Heliyon 6 (2020) e04703

Contents lists available at ScienceDirect

Heliyon

journal homepage: www.cell.com/heliyon

Research article

Major As species, lipid peroxidation and protein carbonylation in rice plants exposed to increasing As(V) concentrations

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ARTICLE INFO

Keywords:
Environmental science
Chemistry
Soil pollution
Environmental chemistry
Environmental pollution
Bioaccumulation
Agroecosystem arsenate
Arsenite
Toxicity
Rice
Oxidative stress
Protein oxidation

ABSTRACT

Arsenic (As) uptake by plants is mainly carried out as arsenate (As(V)), whose chemical analogy with phosphate is largely responsible for its elevated toxicity. Arsenate is known to stimulate reactive oxygen species (ROS) formation in plants that provoke oxidative stress. This manuscript reports the results of a hydroponics study using rice (*Oryza sativa* L.) seedlings as a test plant, where the effects of increasing arsenate concentrations (0–10 mg L^{-1}) on both lipid and protein oxidation, as well as As accumulation and speciation in plant roots and shoots were examined. Plant yield was negatively affected by increasing As concentration. Accumulation in plant roots was higher than in shoots at low arsenate doses (0.5–2.5 mg L^{-1}), while root to shoot transport was drastically enhanced at the highest doses (5 and 10 mg L^{-1}). Moreover, As(V) was the dominating species in the shoots and As(III) in the roots. Rice leaves in the 10 mg As L^{-1} treatment showed the highest lipid peroxidation damage (malondialdehyde concentration), whilst protein oxidation was not remarkably influenced by As dose. Lipid peroxidation seems to be therefore conditioned by As accumulation in rice plants, particularly by the presence of high As(V) concentrations in the aerial part of the plants as a consequence of unregulated translocation from roots to shoots above a threshold concentration (1.25–2.5 mg L^{-1}) in the growing media. These results provide relevant information regarding As(V) toxic concentrations for rice plants, highlight the importance of major As species analysis in plant tissues regarding As toxicity and contribute to better understand plants response to elevated As concentrations in the growing media.

1. Introduction

Arsenic (As) is a ubiquitous toxic metalloid for which concern about its possible chronic and epidemic effects on human health has recently increased (Rahman et al., 2007; Singh et al., 2015). The entrance of As into human beings occurs primarily from food and water. Arsenic contamination in drinking water (>10 μg L^{-1} according to the World Health Organization) threatens directly more than 150 million people all over the world, most of whom live in South Asia (Singh et al., 2015).

Environmental contamination with As occurs through both geogenic sources and anthropogenic activities (Xu et al., 2007). Groundwater contamination is commonly a consequence of As release from solid phases into pore water under anaerobic conditions (Polizzotto et al., 2008). But, in addition to the natural occurrence of As, indiscriminate use of arsenical pesticides, pumping contaminated groundwater for irrigation or mining activities have caused the presence of elevated As concentrations in soil and water (Manirul et al., 2016; Wang et al., 2010), mainly in the form of As(V) in aerobic conditions (Wenzel, 2013). The widespread use of As contaminated groundwater for irrigation has led to the contamination of agricultural soils, from where it can be then taken up by crop plants and enter the food chain through a water-soil-plants (edible parts) pathway (Arco-Lázaro et al., 2018; Manirul et al., 2016; Shri et al., 2009).

Arsenic can be found in both inorganic (arsenite and arsenate) and organic (methylated) forms in anaerobic and aerobic soil/water environments (Liu et al., 2004; Wang et al., 2010). In soils, inorganic As species are the predominant forms, as well as the most phytoavailable and, therefore, toxic species (Panda et al., 2010). Under soil oxidizing conditions, arsenate (As(V)) is the predominant form of As and is less mobile and toxic than As(III) because it is strongly sorbed to Fe (hydr) oxides of soil (Honma et al., 2016). Only in non-aerated waterlogged soils, arsenite (As(III)) becomes the predominant species because of the reducing anaerobic conditions, and cultivation under oxidizing aerobic

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https://doi.org/10.1016/j.heliyon.2020.e04703
Received 28 March 2020; Received in revised form 8 May 2020; Accepted 10 August 2020

conditions has been recommended in fields suffering from As contamination (Honma et al., 2016; Panda et al., 2010; Yamaguchi et al., 2011).

Nevertheless, As is taken up by plants mainly as arsenate (Garg and Singla, 2011), as it is a chemical analogue to phosphate and finds its way into plants through phosphate transporters (PHT), which are strongly expressed in roots and are also normally involved in root-to-shoot As(V) transport (Wang et al., 2015). Plants accumulate As primarily into their roots and subsequently, translocate it to the above-ground parts (Rahman et al., 2008). Once inside root cytoplasm, As(V) is detoxified through rapid reduction to As(III) by arsenate reductase (AR) using glutathione (GSH) as a reductant. Then, As(III) is complexed with thiol-rich peptides, such as phytochelatins (PC), and sequestered in the vacuoles (Tripathi et al., 2007).

