Mezes and Balogh, 2009) it has also been found to reduce the toxic effects of other elements like cadmium, mercury, lead, antimony and arsenic in plants (Srivastava et al., 2009; Sun et al., 2010; Malik et al., 2012; Filek et al., 2008; He et al., 2004; Shanker et al., 1996; Feng et al., 2011). However, at concentrations above 20 μmol/L selenium has been found to induce toxic effects in plants. The toxic effects of selenium, which include stunted growth, chlorosis, leaves withering, decreased protein synthesis and death of plants (Terry et al., 2000; Hawrylak and Szymańska, 2004; Martin, 1936) are similar to toxicity symptoms from other toxic elements like cadmium, mercury, lead and arsenic (Spallholz and Hoffman, 2002; Mascher et al., 2002; Hartley-Whitaker et al., 2001; Hall, 2002) which suggest similar biochemical interactions in the plants. Most of the toxic

* *Corresponding authors.* E-mails: faborode@hotmail.com (Fatai Adigun Aborode), j.feldmann@abdn.ac.uk (Joerg Feldmann).

http://dx.doi.org/10.1016/j.jes.2016.08.009

effects of elements like cadmium, mercury, antimony, lead and arsenic, have to do with the generation of reactive oxygen species which damage cellular functions and are closely linked with their interactions with catalytically active thiols (Hawrylak and Szymańska, 2004; Hall, 2002; Sharma and Dietz, 2009; Gill and Tuteja, 2010; Stohs and Bagchi, 1995). For selenium in its oxyanion forms (selenite and selenate), its toxic effects are thought to be due to its non-specific incorporation in the plant's protein body by replacing sulphur in cysteine and methionine (Terry et al., 2000). However, toxic effects due to its interaction with essential sulfhydryl-containing enzymes and structural proteins leading to oxidative stress at high concentrations have also been widely reported (Mezes and Balogh, 2009; Hawrylak and Szymańska, 2004; Spallholz and Hoffman, 2002; Hartkainen et al., 2000; Chen et al., 2007; Spallholz, 1994, 1997). The first indication of the interaction of selenium with thiols leading to its toxic effects already reported in 1941 (Painter, 1941) and was later corroborated by Ganther (1968), who elucidated the reaction of selenite with glutathione.

Generally speaking, plants have developed different mechanisms for toxic element detoxification and tolerance (Hall, 2002; Zenk, 1996; Memon et al., 2001). The most common tolerance and detoxification mechanisms used by plants for toxic metals and metalloids involve the activation of a self-regulatory and inactivating enzyme commonly known as phytochelatin (PC) synthase (Memon et al., 2001; Ha et al., 1999; Loeffler et al., 1989). Phytochelatins are a class of metal/metalloid binding peptides formed by a wide variety of plant species in response to uptake of toxic metals/metalloids (Ha et al., 1999; Maitani et al., 1996; Ogawa et al., 2011). They are known for detoxification of toxic elements in plants and have a basic structure (ɣ-Glu-Cys)*n*-Gly, where *n* is in the range 2–11 but typically *n* = 2–4 (Wood and Feldmann, 2012). The terminal glycine can also be replaced with serine, alanine and glutamate (Maitani et al., 1996; Cobbett, 2000).

The mechanisms of plant tolerance to selenium oxyanions (common selenium forms in the soil) are not established, and in particular with regard to the roles of glutathione (GSH) and PC. Even though GSH undoubtedly plays significant roles in plant detoxification systems (Dixon et al., 1998; Guo et al., 2008), there have been contradicting reports on the activation/induction of GSH and PCs by selenite in plants. Grill et al. (1987) reported the induction of PCs by a wide variety of metal ions including selenite in cell suspensions of *Rauvolfia serpentina*, another study later reported that selenite did not induce PC production (Maitani et al., 1996; Zenk, 1996).

The reason for the contradictory results between Grill et al. and Zenk et al. might be as a result of different analytical methods used. Whilst Grill et al. used high-performance liquid chromatography (HPLC), Zenk et al. used extended X-ray absorption fine structure (EXAFS). The presence of various complexes of GSH with selenium in a selenized yeast extract (Dernovics and Lobinski, 2008a, 2008b; Lindemann and Hintelmann, 2002) and incubation of end-capped PC2 with selenite leading to formation of a stable selenotrisulfide (Spain and Rabenstein, 2004) suggest the possibility of formation of selenium-PC complex(es) in plants. The first identification of selenium-PC complex (Se^{II}-PC2) in plants was reported by our group in previous studies on *Thunbergia alata*, a plant which is known to produce PCs at low levels of exposure to arsenate (Bluemlein et al., 2009a, 2009b, 2009c; Aborode et al., 2015).

Factors that favour accumulation or uptake and translocation of selenium in plants need to be understood because of its essentiality in human diets. Therefore, in order to gain a better understanding about the roles of GSH and PCs in selenium detoxification in plants, we used *Arabidopsis thaliana*. The Columbia wild-type (WT) was used as a reference plant for selenite and arsenate sensitivity tests, and its derivative mutants were used to confirm the role of GSH and/or PCs in selenium detoxification. One of the mutants is a GSH-deficient mutant Cad 2–1 (Howden et al., 1995a; Meinke and Koornneef, 1997), which is cadmium sensitive owing to the decrease in its ability to biosynthesis GSH, which is about 15% to 30% of that of the WT (Howden et al., 1995a). The other mutant used in this study is a PC deficient mutant Cad 1–3, which has its PC synthase gene deleted and hence has lost its capacity to produce PCs. It has a PC synthesis capacity of about 8% of that of the WT and hence its hypersensitivity to cadmium despite having almost the same level of GSH (Howden et al., 1995b). Both Cad 1–3 and Cad 2–1 have been used to prove the role of PCs in metal/metalloid detoxification (particularly for cadmium and arsenic) (Howden et al., 1995b; Liu et al., 2010). Since PCs are known to be about 1000 fold more effective than GSH in metal detoxification and reactivation of poisoned enzymes (Kneer and Zenk, 1992), the Cad 1–3 mutant is particularly important in this study because it allows a direct test of the hypothesis regarding the role of PCs in the selenium detoxification mechanism.

The aims of this study, were (i) to investigate whether GSH and/or PCs play any role in selenium detoxification using the sensitivities of the mutants (Cad 2–1 and Cad 1–3) in comparison with that of the WT to selenite, (ii) to investigate whether selenium induces the production of PCs and compare this to the arsenate exposure and (iii) to investigate if there is competition between selenium and arsenic for PCs when they are co-exposed to plants. Selenite and arsenate were chosen because they are the most abundant species of these elements in aerobic soil.

1. Materials and methods

1.1. Chemicals and reagents

All chemicals used were of analytical grade or better. Deionised water (18 MΩ cm) was used throughout (Elga UK). Formic acid (90%), methanol (HPLC grade) and hydrogen peroxide (32%) were from Fisher Scientific UK. Potassium sulphate and sodium dihydrogen orthophosphate dihydrate were supplied by BDH. Gallium used as internal standards was from High Purity Standards, Charleston (USA). Magnesium sulphate heptahydrate, calcium nitrate tetrahydrate and potassium nitrate were supplied by Sigma Aldrich (UK). Sodium selenite (Se(IV)) and sodium hydrogen arsenate heptahydrate (As(V)) was obtained from Alfa Aesar (Germany). Sodium dimethylarsinic acid (98%) used as calibration standard for inductively coupled plasma mass spectrometry (ICP–MS) was obtained from ChemService (USA). Nitric acid

(65%), used for the analysis of total selenium and arsenic was obtained from Fluka (UK).