Arsenate toxicity, as well as other inorganic contaminants or environmental stresses, has been proved to stimulate reactive oxygen species (ROS) formation (such as $O_2^{\cdot-}$, $OH^{\cdot}$, H_2O_2), and an imbalance between their production and scavenging will cause oxidative stress damage to DNA, carbohydrates, lipids and proteins (Abbas et al., 2018; Hartley-Whitaker et al., 2001; Sharma et al., 2012). In addition, ROS levels, through linking to redox proteins like thioredoxins, peroxiredoxins, and glutaredoxins, may perturb redox balance upon stress, which in turn activates downstream ROS signaling (Sevilla et al., 2015; Sewelam et al., 2014). Arsenic-induced ROS formation might occur through electron leakage during As(V) reduction to As(III) (Meharg and Hartley-Whitaker, 2002). The oxidation of lipids is known to be one of the most damaging processes that As can provoke in plants, mainly because of the formation of lipid peroxidation byproducts, like malondialdehyde (MDA), which can conjugate with both DNA and proteins (Tripathi et al., 2007). In addition, protein's activity is affected following amino acids oxidation and free carbonyl groups formation (Abbas et al., 2018). Unlike antioxidant enzymes like superoxide dismutase (SOD) or catalase (CAT), whose concentration increases as a response to trace element stress, MDA concentration decreases when the toxicity caused by trace element induced ROS is decreased (Pandey and Bhatt, 2016; Pramanik et al., 2017, 2018). For that reason, quantifying MDA or oxidized proteins can be considered as a useful As toxicity biomarker (Dave et al., 2013).

However, relating the toxic effects of As with its chemical forms inside the different parts of the plant is not always possible. As plants exposed to elevated levels of toxicity are normally severely damaged and do not grow well, the comparison with healthy plants is often not conclusive. This limitation might be overcome studying wide gradients of As concentration and toxicity in the growing media. Arsenic uptake and accumulation mechanisms have been widely studied in rice (*Oryza sativa* L.) plants (Abbas et al., 2018; Abedi and Mojiri, 2020). Arsenic (V) is the major form entering the plants (Garg and Singla, 2011) and the dominating species in well aerated and oxidized conditions (Wenzel, 2013), and may provoke higher toxic effects (Shakoor et al., 2019) than As(III). However, As toxicity when provided to the growing media in the oxidized (As(V)) form has been scarcely studied.

Therefore, the aim of the study was to evaluate the effects of increasing As(V) concentrations on oxidative stress parameters, and their relation with major As chemical forms in rice plants grown in hydroponic conditions. We hypothesized that this would provide original and useful information regarding the response of the plants to a gradient of toxicity (increasing As(V) concentrations in the growing media) from a broader non-mechanistic point of view, compared to conventional As(III) and fixed concentration experiments reported till date. This approach may also allow relating the toxic effects with As accumulation and speciation in the plants. With this aim, lipid peroxidation and protein carbonylation in plant leaves, together with As and its major chemical forms concentration in the plants were determined. The characteristics of the nutrient solution were first optimized in a preliminary study.

2. Materials and methods

2.1. Optimization of the experimental conditions

A preliminary hydroponics experiment (Exp. 1) was carried out in order to establish the most favorable conditions for the development of the main experiment (Exp. 2). Rice seeds (*Oryza sativa* L. cv. J SENDRA) were surface sterilized in 10 % NaClO followed by thorough washing with deionized water, and then were wrapped in moistened ashless filter paper for germination in the darkness. After 5 days, plant seedlings were transferred to 1.7 L opaque plastic hydroponic pots (15 cm × 15 cm, 10–12 plants per pot) and grown in 'half-strength' modified Hoagland nutrient solution (Table 1) for three weeks to develop an adequate size for the preliminary experiment. After that, only five uniform size seedlings were left in the individual pots and the treatments were initiated.

From that moment and until the end of Exp. 1, pots were filled with 1.5 L of 'full-strength' modified Hoagland nutrient solution (Table 1). The nutrient solution was renewed in the pots weekly and continuously aerated to prevent anoxic conditions in the growing medium.

Different pH values (5, 6 and 7) were tested every 2–3 days and adjusted weekly (if necessary) using 0.5 M NaOH or 0.5 M HCl on the different treatments. This pH range (5–7) is not expected to affect plant growth but may possibly alter As forms in the growing solution and plant uptake. In order to simulate soil conditions and to elucidate any possible influence of organic matter (OM) on As chemical forms and availability, water extracts (1:10 w/v) of a mature olive-mill waste compost were added as a source of dissolved organic carbon (DOC, 4.13 g L^{-1}). Two different rates (100 and 235 mg DOC L^{-1}) were tested to simulate low and high DOC concentrations in soil solution, respectively, according to the values previously found in compost amended soils (Pardo et al., 2014; Arco-Lázaro et al., 2018). Arsenic was added in the form of $Na_2HAsO_4{\cdot}7H_2O$ (Sigma-Aldrich), as acquired commercially, in order to obtain a concentration of 1 mg As L^{-1} in the growing media. Seven treatments (with three replicates) were tested in this preliminary experiment (Exp. 1), distributed in a completely randomized design:

(i) Control: pH 6.0;
(ii) Control-OM: pH 6.0, 100 mg L-1 DOC;
(iii) pH5-As: pH 5.0, 1 mg L-1 As;
(iv) pH6-As: pH 6.0, 1 mg L-1 As;
(v) pH7-As: pH 7.0, 1 mg L-1 As;
(vi) pH6-OM-As: pH 6.0, 100 mg L-1 DOC, 1 mg L-1 As;
(vii) pH6-OM2-As: pH 6.0, 235 mg L-1 DOC, 1 mg L-1 As.

The experiment was run in a growth chamber with a standard 12 h day/night cycle of 25/18 °C temperature and 58/70 % relative humidity,

Table 1. Composition of the modified Hoagland nutrient solution used in Exp. 1 and Exp. 2 (based on Rivera, 2014).

Nutrient	Concentration
KNO_3	1.50 mM
$Ca(NO_3)_2$	1.28 mM
$MgSO_4$	0.37 mM
KH_2PO_4	0.17 mM
NaCl	0.15 mM
Fe-EDDHA	24.7 μM
H_3BO_3	16.7 μM
$MnSO_4$	2.37 μM
$ZnSO_4$	0.92 μM
$CuSO_4$	0.63 μM
$(NH_4)_6Mo_7O_{24}$	0.63 μM

respectively. The length of the aerial part of the plants was measured after 0, 7 and 21 days of exposure to the different treatments. Individual roots were marked with a permanent marker 1 cm above the tip at day 0 and the length increase was measured at day 2 and 7 of the experiment. Rice plants were finally harvested after 21 days of treatment exposure. Shoots and roots were separated, weighed (fresh weight), thoroughly rinsed first with tap and then with deionized water, oven-dried (65 °C) until constant (dry) weight (48 h) and finally ground to a fine powder in an electric mill (A10 IKA-Labortechnik, Staufen, Germany).

2.2. Rice-As accumulation experiment (Exp. 2)

Rice seeds were germinated as in Exp.1 and grown in this case for two weeks in half-strength nutrient solution (as described in 2.1) to achieve an adequate size for the development of the main experiment (Exp. 2). Then, twenty plants of uniform size were placed in each pot (1.5 L) and the different treatments applied. Full-strength modified Hoagland nutrient solution was also used in Exp. 2, with the pH value and DOC concentration fixed at 6.0 and 100 mg L^{-1} for all treatments, respectively, as optimized in Exp. 1. For the increasing dose treatment, As was added (as $Na_2HAsO_4{\cdot}7H_2O$) to final concentrations of:

(i) 0 mg As L^{-1} (Control);
(ii) 0.5 mg As L^{-1};
(iii) 1.25 mg As L^{-1};
(iv) 2.5 mg As L^{-1};
(v) 5.0 As mg L^{-1};
(vi) 10.0 mg As L^{-1}.

The growing conditions and the treatments exposure time were the same as in Exp. 1. The length of plants shoots and roots were equally determined throughout the experiment, and finally harvested and weighed fresh as in Exp. 1. However, in Exp. 2 part of the plants (around 50 %, both roots and shoots) was immediately frozen in liquid N_2 (after washing with deionized water) for further analyses, while the rest was processed as in Exp. 1.

2.3. Analytical methods

Total organic carbon (TOC) and total nitrogen (TN) concentrations were determined in an automatic microanalyser (EuroEA3000, Eurovector, Milan, Italy). Trace element and nutrient concentrations were determined in dried plant materials by ICP-OES (ICP-OES; ICAP 6500 DUO + ONE FAST, Thermo Scientific, Waltham, MA USA) after microwave (ETHOS1, Milestone, Sorisole, Italy) assisted acid digestion with H_2O_2/HNO_3 (1:4 v/v)); the analytical accuracy was checked with a certified reference material (NCS DC 73349).

Frozen shoots and roots samples from Exp. 2 were rapidly ground in a mortar with liquid N_2 and individual aliquots (0.2–0.5 g) were extracted in duplicate with 20 ml of phosphate-buffered saline (PBS; 2 mM NaH_2PO_4 and 0.2 mM Na_2-EDTA, pH 6.0) for 1 h under sonication (Ultrasons Medi, JP Selecta, Barcelona, Spain). The extracts were then filtered through 0.45-μm nylon filters before being analyzed for As speciation (determination of major As species: As(III), As(V), monomethylarsinic acid (MMA) and dimethylarsinic acid (DMA)) using high performance liquid chromatography coupled to an atomic fluorescence spectrophotometer (HPLC-AFS, Millennium Excalibur, PSAnalytical, Orpington, UK) as described in Xu et al. (2007).

Rice shoots from experiment 2 were analyzed for oxidative stress related parameters through the determination of malondialdehyde (MDA, lipid peroxidation) and carbonyl proteins (protein oxidation) concentration. Briefly, frozen ground samples were homogenized in a mortar using 50 mM extraction phosphate buffer at pH 7.8 (2g/4ml) as described by Camejo et al. (2007). The extent of lipid peroxidation in the shoots (leaves) was estimated measuring the concentration of MDA in the extracts (Martí et al., 2009), while carbonyl protein content was measured by reaction with 2,4-dinitrophenylhydrazine (DNPH), as described by Levine et al. (1990). The results of protein carbonylation were referred to total protein concentration, for what soluble total proteins concentrations were measured according to the protein dye-binding method (Bradford, 1976), using bovine serum albumin (BSA Fraction V, Roche) as calibration standard.

Nutrient solution samples from Exp. 1 were analyzed for major As chemical species through HPLC-AFS, which confirmed that As(V) had not been reduced to As(III) and arsenate was the only species present in the growing solution. pH was also directly analyzed in the different nutrient solutions and growing media using a Crison BasiC 20 pH-meter. Dissolved organic carbon (DOC) content in the mature compost extract was determined in an automatic analyzer for liquid samples (TOC-V CSN + TNM-1 Analyser, Shimadzu, Tokyo, Japan), after nylon-membrane (0.45 μm) filtration.

2.4. Statistical analysis

The statistical data analysis was performed using IBM SPSS Statistics Version 24.0 software (IBM Corporation, New York, USA). The analysis of variance (ANOVA), followed by Tukey's HSD test, was carried out to assess the significant differences among treatments ($P < 0.05$). A Principal Component Analysis (PCA) was run considering all the determined parameters to reveal general tendencies. The chemical forms of the trace elements (apart from As) present in the nutrient solution in Exp. 1 were estimated using full characterization data (pH, and organic-C, total-N, nutrient, trace element and dissolved anions concentrations) in an equilibrium speciation model using the software Visual MINTEQ 3.0, in order to assess any possible modification of relevance in the growing media.

3. Results and discussion

3.1. Response of the plants at different pH and OM concentration

No significant differences in shoots length were observed among treatments throughout Exp. 1 (Figure 1). However, at the end of the experiment shoots from pH 6-OM-As treatment were on average shorter than those from pH 5-As treatment and, especially, than those in the controls with no As. In any case, only plants from pH 7-As treatment showed significantly lower dry weight production (0.139 ± 0.017 g per pot) than the controls (0.249 ± 0.041and 0.260 ± 0.041 g per pot with and without OM, respectively; Table 2).