1.2. Plants germination

The *Arabidopsis thaliana* lines used in this study were WT (Columbia-0) (Howden and Cobbett, 1992), Cad 1–3 (PC synthase-deficient mutant), and Cad 2–1 (GSH-deficient mutant) (Howden et al., 1995a, 1995b). The method used for seed germination was adopted from our previous work (Liu et al., 2010) and is described briefly here. Seeds were germinated on 0.8% (W/V) agarose solution in 0.5 mL Eppendorf vials (one seed per vial) that were cut at their V ends to enable the roots to grow into the nutrient solution. The nutrient solution was 10% of the strength of Hoagland recipe and buffered at pH 5.5 with 2 mmol/L 2-N-Morpholino ethane sulfuric acid. It was contained in 600 mL hydroponic plastic boxes with lids that have holes to support the Eppendorf vials. Eight boxes were maintained for each line of seedlings (WT, Cad 2–1 and Cad 1–3) and each box accommodated 60 seeds. The boxes (24 altogether) were placed in the dark in an incubator maintained at 4°C for 4 days to ensure uniform seed germination. After 4 days, the boxes were transferred to the growth chamber (phytotron) and maintained at 20°C and 70% relative humidity, with a 12 hr light period per day using fluorescent light bulbs. The nutrient solution in the boxes was maintained at a constant volume by regularly topping up with fresh nutrient solution.

1.3. Toxicity tests

In order to be able to monitor the sensitivity of each mutant, Cad 2–1 and Cad 1–3, and wild-type, plants were exposed to varying concentrations of selenite and arsenate separately and co-exposed to both elemental species. At the end of the exposure regimes, root growth measurements were carried out. It should be stated that the chosen concentration range was relatively high for environmental settings and would only reflect porewater from highly polluted soil. The concentration range was chosen for both elemental species since it had previously been shown that arsenate inflicted measureable toxicity in Arabidopsis at this concentration range (Liu et al., 2010).

1.3.1. Exposure to selenite

Four days after germination, root lengths were measured and seedlings with root length between 2 and 4 cm were transferred with their vials into nutrient solution of the same strength as that used for germination. The solutions were supplemented with 0, 5, 10, 25, 50, and 100 μmol/L selenite concentrations. Ten seedlings were maintained per selenite concentration for each of the WT, Cad 2–1 and Cad 1–3 lines. Each treatment was duplicated and the boxes with the seedlings were kept in the phytotron and maintained for 10 days under the same conditions as those described for germination. The solutions in the boxes were renewed twice (*i.e.* every 3 days) during the period of exposure to selenite while that of the control (zero selenium) was maintained at constant volume with regular topping up. After 10 days, the seedlings were carefully washed with tap water, blot dried and their root length was measured. The sensitivity of each line to selenite was compared by determining the concentration (EC_{50}) that caused 50% inhibition of root growth in comparison with the control (no added selenite). The root lengths were measured at the beginning of exposure (d-0), and on the last day of exposure (d-10), from the root-shoot junction to the tip of the root. The determined root growths were used to compute the % root tolerance index (% RTI) by dividing the root growth for the treated plants by that for the control and multiply by 100. The untreated plants (control) therefore become the reference with a % RTI of 100.

1.3.2. Exposure to arsenate

The same germination, exposure and measurement protocols described above for selenite was employed for arsenate. In this case, however, the nutrient solution was supplemented with 0, 5, 10, 25, 50, 100, and 200 μmol/L arsenate.

1.3.3. Co-exposure to selenite and arsenate

Arsenate detoxification is believed to be through thiolate coordination of PC to arsenite after reduction of arsenate. If selenium also binds to form the PC complex such as Se^{II}-PC2 as reported previously in *Thunbergia alata* (Bluemlein et al., 2009a, 2009b, 2009c; Aborode et al., 2015) then, there is a strong possibility of competition between As and Se for PC complexation. Hence, not all arsenic would be complexed and detoxified if no extra PCs are formed. However, selenite at low concentrations is also known to increase the amount of non-protein thiols (Srivastava et al., 2009; Malik et al., 2012; Hawrylak and Szymańska, 2004), which include GSH and PCs. Therefore, if selenite is present at low concentrations, there is a possibility that arsenic might benefit from the increased concentration of thiols for its detoxification. To test the two hypotheses; (i) that competition between selenium and arsenic for thiols might increase arsenate toxicity to plants, and (ii) that selenite at low concentrations induces production of GSH (Srivastava et al., 2009; Hawrylak and Szymańska, 2004; Cui et al., 2008), which can further enhance production of PC for eventual detoxification of arsenate, hence the two mutants and the WT were subjected to co-exposure experiments.

In order to be able to determine the effect of selenite on the sensitivity of the two mutants and the WT to arsenate, uniform seedlings were exposed to varying concentrations of arsenate at a fixed concentration of selenite following the same exposure and measurement protocols as those described above for the separate exposure regimes. The arsenate concentrations were 0, 5, 10, 25, 50, 100 and 200 μmol/L in addition to fixed concentration of 5 μmol/L selenite to all the treatments except the control. The concentration of 5 μmol/L selenite was chosen because it was the concentration with the least root growth inhibition when the seedlings were exposed to varying concentrations of selenite (exposure to selenite).

1.4. Uptake and speciation experiments

In order to be able to determine whether phytochelatins are induced by selenite and to understand what role they play in selenite metabolism and detoxification, fully grown seedlings (8 weeks old) were exposed to 5 μmol/L concentration of selenite for 10 days. The solution was later changed to

15 μmol/L (EC_{50} for Cad 1–3) for a further 4 days using the same protocol as that used for the sensitivity tests described above. 10 seedlings were used for each of WT and Cad 1–3 plants.

After the last 4 days, the plant roots were separated from the shoots and washed with tap water for about 5 min, rinsed in an ice-cold phosphate solution (10 mmol/L K_2HPO_4) for 10 min followed by a final rinse in distilled water. Washed roots were blotted dry, ground under liquid nitrogen. One part was lyophilised in a Thermo Heto PowerDry LL3000 Freeze dryer at −52°C and selenium concentrations measured in roots and in shoots as described below. The other part was used for speciation analysis using a modified method adapted from our previous studies (Aborode et al., 2015; Raab et al., 2004, 2005).

For the extraction, 1.0 ± 0.2 g of each sub-sample was extracted with 3 mL of 1% (V/V) formic acid in an ice bath for 90 min. Extracted samples were subsequently centrifuged at 3500×*g* for 10 min. Thereafter, approximately 1 mL of supernatant was syringed into a 1.5 Eppendorf vial and further centrifuged at 13,000×*g* for 5 min and transferred into 1.8 mL HPLC vials for HPLC–ICP–MS and Electrospray ionisation mass spectrometry (ESI–MS) analysis. This procedure ensured that the analyses were performed within 4 hr of harvests and extraction.

The speciation protocol described above for selenite was used for arsenate (with exposure to 5 μmol/L arsenate for 10 days followed by 15 μmol/L arsenate for 4 days).

For the question regarding competition between selenium and arsenic for thiols, the mutants and the WT were co-exposed to the two element species. For this experiment the same protocol as for individual exposures was employed.

1.5. Analytical methods

1.5.1. Sample preparation

Lyophilised roots and shoot samples (n = 3) were weighed into a 50 mL plastic centrifuge tube (120 ± 115 mg), 2 mL of concentrated nitric acid was added and left to stand overnight at 25°C. Hydrogen peroxide (3 mL) and 250 μL of 20 μg/g of indium were added and the samples digested in a Mars 5 microwave oven (Matthews Inc., USA) as previously described (Aborode et al., 2015). Samples were cooled and diluted with deionised water to a final concentration of 2% (V/V) nitric acid. Selenium and arsenic were then measured by high-resolution ICP–MS (Element 2, Thermo Fisher Scientific) at *m/z* 77, 78 and 75 respectively. In order to correct for the effects of possible fluctuation in the plasma conditions or instrumental drift, an internal standard gallium (10 μg/g) was added continuously before nebulisation and its signal measured at *m/z* 69.

1.5.2. Quality control

For quality control, the certified reference material (rice flour, National Institute of Standards and Technology (NIST) 1568a) was digested in quintuplicate along with 5 reagent blanks to validate the accuracy of the total selenium and arsenic measurements. The measured selenium concentration in NIST 1568a was 0.38 ± 0.03 μg/g dry weight (d.w.) (n = 5) which is in agreement with the certified value of 0.38 ± 0.04 μg/g d.w. Similarly the measured arsenic concentration in NIST 1568a was 0.29 ± 0.04 μg/g d.w. (n = 5) which is also in agreement with the certified value of 0.29 ± 0.03 μg/g d.w. The limits of detection were calculated as 3 times standard deviation of the blank signal and gave 0.01 μg Se/g and 0.001 μg As/g d.w.