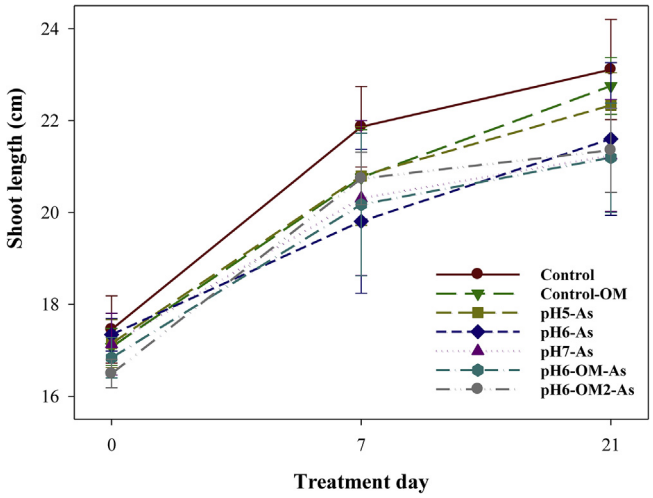

Figure 1. Length of the aerial part (mean ± SE) of *O. sativa* plants at the beginning (day 0), after one week (day 7) and at the end (day 21) of treatments exposure in **Experiment 1**.

Table 2. Dry weight (g per pot) and concentration (g kg^{-1}) of nutrients in the plants from **Experiment 1** (mean ± SE).

		DW	N	P	K	Ca	Mg
Shoots	Control	0.260 ± 0.041a	41.9 ± 1.8	6.69 ± 0.17	39.19 ± 0.83	9.172 ± 0.076a	5.69 ± 0.26a
	Control-OM	0.249 ± 0.041ab	44.1 ± 2.8	7.02 ± 0.26	39.6 ± 1.6	6.23 ± 0.79abc	5.10 ± 0.22ab
	pH5-As	0.1950 ± 0.0049abc	37.9 ± 1.1	6.24 ± 0.31	41.47 ± 0.76	6.72 ± 0.30abc	4.669 ± 0.052bc
	pH6-As	0.160 ± 0.025bc	37.9 ± 1.3	7.27 ± 0.40	41.36 ± 0.99	8.61 ± 0.82ab	4.85 ± 0.19ab
	pH7-As	0.139 ± 0.017c	38.6 ± 1.7	6.754 ± 0.096	40.7 ± 1.2	7.9 ± 1.2ab	5.38 ± 0.26ab
	pH6-OM-As	0.189 ± 0.028abc	42.35 ± 0.28	7.41 ± 0.34	43.8 ± 1.8	5.99 ± 0.50bc	4.86 ± 0.16ab
	pH6-OM2-As	0.1533 ± 0.0090bc	40.5 ± 2.0	7.02 ± 0.32	42.7 ± 1.8	4.36 ± 0.17c	3.85 ± 0.14c
	ANOVA	*	NS	NS	NS	**	***
Roots	Control	0.096 ± 0.011	24.8 ± 1.1	3.90 ± 0.22	14.6 ± 1.8ab	4.258 ± 0.024	2.058 ± 0.091
	Control-OM	0.119 ± 0.020	29.5 ± 1.6	3.82 ± 0.36	17.4 ± 1.8a	3.78 ± 0.42	1.933 ± 0.084
	pH5-As	0.071 ± 0.023	23.78 ± 0.63	3.20 ± 0.17	11.29 ± 0.42ab	3.65 ± 0.16	1.68 ± 0.15
	pH6-As	0.079 ± 0.012bc	21.45 ± 0.78	3.11 ± 0.23	8.75 ± 0.43b	4.17 ± 0.67	1.780 ± 0.020
	pH7-As	0.1103 ± 0.0095	22.15 ± 0.75	3.22[1]	8.70[1]	3.69[1]	1.96[1]
	pH6-OM-As	0.0841 ± 0.0051	24.55 ± 0.75	3.08[1]	12.43[1]	3.91[1]	1.55[1]
	pH6-OM2-As	0.0926 ± 0.0029	26.0 ± 3.5	3.34[1]	11.09[1]	4.06[1]	1.51[1]
	ANOVA	NS	NS	NS	*	NS	NS

*, **, ***: significant at $P < 0.05$, 0.01 and 0.001, respectively. NS: not significant. Values followed by the same letter in each column and for each plant part do not differ significantly according to Tukey's test ($P < 0.05$).
[1] Single replicate sample.

Arsenic concentration was below detection limits (1 mg kg^{-1}, plant dry weight) in control treatment shoots, both with and without OM addition, and was between 20-40 mg kg^{-1} in plant shoots from all the As treatments (Table 3), with no significant differences between samples from the different pH and OM concentration treatments. Arsenic concentrations in the roots (100–180 mg kg^{-1}; Table 3) were higher than in the shoots; this indicates a low rate of translocation. Shaibur et al. (2006) reported that As was mostly accumulated in the roots and a reduced translocation rate in rice plants grown hydroponically in the presence of 13.4 μmol L^{-1} As(III). These authors also suggested that the elevated levels of As found in the roots (>50 mg kg^{-1}) were a consequence of the strong adsorption of As(V) on the membrane surface of the roots.