1.5.3. Speciation

For the speciation experiments, the instrumentation used consisted of a Thermo Accela HPLC system coupled online with a high-resolution ICP–MS (Element 2, Thermo Fisher Scientific) and in parallel to a high-resolution ESI–MS (LTQ Orbitrap Discovery, Thermo Fisher Scientific). For the separation of the species, a Zorbax Eclipse XDB C-18 (4.6 mm × 150 mm, 5 μm) column was used with a gradient program; a flow rate of 1 mL/min, injection volume of 100 μL, and the auto-sampler was held at 4°C. Mobile phase A was 0.1% (V/V) aqueous formic acid and mobile phase B 0.1% (V/V) formic acid in methanol as described in more detail elsewhere (Aborode et al., 2015). The ICP–MS was used in medium resolution mode (R = 4000) for the measurement of selenium (*m/z* 77 and 78), arsenic (*m/z* 75) and sulphur (*m/z* 32) to avoid the $(^{16}O)_2^+$ interference. For correction of the effects of possible fluctuation in the plasma conditions and instrumental drift, gallium (10 μg/g) was added post-column as an internal standard. Quantification (peak area *vs.* concentration) was performed using species independent external calibration with sodium selenite, DMA^V and sulphate as calibration species. As methanol in the mobile phase is known to enhance ionisation and hence change ICP–MS signal intensities for selenium and arsenic (Gammelgaard and Jøns, 1999; Bluemlein et al., 2008), quantification was performed corresponding to the changes caused by the methanol gradient as discussed in our previous publication (Amayo et al., 2011). The ESI–MS was used in positive mode with an ESI-MS in high-resolution mode and mass range of 100 to 2000 *m/z*, resolution of 30,000, capillary voltage of 4.5 kV and 35% collision energy.

1.5.4. Data analysis

All statistical analysis were performed in Excel using the means and a two-tailed Student's *t*-test if otherwise not explicit mentioned and the *P*-values were reported accordingly.

2. Results and discussion

2.1. Toxicity tests

2.1.1. Sensitivity to selenite

The *Arabidopsis thaliana* accession widely used for genetic and molecular studies is the Columbia ecotype (Meinke et al., 1998). In order to be able to ascertain whether GSH and/or PCs play a significant role in detoxification of selenium in plants, the WT and its mutants; one deficient in GSH (Cad 2–1) and the other lacking PC synthase (Cad 1–3) were subjected to varying concentrations of selenite.

The sensitivities or EC_{50} of the WT and the two mutants were determined as 16.5 ± 0.7, 17.5 ± 0.4, and 14.5 ± 0.5 μmol/L selenite for the WT, Cad 2–1, and Cad 1–3 respectively (Fig. 1a). The observed sensitivities indicated that WT and the mutants Cad 2–1 showed similar sensitivity to selenite. However, the

observed sensitivity of Cad 1–3 to selenite was lower and statistically different ($p < 0.05$) from those of WT and the Cad 1–2.

2.1.2. Sensitivity to arsenate

Since the sensitivities of the WT and the mutants to selenite were similar, the experimental protocol used for selenite was repeated for arsenate at concentrations 0–200 μmol/L. This approach was chosen to compare the results with a previous study (Liu et al., 2010) for direct comparison to selenite, and to be sure that the mutants were indeed deficient in the genes responsible for metals/metalloids detoxification. The EC_{50} for the exposure to arsenate has been determined to be 140 ± 14.2, 6.5 ± 0.4, and 6.0 ± 0.3 μmol/L for the WT, Cad 2–1, and Cad 1–3 respectively (Fig. 1b). Previous studies (Ha et al., 1999; Liu et al., 2010) on the two mutants and the WT had shown that the mutants are about 20-fold more sensitive to arsenate than the WT, and this sensitivity was justifiably attributed to their deficiency in GSH and PC biosynthesis. Our EC_{50} data are very similar to those previously reported EC_{50} values for WT, Cad 2–1, and Cad 1–3 (123.1 ± 14.2, 7.1 ± 0.2, and 5.9 ± 0.5 μmol/L) respectively (Liu et al., 2010).

Here with regards to the sensitivity to selenite the WT was 8 fold more sensitive to selenite (16.5 ± 0.7 μmol/L) than to arsenate (140 ± 14.2 μmol/L) whereas the mutants were 2–3 times less sensitive to selenite (14.5–17.5 μmol/L) than to arsenate (6.0–6.5 μmol/L). This experiment indicates that GSH and PC are instrumental in the detoxification of arsenate but GSH and PCs have no or only a small influence on the detoxification of selenite respectively.

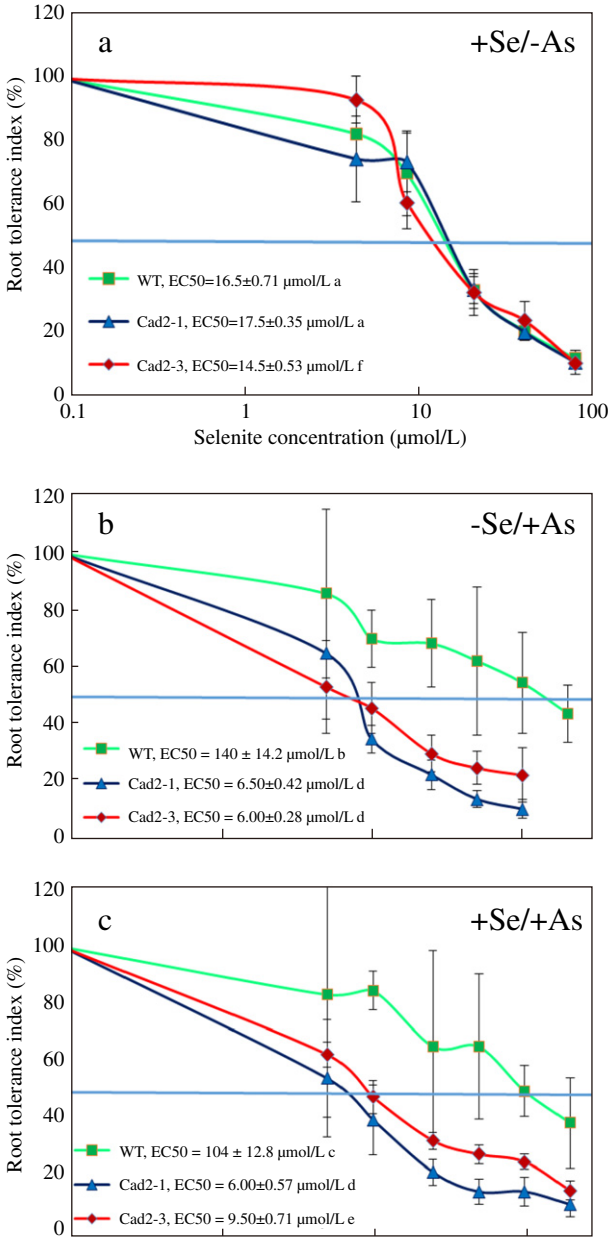

Fig. 1 – Toxicity test measuring the root tolerance index compared to the unexposed controls of *Arabidopsis thaliana* seedlings (wild-type (WT) and its mutants Cad 2–1 and Cad 1–3) exposed to varying concentrations of selenite (a) or arsenate without selenite (b) and with co-exposure to 5 μmol/LM selenite (c) for 10 days. Data are RTI mean ± SD ($n = 20$ seedlings). EC_{50} is the effective concentration of selenite and, or arsenate causing 50% inhibition of root growth. a–f means followed by different letters are significantly different ($p < 0.05$, two-tailed *t*-test).

2.1.3. Effect of selenite on the sensitivity to arsenate

For the co-exposure of the plants to both arsenate and selenite, the EC_{50} values have been measured to be 104 ± 12.8, 6.0 ± 0.6, and 9.5 ± 0.7 μmol/L arsenate for the WT, Cad 2–1, and Cad 1–3 respectively (Fig. 1c). The sensitivity (EC_{50}) of the WT plants to arsenate shifted from average of 140 μmol/L in the single exposure to arsenate to average of 104 μmol/L in the plants co-exposed to a small concentration of selenite. This change in sensitivity was statistically significant ($p < 0.01$) and implies that selenite increased the toxicity of arsenate in the plants even at a low concentration of 5 μmol/L which in a sole selenite exposure did not show any significant effect on the WT.

This is a small but significant synergetic effect of selenite on arsenate. Arguably, in the presence of selenite, and probably because of competition, less PCs and/or GSH may have been available for complexation of arsenic and hence the increased toxicity. This needs confirmation by determining the complexed peptide species of both elements in roots of the plants which is discussed later.

There was no significant change ($p = 0.4$) in the sensitivity of Cad 2–1 to arsenate in the presence of selenite (Fig. 1). However, a very significant change ($p = 0.01$) in the sensitivity of Cad 1–3 was observed from an EC_{50} value of 6.0 in single exposure to 9.5 μmol/L in the co-exposed plants which indicates that the PC deficient mutant becomes less sensitive to arsenate when co-exposed to low concentration of selenite (Fig. 1).

But the PC deficient mutant is still 10 fold more sensitive to arsenate than the WT when co-exposed to selenite. In contrast to the WT with high level of PCs in which a synergistic effect was recorded, in Cad 1–3 selenite shows a small but significant antagonistic effect to arsenate exposure ($p < 0.05$). Such antagonistic effect could be explained either through the influence of selenium on the uptake or efflux of arsenic or by the identification of a selenium compound

which complexes arsenic. Such an adduct has previously been identified as $(GS)_2AsSe^-$ in the erythrocytes of an selenium and arsenic exposed mammal which the authors claim is the reason for the selenium/arsenic antagonism (Gailer, 2009). In order to answer these questions an uptake experiment was conducted in which the total accumulated selenium and arsenic were measured in roots and shoots and the elemental species in the extracts identified.

2.1.4. Accumulation of selenium and arsenic in roots and shoots

The roots of the WT plants exposed to arsenate accumulated 17.8 μmol As/g d.w. in their roots compared with 11.0 μmol As/g d.w. determined for the Cad 1–3, a PC deficient mutant (Table 1). For the selenite exposed roots; the WT plants accumulated 7.70 μmol Se/g d.w. whilst the Cad 1–3 plants took up 7.62 μmol Se/g d.w. (Table 1). Whilst there was 60% reduction in arsenic accumulation in Cad 1–3 compared to the WT plants exposed to the same levels of arsenate, the selenium accumulation in the WT and the mutant Cad 1–3 remained the same for the same level of exposure to selenite. The shoots show detectable levels for the arsenic exposed plants but the selenium concentration was below the detection limit of the method used. The translocation factors (ratio of shoot concentration over the root concentration) show that for arsenic in the PC deficient mutant Cad 1–3 was higher than in WT. These results are consistent with our previous findings (Liu et al., 2010). Clearly, PCs play a significant role in arsenic accumulation, illustrated through the reduced accumulation in the absence of PCs in Cad 1–3 compared to WT. The accumulation results for selenium however, suggest that PCs do not play any role in selenium accumulation of these plants. So far no evidence exist that there are specific transporters for Se^{II}-PC complexes into vacuoles. This result confirms also that the sensitivity of the Arabidopsis species to selenite did not show any large differences between the WT and the mutant plants, as the three lines have almost the same level of sensitivity to selenite.

The reduction of arsenic accumulation in Cad 1–3 as compared to WT, might be due to enhanced efflux of As^{III} from the cytosol because of the impaired capability of Cad 1–3 to produce arsenic-PC complexes.

The roots of the WT plants co-exposed to selenite and arsenate accumulated 9.23 ± 0.12 μmol As/g (Table 1) which was about half of the arsenic accumulated when the plants were exposed to only arsenate (17.8 μmol As/g). Similarly reduction of arsenic was also established for the Cad 1–3 (Table 1). Since the arsenic accumulation in WT in both exposure regimes is significantly higher in the WT compared to the Cad 1–3, it means that in the presence of PCs more arsenic is accumulated in the roots. This has been shown before for arsenic exposure and it was suggested that arsenic-PC complexes transport the arsenic into the vacuoles (Liu et al., 2010). Additionally, it seems that selenite shows a mitigating effect on the accumulation of arsenic in both the WT and the Cad 1–3 mutants. The reason for this effect is unclear.

The amount of selenium accumulated in the roots of WT plants co-exposed to selenite and arsenate was 7.06 μmol Se/g, which was not significantly different from the amount accumulated when the plants were exposed to only selenite (Table 1). However, selenium accumulation (11.4 μmol Se/g) in the roots was enhanced by arsenate in the absent of PCs in Cad 1–3. The selenium concentration was 1.5 times higher ($p < 0.05$) than the amount accumulated when the plants were exposed to only selenite (7.62 μmol Se/g). The reason for the increased accumulation of selenium in the presence of arsenic in Cad 1–3 is not clear. But it seems that plants co-exposed to arsenic and selenium accumulate more selenium and less arsenic when there is shortage or no supply of PCs as the case with Cad 1–3. This could explain why the Cad 1–3 were less sensitive to the arsenic when co-exposed to selenite and hence arsenate and selenite show a small but significant antagonistic effect with regards to the arsenate toxicity. This however needs to be substantiated in further experiments

Table 1 – Total concentration of arsenic and selenium in roots and shoots of the wild-type and PC deficient mutant Cad 1–3 after exposed to 15 μmol/L arsenate with and without co-exposure to 5 μmol/L selenite. TF is the translocation factor defined as concentration ratio of shoots to roots. The concentrations are from pooled samples ($n = 10$). The analytical SD based on repeats are found in parentheses.

Arsenic	WT (μmol As/g d.m.)			Cad 1–3 (μmol As/g d.m.)		
	Root	Shoot	TF	Root	Shoot	TF
As	17.8[a] (0.52)	0.20 (0.01)	0.011	11.0[c] (0.05)	0.28 (0.01)	0.025
As + Se	9.23[b] (0.12)	0.06 (0.02)	0.006	5.77[d] (0.11)	0.54 (0.01)	0.093
Selenium	**WT (μmol Se/g d.m.)**			**Cad 1–3 (μmol Se/g d.m.)**		
	Root	Shoot	TF	Root	Shoot	TF
Se	7.70[a] (0.04)	<0.5	<0.06	7.62[a] (0.03)	<0.5	<0.06
As + Se	7.06[b] (0.05)	<0.5	<0.07	11.4[c] (0.1)	<0.5	<0.04

Means followed by different letters (a–d) are significantly different ($p < 0.05$, two-tailed *t*-test). l.o.d. 0.05 μmol As/g d.m.; 0.5 μmol Se/g d.m. (dry matter).

with variable selenite concentrations. Whether specific selenium species bind directly to arsenic might be revealed by performing speciation analysis of the extracts from exposed roots.

2.2. Speciation analysis

2.2.1. Exposure to individual species

The speciation method employed in this study as described in the method section showed the complexation of arsenic and selenium with PCs as previously reported in other studies with plants (complexation of arsenic with PCS, Liu et al., 2010; Bluemlein et al., 2008, 2009a, 2009b) and (with selenium, Bluemlein et al., 2009a, 2009b, 2009c; Aborode et al., 2015). The identification of the individual selenium, sulphur and arsenic species was done by simultaneous detection of the elements using the HR–ICP–MS and the parallel detection of the molecular peak with the fragmentation pattern indicating the isotopic fingerprint (in case of selenium) and the accurate mass measurement from the Orbitrap. The HPLC–ICP–MS chromatograms of the arsenate exposed plants are shown in Fig. 2, whereas the extracted ion chromatograms of the WT exposed to both selenite and arsenate are shown in Fig. 3. All other mass spectra including fragmentation pattern and chromatograms are shown Appendix A Figs. S1–S8. The list of the identified species and their concentrations in the WT and the mutant Cad 1–3 for the different exposure regimes are listed in Tables 2 and 3. (See Fig. 4.)

Analysis of the root extracts of arsenate exposed WT plants, using the HR–ICP–MS, revealed the presence of 5 distinct arsenic species (Fig. 2). The first peak, which eluted in the void volume with a retention time of 2 min, was the hydrophilic non-complexed arsenite, while the other peaks that eluted after more than 6 min were the hydrophobic fractions, and represented the complexed/bound arsenic species.