Phosphorous concentrations in the shoots were within a rather narrow range (6.2–7.4 mg kg^{-1}) and did not show significant differences between the different treatments (Table 2). This suggested that As presence did not affect P concentration in the aerial parts, probably because no competition for the same transporters took place in the plants at low As concentration, which agrees with the results reported by Shaibur et al. (2016) in the xylem tubes of rice. However, significant negative correlations were found between P concentration in the roots and those of As in both roots ($r = -0.713$; $P < 0.05$) and the aerial part ($r = -0.761$; $P < 0.01$), which indicates competition between phosphate and arsenate for the adsorption sites on the roots of the plants (Pardo et al., 2016). It is interesting to notice that Fe concentrations in roots exposed to DOC were lower than those in the treatments with no DOC added, which may indicate Fe-organic matter chelation in the nutrient solution (>99 % Fe in the solution was associated with DOC in both DOC treatments according to the speciation model; results not shown). However, this did not affect Fe shoot concentration.

Due to the scarce differences between the different conditions studied, intermediate conditions (pH 6.0 and 100 mg L^{-1} DOC) were selected for the development of Exp. 2.

3.2. Effects of increasing As concentrations on the plants

3.2.1. Plant growth and As accumulation

Plants exposed to 0.5 mg As L^{-1} showed the highest shoot (28.150 ± 0.029) and root (11.1 ± 1.3) length (cm) and significant differences in length increase with the rest of the treatments were found during all the exposure time (Figure 2). This seems to indicate that low concentrations of As did not provoke toxic effects in rice plants, but slightly stimulated

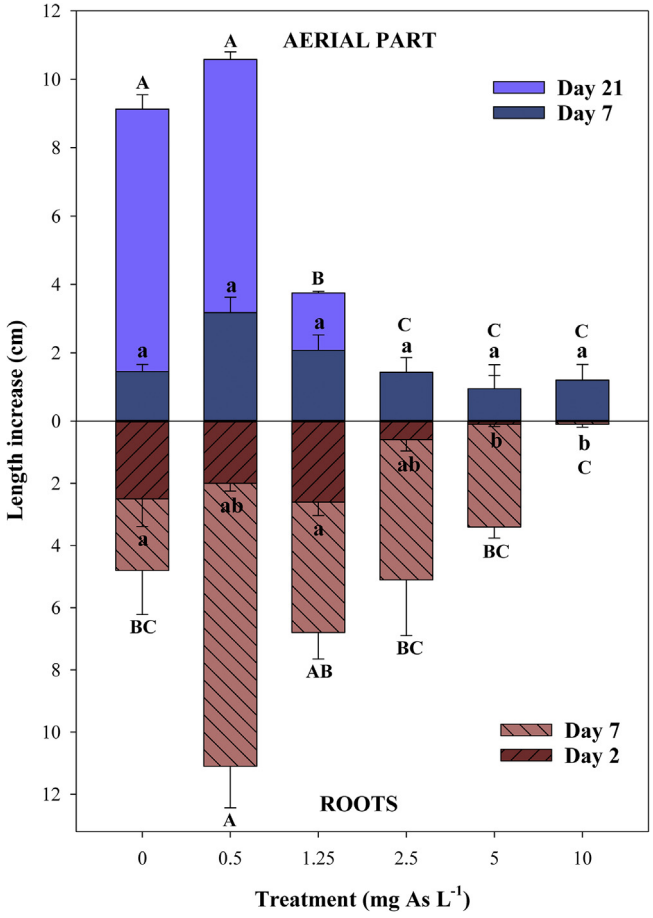

Figure 2. Length increase of the aerial part and roots (mean ± SE) of *O. sativa* plants, after 7 and 21 days and after 2 and 7 days of treatments exposure, respectively, from **Experiment 2**. Bars marked with the same letter (upper-case for days 21 and 7, and lower case for days 7 and 2 for aerial part and roots, respectively) do not differ significantly according to Tukey's test ($P < 0.05$).

Table 3. Concentration (mg kg^{-1}, DW) of micronutrients and As in the plants from **Experiment 1** (mean ± SE). bdl: below detection limit.

	Treatment	As	Cu	Fe	Mn	Zn
Shoots	Control	bdl	26.47 ± 0.78a	243 ± 57	1241 ± 88a	630 ± 105
	Control-OM	bdl	26.9 ± 1.1a	146 ± 18	1007 ± 108ab	486 ± 26
	pH5-As	26.7 ± 2.9	16.46 ± 0.25c	229 ± 55	1153 ± 168a	492 ± 15
	pH6-As	39.2 ± 6.4	18.3 ± 1.7c	133 ± 17	1197 ± 55a	608 ± 56
	pH7-As	28.3 ± 6.9	18.10 ± 0.52c	218.8 ± 6.8	1220 ± 59a	529 ± 55
	pH6-OM-As	30.3 ± 6.3	24.1 ± 1.7ab	127 ± 12	1076 ± 38a	472 ± 35
	pH6-OM2-As	20.1 ± 6.6	21.15 ± 0.68bc	137 ± 27	609 ± 34bc	417 ± 15
	ANOVA	NS	***	NS	**	NS
Roots	Control	7.82 ± 0.22b	96.0 ± 3.1	3441 ± 184a	137 ± 33	702 ± 171
	Control-OM	4.77 ± 0.18b	66.2 ± 1.4	1502 ± 250b	121 ± 27	468 ± 60
	pH5-As	184 ± 24a	71.0 ± 6.9	3720 ± 488a	171 ± 11	646 ± 98
	pH6-As	156 ± 19a	56 ± 13	3192 ± 160ab	202 ± 15	708 ± 149
	pH7-As[1]	142	68.8	2647	228	876
	pH6-OM-As[1]	101	54.6	973	140	517
	pH6-OM2-As[1]	101	41.5	832	123	509
	ANOVA	**	NS	*	NS	NS

*, **, ***: significant at $P < 0.05$, 0.01 and 0.001, respectively. NS: not significant. Values followed by the same letter in each column and for each plant part do not differ significantly according to Tukey's test ($P < 0.05$).