Of the 4 complexed/bound species, one species was not identifiable. The other 3 were identified by their accurate masses, their retention time using both the HR–ICP–MS of the arsenic trace at m/z 75 and the HR–ESI–MS of the protonated masses $[M + H]^+$. Full scan spectrum (HPLC–HR–ESI–MS) of the extracts of the exposed WT plants (Fig. S1) helped to identify the complexed species as (peak 3) AsPC3(OH) ($C_{26}H_{41}N_7O_{15}S_3As^+$, elution time 13.5 min, experimental $[M + H]^+$ 862.1044, theoretical 862.1034, Δm = 1.2 ppm), (peak 4) AsPC2(GS) ($C_{28}H_{44}N_8O_{16}S_3As^+$, elution time 14.7 min, experimental $[M + H]^+$ 919.1260, theoretical 919.1248, Δm = 1.3 ppm), and (peak 5) AsPC3 ($C_{26}H_{39}N_7O_{14}S_3As^+$, elution time 17.3 min, experimental $[M + H]^+$ 844.0939, theoretical 844.0928, Δm = 1.3 ppm). In addition to the complexed As, a number of free thiol compounds; reduced GSH, oxidised PC2 and PC3 were identified.

The Cad 1–3 mutant is a PC deficient mutant and, as expected, no PCs were detected in the extracts of the arsenate exposed roots (Fig. 2 and Appendix A Fig. S2). Root extracts of the arsenate exposed plants using the HR–ICP–MS, revealed the presence of 3 distinct arsenic species (Fig. 2). A full scan spectrum (HPLC–HR–ESI–MS) of the extracts helped to identify one complexed species as (peak 4) $As(GS)_3$ ($C_{30}H_{49}N_9O_{18}S_3As^+$, elution time 10.5 min, experimental $[M + H]^+$ 994.1586, theoretical 994.1569, Δm = 1.7 ppm). Reduced and oxidised GSH was also detected.

Fig. 3 shows superimposed the HPLC signals for sulphur, selenium and arsenic and the simultaneously measured protonated molecular masses of the elemental species. The ICP–MS can be used to quantify all elemental species by species independent calibration as shown previously (Bluemlein et al., 2008, 2009a, 2009b, 2009c). This led to identification and quantification of a number of complexed and free thiols as listed in Table 3.

In order to establish whether selenite induces PC formation, fully grown plants (WT and Cad 1–3) were exposed to 15 μmol/L selenite for 4 days. The HR–ICP–MS results of the extracts of the exposed roots revealed the presence of 5 distinct selenium species in both the WT and the Cad 1–3 mutant (Fig. 2c and d and Appendix A Figs. S3–S6). However, only one species was identified as Se^{II}Cysteinyl-2,3-dihydyroxylproprionyl glutathione (Se^{II}CysDHP-GSH) in both WT and Cad 1–3. The identification of this selenopeptide has been reported in *Thunbergia alata* in our previous study (Aborode et al., 2015). In addition to the identified selenopeptide, a number of free thiols were also identified in the extracts of the roots exposed to selenite. Reduced and oxidised GSH were identified in both WT and Cad 1–3. Oxidised PC2 was identified in the extracts of the WT but not in the Cad 1–3. This is consistent with the expectation of the experiment, since the mutant lacks the PC synthase for synthesis of PCs.

The PCs observed in the selenite exposed WT plants terminated at PC2 whereas both PC2 and PC3 were formed in the arsenate exposed plants. The inability to elongate beyond PC2 in the selenite exposed plants might be as a result of the preference of selenium to use the available GSH for transformation into selenopeptides through selenocysteine (see the schematic description of the proposed selenite transformation in Fig. 3).

The transformation route into selenopeptides using glutathione as reductant and precursor for incorporation through selenocysteine is thought to be responsible for the toxic effects of selenium. The GSH/GSSG ratio is usually a measure of the oxidative stress of cells and, the higher the GSSG, the lower the ratio and the more pronounced is the oxidative damage to biological cells (Cui et al., 2008). Only GSH was identified in the extracts of the arsenic exposed plants (WT) with GSSG levels too low to be detectable (Appendix A Fig. S1), whereas the selenite exposed plants (WT) has a considerable level of GSSG compared with GSH (Fig. S3) indicative of higher oxidative stress.

The very high level of GSSG (Appendix A Fig. S3) in the selenite exposed plants compared with the arsenate exposed plants where only GSH was observed (Appendix A Fig. S1) might explain why WT plants were more sensitive to selenite than to arsenate. It is possible that the interaction of selenium with GSH to form selenopeptides increased the oxidative stress in plants far more than the interaction of arsenic with glutathione to form both free PCs and their complexed species. In the selenite exposed plants there was no difference between the WT and Cad 1–3 in the utilisation of GSH which in both lines was used in the formation of the selenopeptide Se^{II}CysDHP-GSH.

2.2.2. Competition for PC between selenium and arsenic

In order to be able to investigate the hypothesis regarding complexation of PC with selenite on one hand and

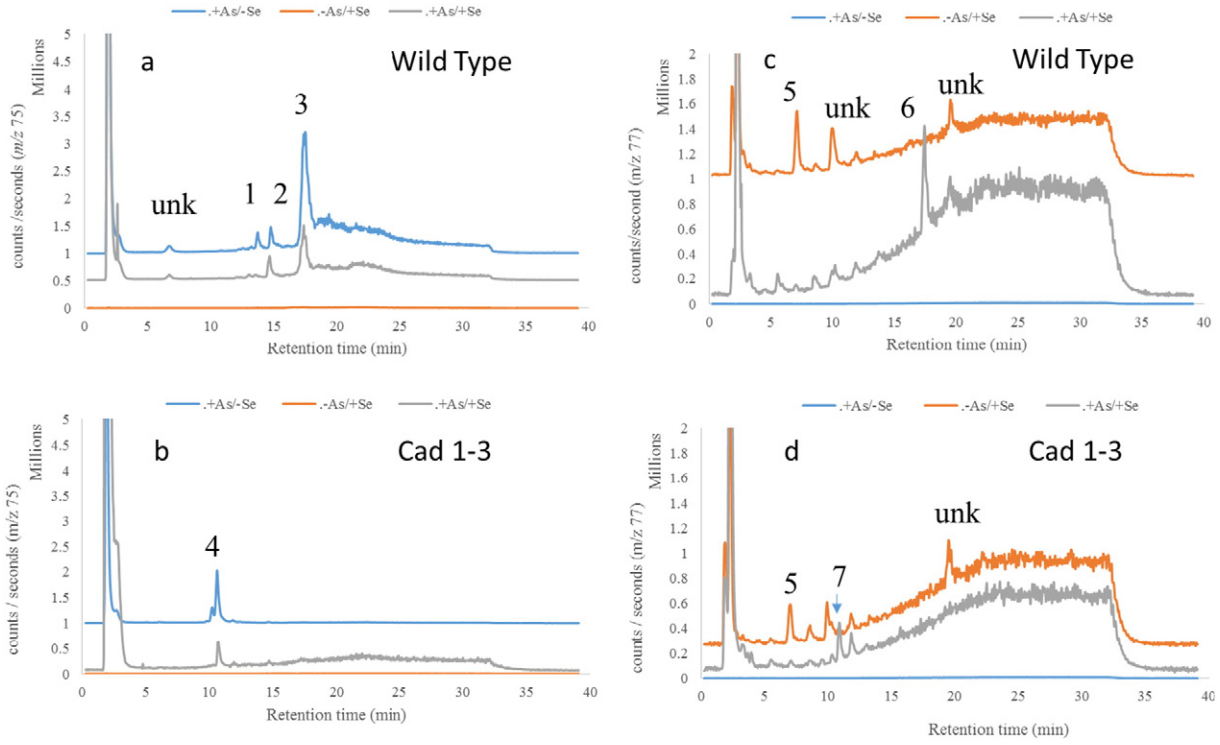

Fig. 2 – Chromatograms of root extracts from WT and Cad 1–3 from different exposure regimes using RP–HPLC–ICP–MS/ESI–MS; (a) and (b) show the specific arsenic detection (*m*/*z* 75) and (c) and (d) show selenium detection (*m*/*z* 77). The second traces for a, b, c and d show specific sulphur detection at *m*/*z* 32. The compounds 1–6 were identified using the ESI–MS data, the respective chromatograms and mass spectra are in the supplementary material. peak 1; AsPC3(OH), peak 2; AsPC2(GS), peak 3; AsPC3, peak 4; $As(GS)_3$, peak 5; Se^{II}cysDHP(GSH), peak 6; $Se^{II}PC2$, peak 7; $Se^{II}(GS)_2$ unk = unknown As and Se species.