[1] Single replicate sample due to low biomass.

plant growth. Shoot length was strongly affected at the end of the experiment in the treatments with 2.5, 5.0 or 10.0 mg As L^{-1} in the nutrient solution (Figure 2). Root growth was also negatively affected by high As concentrations (above 2.5 mg L^{-1}) and almost completely inhibited at 10.0 mg As L^{-1} exposure (Figure 2). Similarly, Abedin et al. (2002) observed that plant height in rice decreased significantly with increasing As(V) concentration in irrigation water (above 2.0 mg As L^{-1}) in a greenhouse soil pot experiment. In addition, As(V) toxicity was tested in different rice varieties by Abedin and Meharg (2002) in an early seedling growth experiment and resulted in a significant reduction in root length when the plants were exposed to concentrations within the range 2.0–8.0 mg As L^{-1}.

Dry weight results were in good agreement with those of plant length, following a very similar trend (Figure 3), with the highest shoots and roots weight in the 0.5 mg As L^{-1} treatment, which decreased significantly when As dose was above 1.25 mg L^{-1} ($P < 0.001$). Dry weight reduction in rice plants due to As(III) toxicity has been previously reported (Shaibur et al., 2016; Wang et al., 2010), and toxic effects have been found in rice seedlings grown in nutrient solutions with low As(III) concentrations (≤0.5 mg L^{-1}; Shaibur et al., 2006).

Phosphorus concentrations in the roots decreased significantly with increasing As concentration (Table 4). This is likely a consequence of As being preferentially uptaken using phosphate carriers in the root plasmalemma, as plants have developed As(V) resistance suppressing those transporters to reduce As influx into the roots, and this may also affect phosphorus concentrations in plant roots (Meharg and Hartley-Whitaker, 2002; Abedi and Mojiti, 2020). Contrastingly, P concentrations in the shoots were only significantly decreased in the high As treatments (>5 mg L^{-1}) compared to the low As ones (<2.5 mg L^{-1}; Table 4). Phosphorus has higher mobility regarding translocation in comparison with As, except for hyperaccumulators (Zhao et al., 2009), and this seems to allow plants to maintain proper P levels on the aerial parts at low As doses. This indicates that phosphate transporters involved in translocation into aerial parts were not significantly affected at low As doses (as most As was found as As(III) in the roots while the remaining As(V) is root-to-shoot transported with no interference with P). Similarly, the concentrations of K decreased significantly in roots with increasing As dose treatment ($r = -0.737$; $P < 0.01$), showing some kind of interaction between the presence of As in the growing media and K absorption by the plants, although K accumulation in the shoots was not affected (Table 4). Similarly, Tu and Ma (2005) reported that As-induced an increase in P

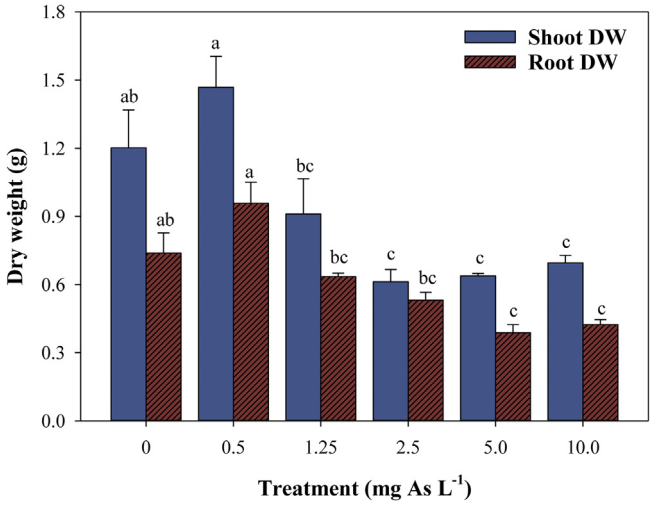

Figure 3. Dry weight (g per pot) of shoots and roots (mean ± SE) of As treated *O. sativa* plants from **Experiment 2**. Bars of each plant part marked with the same letter do not differ significantly according to Tukey's test ($P < 0.05$).

and K concentrations in the fronds of the As hyperaccumulator *Pteris vittata* at low As(V) levels in the soils (5–30 mg kg^{-1}). Regarding N and Mg their concentrations were significantly decreased with increasing As concentrations, this being more relevant in the roots than in the aerial parts, and stronger at higher As concentrations (Table 4). In a previously reported hydroponic experiment with rice, Mg concentrations in shoots and roots decreased at As(III) concentrations above 1 mg L^{-1} in the growing medium (Shaibur et al., 2006), and was assumed to be a consequence of the toxic effect of As on the nutritional status of the plant. In the case of micronutrients, only Cu concentrations decreased significantly with increasing As dose in both roots and shoots, while Mn concentrations increased in the roots in the treatments with the highest As concentrations (>5 mg l^{-1}; Table 4). Iron concentrations fluctuated in the different treatments and showed a tendency to increase with As presence in the growing media, especially in plant roots. Shaibur et al. (2006) also indicated that As (1–2 mg L^{-1}) provoked the retention of Fe in the roots and blocked Fe translocation to shoots. Increased Fe and Mn concentrations in rice roots with increasing As dose could be a

Table 4. Macro and micronutrient concentrations (mean ± SE) in rice plants exposed to different As treatments from **Experiment 2** and Pearson correlation coefficient between As concentration in the nutrient solution and nutrient/trace element concentration in the plants.