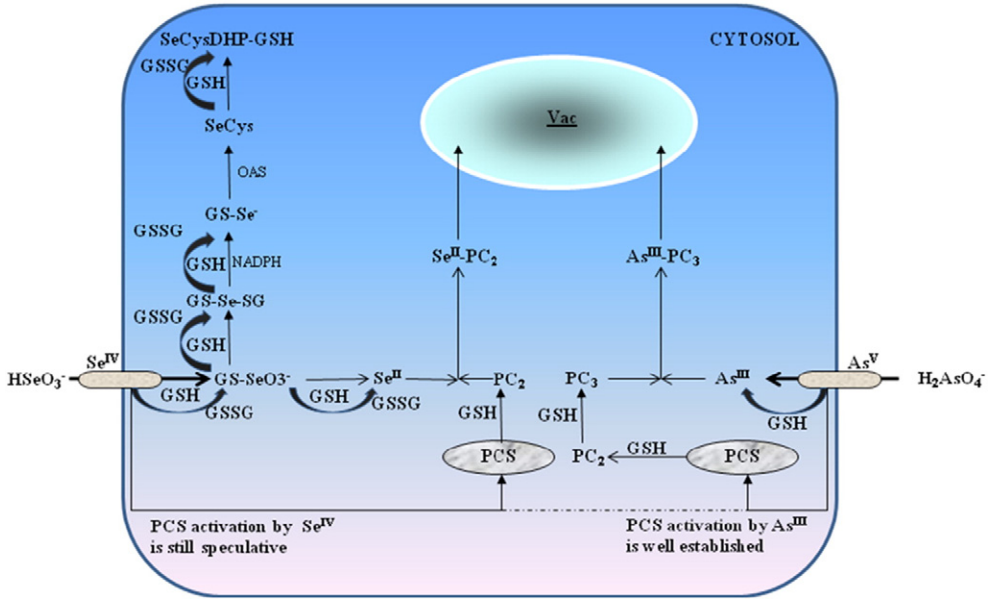

Fig. 3 – Proposed mechanisms for utilisation of glutathione by selenite and arsenate in *Arabidopsis thaliana*. Selenium and arsenic ions entering the cell activate the constitutive PC synthase that catalyses the synthesis of PCs from GSH. The synthesised PCs are complexed with selenide and arsenite and the complexed species are transported into the vacuole where they are stored as detoxified species. It is thought that selenite induced synthesis of glutathione and extensively uses it for its biotransformation into selenopeptides at the expense of the PC biosynthesis. Arsenite effectively uses glutathione for PC synthesis and the plant can benefit from enhanced glutathione synthesis in the presence of selenite for synthesis of PCs to enhance arsenic detoxification.

Table 2 – List of identified arsenic, selenium and sulphur species in the root extract of the WT and the PC deficient mutant Cad 1–3. See the supplementary data for the identification of those species.

WT	+As/−Se		−As/+Se		+As/+Se		
	S	As	S	Se	S	As	Se
	GSH ox-PC2 red-PC2 ox-PC3	U1 (7 min) AsPC3(OH) AsPC2(GS) **AsPC3**	**GSH** GSSG ox-PC2	**Se^{II}cysDHP(GSH)** U1 (5.4 min) U2 (10 min) U3 (20 min)	**GSH** GSSG ox-PC2 red-PC2	 AsPC2(GS) **AsPC3**	 **Se^{II}PC2**
Cad 1–3	S	As	S	Se	S	As	Se
	GSH GSSG	**As(GS)3**	**GSH** GSSG	**Se^{II}cysDHP(GSH)** U1 (5.3 min) U2 (10 min) U3 (20 min)	GSH GSSG	**As(GS)3**	**Se^{II}(GS)2**

Bold compounds indicate that they were the major compounds for the element in the extract.

competition with arsenite for the PCs on the other, the root extracts of the co-exposure experiment were analysed. The speciation of both the WT and Cad 1–3 were compared with those of the individually exposed plants. Whilst the extracts of the plant (WT) roots exposed to arsenate revealed 3 complexed arsenic species (AsPC2(GS), AsPC3(OH), and AsPC3), the extracts of the co-exposed WT roots revealed only 2 complexed species (AsPC2(GS) and AsPC3) (Appendix A Fig. S5). However, for the selenium speciation, there was a marked difference between the co-exposed and the selenite exposed roots; while the WT roots exposed to only selenite showed the presence of as Se^{II}CysDHP-GSH, the co-exposed roots revealed the presence of Se^{II}PC2 (Appendix A Fig. S5).

The selenium phytochelatin complex (Se^{II}PC2) was detected from the accurate masses from the HR–ICP–MS and ESI–MS in addition to the characteristic isotopic fingerprint of a mono-selenium compound (Appendix A Figs. S6–7). The species was detected at 17.3 min on the HR–ICP–MS and the ESI–MS and the structure was established from the fragments of the MS/MS spectrum of the protonated mass at *m/z* 618 (^{80}Se). On the MS/MS spectrum, the signals at *m/z* 489 and 414 corresponded to a loss of ɤ-glutamic acid and glycine respectively.

The quantification showed that less arsenic is complexed in the co-exposed WT than when only exposed to arsenate. Hence, the speciation analysis confirmed a competition between arsenic and selenium for the PCs and that would explain the higher sensitivity of the WT when it is co-exposed to arsenate and selenite.

The speciation results for Cad 1–3 exposed roots also lend credence to the hypothesis of competition between selenite and arsenite for thiols other than PCs. As expected no PC was detected in neither the individual exposures to selenite and

Table 3 – Quantification of the complexed species and comparison of the total element concentrations in the roots of the WT and the mutant Cad 1–3 after the different exposure regimes. The concentrations are given in micromole/g d.w. or μmol/g d.w. respectively. The data for sulphur compounds are only given in relative amounts in counts per second measured on *m/z* 32 for ^{32}S on the ICP–MS.

Arsenic	Sum (all As (Unk))		As(GS)3		AsPC3(OH)		AsPC2(GS)		AsPC3		% complexed	
	WT	Cad 1–3	WT	Cad 1–3	WT	Cad 1–3	WT	Cad 1–3	WT	Cad 1–3	WT	Cad 1–3
+As/−Se	0.45	–	–	0.59	0.23	–	0.28	–	3.96		27.6	5.4
+As/+Se	0.12	0.02	–	–	–	–	0.13	–	0.45	–	7.6	0.4
Selenium	Sum (all Se (Unk))		SeCysDHPGSH		Se(GS)2		SePC2		% complexed			
	WT	Cad 1–3	WT	Cad 1–3	WT	Cad 1–3	WT	Cad 1–3	WT	Cad 1–3		
−As/+Se	2.32	1.16	1.14	0.51	–	–	–	–	44.9	21.9		
+As/+Se	0.69	1.65	–	–	–	0.52	0.26	–	13.5	11.4		
Sulphur	GSH		GSSG		ox-PC2		red-PC2		ox-PC3			
	WT	Cad 1–3	WT	Cad 1–3	WT	Cad 1–3	WT	Cad 1–3	WT	Cad 1–3		
+As/−Se	300	300	–	40	80	–	80	–	20	–		
−As/+Se	40	40	20	5	5	–	–	–	–	–		
+As/+Se	40	40	30	45	10	–	5	–	–	–		