	As dose (mg L^{-1})	N (g kg^{-1})	P (g kg^{-1})	K (g kg^{-1})	Ca (g kg^{-1})	Mg (g kg^{-1})	Cu (mg kg^{-1})	Fe (mg kg^{-1})	Mn (mg kg^{-1})	Zn (mg kg^{-1})
Shoots	Control	42.1 ± 1.3ab	6.68 ± 0.14ab	39.8 ± 1.2b	9.516 ± 0.203ab	7.99 ± 0.13a	28.383 ± 0.901a	114.6 ± 4.5b	396 ± 29bc	480 ± 30ab
	0.5	44.60 ± 0.71ab	7.40 ± 0.42a	41.24 ± 0.27ab	8.4148 ± 0.4998bc	7.36 ± 0.15ab	25.2 ± 1.2ab	125 ± 16b	339 ± 32c	404 ± 33b
	1.25	40.3 ± 1.8b	7.93 ± 0.76a	41.37 ± 0.66ab	11.11 ± 0.56a	7.084 ± 0.403bc	23.2 ± 1.56b	291 ± 19a	504 ± 40ab	600 ± 81a
	2.5	46.2 ± 1.2a	7.33 ± 0.19a	47.3470 ± 0.9001a	8.032 ± 0.309bc	6.212 ± 0.088cd	17.70 ± 0.46c	132 ± 10b	478 ± 41ab	392 ± 30b
	5	40.2 ± 1.4b	5.42 ± 0.11bc	43.3 ± 1.2ab	9.49 ± 0.33ab	6.07 ± 0.25d	15.49 ± 0.29c	134.8 ± 6.5b	537 ± 18a	514 ± 18ab
	10	31.26 ± 0.94c	4.45 ± 0.46c	41.6 ± 2.9ab	7.59 ± 0.43c	4.93 ± 0.38e	13.67 ± 1.07c	140.9 ± 5.5b	421 ± 17abc	444 ± 20ab
	ANOVA	***	***	*	***	***	***	***	**	*
	Pearson	-0.789**	-0.755***	-	-	-0.867***	-0.828***	-	-	-
Roots	Control	26.97 ± 0.48a	3.3288 ± 0.0895a	26.84 ± 0.94a	2.98 ± 0.17cd	2.70 ± 0.08a	45.0 ± 2.1a	385 ± 12b	54.2 ± 3.2cd	314 ± 45
	0.5	26.06 ± 0.61ab	2.815 ± 0.074b	26.08 ± 0.45a	2.56 ± 0.12d	2.69 ± 0.08a	35.0 ± 1.2b	374 ± 12b	36.7 ± 1.3d	222 ± 17
	1.25	22.73 ± 0.21bc	2.4137 ± 0.0599c	14.96 ± 0.55b	3.27 ± 0.17bc	2.14 ± 0.12b	32.53 ± 0.79bc	475 ± 43ab	60.7 ± 4.8bc	210 ± 38
	2.5	22.84 ± 0.33bc	2.177 ± 0.071c	10.14 ± 0.49cd	3.698 ± 0.074ab	1.77 ± 0.10c	28.56 ± 0.96cd	578.2 ± 8.2a	80.5 ± 5.4b	279 ± 14
	5	21.1 ± 1.8c	1.676 ± 0.071d	6.79 ± 0.17d	4.137 ± 0.094a	1.37 ± 0.01d	23.3 ± 1.5d	612 ± 58a	103.8 ± 10.1a	336 ± 75
	10	21.00 ± 0.64c	1.627 ± 0.057d	8.24 ± 0.29cd	4.03 ± 0.21a	1.40 ± 0.06d	23.6 ± 1.4d	580 ± 29a	106.6 ± 2.9a	249 ± 13
	ANOVA	**	***	***	***	***	***	***	***	NS
	Pearson	-0.696**	-0.824***	-0.737**	0.693**	-0.800***	-0.747***	-	0.792***	-

*, **, ***: significant at $P < 0.05$, 0.01 and 0.001, respectively. NS: not significant. Values followed by the same letter in each column and for each plant part do not differ significantly according to Tukey's test ($P < 0.05$).

consequence of Fe plaque formation on the plants roots as a way to retain As and avoid its transport to the aerial part of the plant (Pardo et al., 2016: Shaibur et al., 2015). This has been considered to be a defense mechanism of the plants to prevent As toxic effects (Moreno-Jiménez et al., 2012; Wang et al., 2015). In fact, As(V) has shown to be strongly retained on the Fe plaque when this is formed in rice seedlings, while As(III) is mostly retained in the plant roots (Liu et al., 2005).

Total As concentrations significantly increased in plants shoots and roots with increasing As dose, reaching very high values (>600 mg kg^{-1}) and showing concentrations within the same range in both plant parts in the treatments with the highest concentrations (>5 mg kg^{-1}; Figure 4). This seems to point out that As absorption and further transport from roots to the aerial parts was not strongly inhibited in the plants in the presence of high As concentrations in the growing media. This indicates that As tolerance mechanisms in the plant have been surpassed at the highest As concentrations and that As is therefore being translocated in a passive way (Pardo et al., 2014.). Plants have been reported to contain both high- and low-affinity P transporters (Abbas et al., 2018) and, in the presence of the highest As levels, it seems that phosphate transporters showed low selectivity towards phosphorus and As transport was not inhibited. Consequently, shoot to root As ratio was higher in plants from Exp. 2 (0.2 in Exp. 1 and 0.4–1 in Exp.2), which was concomitant to a lower shoot to root dry weight ratio (impaired plant growth) in the second experiment (2 in Exp. 1 and 1.2–1.6 in Exp. 2). Regarding As speciation in the plants, only As(V) and As(III) were detected, but a low extraction efficiency (compared to the determined total concentrations) was observed (3–18 % extraction for shoots and 6–10 % for roots). Both As(III) and As(V) concentrations were higher in the roots than in the aerial part of the plants, with the only exception of As(V) in the highest doses treatments (>5 mg As L^{-1}), for which the concentration was higher in the shoots than in the roots (Figure 4). As(V) might be rapidly reduced to As(III) and retained in plant roots, likely complexed with thiol groups (Moreno-Jiménez et al., 2012; Zhao et al., 2009). But at high (toxic) As concentrations, As(V) seemed to saturate arsenate reductase in root cytoplasm, a crucial enzyme in As metabolism, resulting in a higher translocation to the aerial parts of the plants (Tripathi et al., 2007). Membrane composition and functionality might be disrupted after exposure to toxic As concentrations in the xylem (Shri et al., 2009). Therefore, plant cells were not able to completely control As transport,