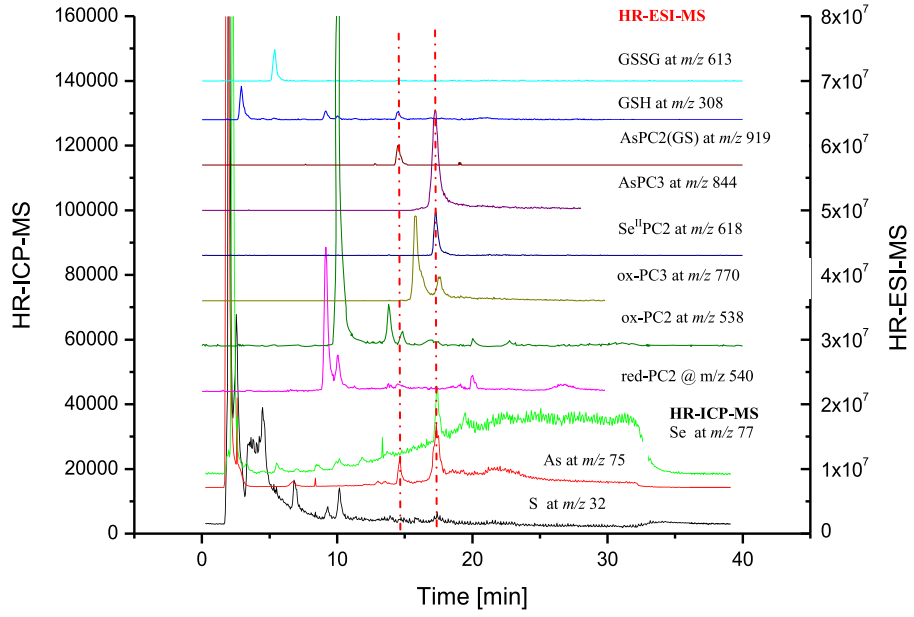

Fig. 4 – HPLC–HR–ICP–MS/ESI-Orbitrap MS of formic acid extract from WT roots co-exposed to 15 μmol/L selenite and arsenate for 4 days, with overlaid ESI–MS (top) $[M + H]^+$ for GSSG at *m/z* 613, GSH at *m/z* 308, AsPC2(GS) at *m/z* 919, AsPC3 at *m/z* 844, Se^{II}PC2 at *m/z* 618, ox-PC3 at *m/z* 770, oxPC2 at *m/z* 538, red-PC2 at *m/z* 540, and traces for selenium, arsenic and sulphur at *m/z* 77, 75 and 32 respectively (bottom).

arsenate nor in the co-exposed roots. In the absence of PCs however, not only $As(GS)_3$ was detected but also $Se^{II}(GS)_2$ in the co-exposure experiment. The $Se^{II}(GS)_2$ was identified through its Se isotopic fingerprint and its fragmentation pattern (Appendix A Fig. S8).

Hence only in the absence of PCs, GSH binds to arsenic and to selenium if the levels of GSH are sufficiently high. Since arsenic is known to induce higher levels of GSH, only in the co-exposure experiment with Cad 1–3 only $Se^{II}(GS)_2$ is preferentially formed instead of the other selenopeptides (Fig. 2). This leads to a hypothesis that the formation of the other selenopeptides such as Se^{II}cysDHP(GSH) is the reason for the high sensitivity of the Arabidopsis species towards selenite. Therefore, it should be noted that the antagonistic behaviour of these elements in the absence of phytochelatins (Cad 1–3) is in contrast to the synergistic effect in the presence of phytochelatins (WT) although more selenium is actually accumulating in the roots. A formation of an As/Se complex such as $(GS)_2AsSe^-$ could not be established probably because of the limitations of the method employed, as this complex is only stable at pH above 7 and rapidly oxidised to elemental selenium (red) in the presence of oxygen. This antagonistic effect could be further investigated in more detail with varying selenite concentrations.

3. Conclusions

From this study, it is evident that either GSH or PCS play only a subordinate role in selenium detoxification in *Arabidopsis thaliana*. Whilst both peptides are necessary for arsenic detoxification, their capability to detoxify arsenic is compromised in the presence of selenium. In the presence of PCs, selenite and arsenate show synergistic effects with regard to the toxicity because they compete for the same biothiol in the cells and selenium does not induce the production of PCs. In the absence of PCs selenite and arsenate show an antagonistic effect. The latter cannot be explained at the moment and needs further investigations. In summary selenium accumulates in *Arabidopsis thaliana* WT not only as PC complexes but forms also selenopeptides. But due to the necessary redox change of selenite during the biotransformation an extra burden on the plant which is not compensated by the induction of the formation of more PCs, show higher sensitivity to selenite than to arsenate in the WT.

Acknowledgment

This study was supported by the Tesla Research Funds. L.M.C. thanks CNPq (201969/2010-6) for her research visit to Aberdeen and M.V. thanks the European Erasmus Exchange programme.

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.jes.2016.08.009.

REFERENCES

Aborode, F.A., Raab, A., Foster, S., Lombi, E., Maher, W., Krupp, E.M., Feldmann, J., 2015. Selenopeptides and elemental selenium in *Thunbergia alata* after exposure to selenite: quantification method for elemental selenium. Metallomics 7, 1056–1066.

Amayo, K.O., Pettursdottir, A., Newcombe, C., Gunnlaugsdottir, H., Raab, A., Krupp, E.M., Feldmann, J., 2011. Identification and

quantification of arsenolipids using reversed-phase HPLC coupled simultaneously to high-resolution ICPMS and high-resolution electrospray MS without species-specific standards. Anal. Chem. 83, 3589–3595.
Bluemlein, K., Raab, A., Meharg, A.A., Charnock, J.M., Feldmann, J., 2008. Can we trust mass spectrometry for determination of arsenic peptides in plants: comparison of LC–ICP–MS and LC–ES–MS/ICP–MS with XANES/EAXAFS in analysis of *Thunbergia alata*. Anal. Bioanal. Chem. 390, 1739–1751.
Bluemlein, K., Klimm, E., Raab, A., Feldmann, J., 2009a. Selenite enhances arsenate toxicity in *Thunbergia alata*. Environ. Chem. 6, 486–494.
Bluemlein, K., Raab, A., Feldmann, J., 2009b. Stability of arsenic peptides in plant extracts: off-line versus on-line parallel elemental and molecular mass spectrometric detection for liquid chromatographic separation. Anal. Bioanal. Chem. 393, 357–366.
Bluemlein, K., Krupp, E.M., Feldmann, J., 2009c. Advantages and limitations of a desolvation system coupled online to HPLC–ICP–qMS/ES–MS for the quantitative determination of sulfur and arsenic in arseno-peptide complexes. J. Anal. At. Spectrom. 24, 108–113.
Chen, J.J., Boylan, L.M., Wu, C.K., Spallholz, J.E., 2007. Oxidation of glutathione and superoxide generation by inorganic and organic selenium compounds. Biofactors 31, 55–66.
Cobbett, C.S., 2000. Phytochelatin biosynthesis and function in heavy-metal detoxification. Curr. Opin. Plant Biol. 3, 211–216.
Cui, S.Y., Jin, H., Kim, J.S., Kumar, A.P., Lee, Y.I., 2008. Interaction of glutathione and sodium selenite in vitro investigated by electrospray ionization tandem mass spectrometry. J. Biochem. 143, 685–693.
Dernovics, M., Lobinski, R., 2008a. Characterization of the selenocysteine-containing metabolome in selenium-rich yeast. Part 1. J. Anal. At. Spectrom. 23, 72–83.
Dernovics, M., Lobinski, R., 2008b. Characterization of the selenocysteine-containing metabolome in selenium-rich yeast. Part 2. J. Anal. At. Spectrom. 23, 744–751.
Dixon, D.P., Cummins, L., Cole, D.J., Edwards, R., 1998. Glutathione-mediated detoxification systems in plants. Curr. Opin. Plant Biol. 1, 258–266.
Feng, R., Wei, C., Tu, S., Tang, S., Wu, F., 2011. Detoxification of antimony by selenium and their interaction in paddy rice under hydroponic conditions. Microchem. J. 97, 57–61.
Filek, M., Keskinen, R., Hartikainen, H., Szarejko, I., Janiak, A., Miszalski, Z., Golda, A., 2008. The protective role of selenium in rape seedlings subjected to cadmium stress. J. Plant Physiol. 165, 833–844.
Gailer, J., 2009. Chronic toxicity of As(III) in mammals: the role of $(GS)2AsSe^-$. Biochimie 91, 1268–1272.
Gammelgaard, B., Jøns, O., 1999. Determination of selenium in urine by inductively coupled plasma mass spectrometry: interferences and optimization. J. Anal. At. Spectrom. 14, 867–874.
Ganther, H.E., 1968. Selenotrisulfides. Formation by the reaction of thiols with selenious acid. Biochemistry 7, 2898–2905.
Gill, S.S., Tuteja, N., 2010. Reactive oxygen species and antioxidant machinery in abiotic stress tolerance in crop plants. Plant Physiol. Biochem. 48, 909–930.
Grill, E., Winnacker, E.L., Zenk, M.H., 1987. Phytochelatins, a class of heavy-metal-binding peptides from plants, are functionally analogous to metallothioneins. Proc. Natl. Acad. Sci. U. S. A. 84 (2), 439–443.
Guo, J., Dai, X., Xu, W., Ma, M., 2008. Overexpressing GSH1 and AsPCS1 simultaneously increases the tolerance and accumulation of cadmium and arsenic in *Arabidopsis thaliana*. Chemosphere 72, 1020–1026.
Ha, S.B., Smith, A.P., Howden, R., Dietrich, W.M., Bugg, S., O'Connell, M.J., Goldsbrough, P.B., Cobbett, C.S., 1999. Phytochelatin synthase genes from Arabidopsis and the yeast *Schizosaccharomyces pombe*. Plant Cell 11, 1153–1163.
Hall, J.L., 2002. Cellular meachanisms for heavy metal detoxification and tolerance. J. Exp. Bot. 53, 1–11.
Hartkainen, H., Xue, T., Piironen, V., 2000. Selenium as an anti-oxidant and pro-oxidant in ryegrass. Plant Soil 225, 193–200.
Hartley-Whitaker, J., Ainsworth, G., Meharg, A.A., 2001. Copper- and arsenate-induced oxidative stress in *Holcus lanatus* L. clones with different sensitivity. Plant Cell Environ. 24, 713–722.
Hawrylak, B., Szymańska, M., 2004. Selenium as a sulphydrylic group inductor in plants. Cell Mol. Biol. Lett. 9, 329–336.
He, P.P., Lv, X.Z., Wang, G.Y., 2004. Effects of Se and Zn supplementation on the antagonism against Pb and Cd in vegetables. Environ. Int. 30, 167–172.
Howden, R., Cobbett, C.S., 1992. Cadmium-sensitive mutants of *Arabidopsis thaliana*. Plant Physiol. 100, 100–107.
Howden, R., Andersen, C.R., Goldsbrough, P.B., Cobbett, C.S., 1995a. A cadmium-sensitive, glutathione-deficient mutant of *Arabidopsis thaliana*. Plant Physiol. 107, 1067–1073.
Howden, R., Goldsbrough, P.B., Andersen, C.R., Cobbett, C.S., 1995b. Cadmium-sensitive, Cad 1 mutants of *Arabidopsis thaliana* are phytochelatin deficient. Plant Physiol. 107, 1059–1066.
Kneer, R., Zenk, M.H., 1992. Phytochelatins protect plant enzymes from heavy metal poising. Phytochemistry 31, 2663–2667.
Lindemann, T., Hintelmann, H., 2002. Identification of selenium-containing glutathione S-conjugatess in a yeast extract by two-dimensional liquid chromatography with inductively coupled plasma MS and nanoelectrospray MS/MS detection. Anal. Chem. 74, 4602–4610.
Liu, W.J., Wood, B.A., Raab, A., McGrath, S.P., Zhao, F.J., Feldmann, J., 2010. Complexation of arsenite with phytochelatins reduces arsenite efflux and translocation from roots to shoots in Arabidopsis. Plant Physiol. 152, 2211–2221.
Loeffler, S., Hochberger, A., Grill, E., Winnacker, E., Zenk, M.H., 1989. Termination of the phytochelatin synthase reaction through sequestration of heavy metals by the reaction product. FEBS Lett. 258, 42–46.
Maitani, T., Kubota, H., Sato, K., Yamada, T., 1996. The composition of metals bound to class III metallothionein (phytochelatin and its desglycyl peptide) induced by various metals in root cultures of *Rubia tinctorum*. Plant Physiol. 110, 1145–1150.
Malik, J.A., Goel, S., Kaur, N., Sharma, S., Singh, I., Nayyar, H., 2012. Selenium antagonises the toxic effects of arsenic on mungbean (*Phaseolus aureus Roxb.*) plants by restricting its uptake and enhancing the anti-oxidative and detoxification mechanisms. Environ. Exp. Bot. 77, 242–248.
Martin, A.L., 1936. Toxicity of selenium to plants and animals. Am. J. Bot. 23, 471–483.
Mascher, R., Lippmann, B., Holzinger, S., Bergmann, H., 2002. Arsenic toxicity: effects on oxidative stress response molecules and enzymes in red clover plants. Plant Sci. 163, 961–969.
Meinke, D., Koornneef, M., 1997. Community standards for Arabidopsis genetics. Plant J. 12, 247–253.
Meinke, D.W., Cherry, J.M., Dean, C., Rounsley, S.D., Koornneef, M., 1998. *Arabidopsis thaliana*: a model plant for genome analysis. Science 282, 679–682.
Memon, A.R., Aktoprakligil, D., Özdemir, A., Vertii, A., 2001. Heavy metal accumulation and detoxification mechanisms in plants. Turk. J. Bot. 25, 111–121.
Mezes, M., Balogh, K., 2009. Prooxidant mechanisms of selenium toxicity — a review. Acta Biol. Szeged. 53, 15–18.
Ogawa, S., Yoshidomi, T., Yoshimura, E., 2011. Cadmium(II)-stimulated enzyme activation of *Arabidopsis thaliana* phytochelatin synthase1. J. Inorg. Biochem. 105, 111–117.

Painter, E.P., 1941. The chemistry and toxicity of selenium compounds, with special reference to the selenium problem. Chem. Rev. 28, 179–213.

Raab, A., Feldmann, J., Meharg, A.A., 2004. The nature of arsenic-phytochelatin complexes in *Holcus lanatus* and *Pteris cretica*. Plant Physiol. 134, 1113–1122.

Raab, A., Schat, H., Meharg, A.A., Feldmann, J., 2005. Uptake, translocation and transformation of arsenate and arsenite in sunflower (*Helianthus annuus*): formation of arsenic-phytochelatin complexes during exposure to high arsenic concentrations. New Phytol. 168, 551–558.

Shanker, K., Mishra, S., Srivastava, S., Srivastava, R., Dass, S., Prakash, S., Srivastava, M.M., 1996. Study of mercury-selenium (Hg–Se) interactions and their impact on Hg uptake by the radish (*Raphanus sativus*) plant. Food Chem. Toxicol. 34, 883–886.

Sharma, S.S., Dietz, K., 2009. The relationship between metal toxicity and cellular redox imbalance. Trends Plant Sci. 14, 43–50.

Spain, S.M., Rabenstein, D.L., 2004. Characterization of the selenotrisulfide formed by reaction of selenite with end-capped phytochelatin-2. Anal. Bioanal. Chem. 378, 1561–1567.

Spallholz, J.E., 1994. On the nature of selenium toxicity and carcinostatic activity. Free Radic. Biol. Med. 17, 45–64.

Spallholz, J.E., 1997. Free radical generation by selenium compounds and their prooxidant toxicity. Biomed. Environ. Sci. 10, 260–270.

Spallholz, J.E., Hoffman, D.J., 2002. Selenium toxicity: cause and effects in aquatic birds. Aquat. Toxicol. 57, 27–37.

Srivastava, M., Ma, L.Q., Rathinasabapathi, B., Srivastava, P., 2009. Effects of selenium on arsenic uptake in arsenic hyperaccumulator *Pteris vittata* L. Bioresour. Technol. 100, 1115–1121.

Stohs, S.J., Bagchi, D., 1995. Oxidative mechanisms in the toxicity of metal ions. Free Radic. Biol. Med. 18, 321–336.

Sun, H.W., Ha, J., Liang, S.X., Kang, W.J., 2010. Protective role of selenium on garlic growth under cadmium stress. Commun. Soil Sci. Plant Anal. 41, 1195–1204.

Terry, N., Zayed, A.M., De Souza, M.P., Tarun, A.S., 2000. Selenium in higher plants. Annu. Rev. Plant Physiol. Mol. Biol. 51, 401–432.

Wood, B.A., Feldmann, J., 2012. Quantification of phytochelatins and their metal (loid) complexes: critical assessment of current analytical methodology. Anal. Bioanal. Chem. 402, 3299–3309.

Zenk, M.H., 1996. Heavy metal detoxification in higher plants — a review. Gene 179, 21–30.