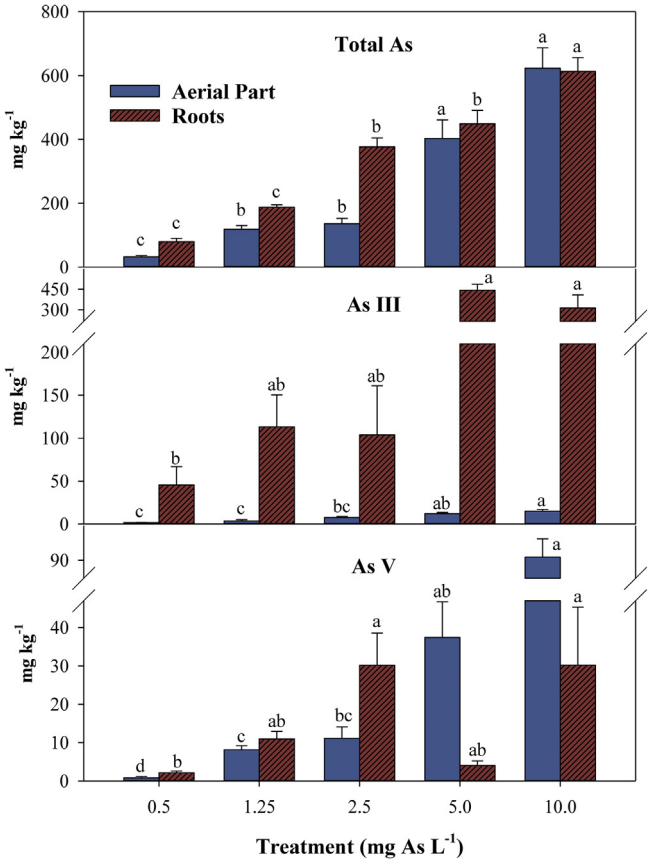

Figure 4. Concentration (mg kg^{-1}) of major As species (mean ± SE) in plant shoots and roots from **Experiment 2**. DMA and MMA were below the detection limit in all the samples. Bars of each plant part marked with the same letter do not differ significantly according to Tukey's test ($P < 0.05$).

and translocation to the aerial parts was increased or facilitated (Figure 4). This enhanced transport to the aerial part may help to explain why As-related toxic effects take place above certain threshold

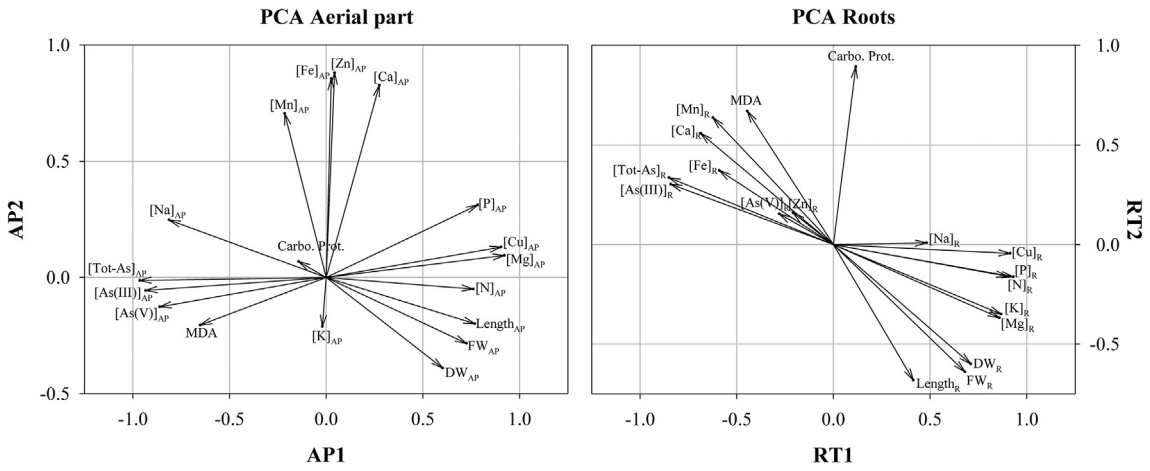

Figure 5. PCAs of *O. sativa* plants data (aerial part, left; roots, right) from **Experiment 2**.

Table 5. Malondialdehyde (MDA) (μmol g^{-1} FW) and carbonyl proteins (nmol mg^{-1} protein) in shoot samples (mean ± SE).

Treatment	MDA	Carbonyl proteins
Control	0.738 ± 0.097ab	2.25 ± 0.23a
0.5	0.550 ± 0.051b	1.115 ± 0.082b
1.25	0.681 ± 0.042ab	1.71 ± 0.37ab
2.5	0.852 ± 0.097ab	1.72 ± 0.17ab
5	0.887 ± 0.062ab	1.88 ± 0.25ab
10	0.9666 ± 0.0048a	2.03 ± 0.58ab
ANOVA	*	*
Pearson	0.554*	-

*: significant at $P < 0.05$. Values followed by the same letter in each column do not differ significantly according to Tukey's test ($P < 0.05$).

concentrations (between 0.5 and 1.25 mg L^{-1} according to our results) in the growing media.

Arsenic species differ in their mobility through rice plant parts, which is greater for the organic (DMA, MMA) than for the inorganic species (Awasthi et al., 2017). Moreover, thiol complexation restricts As(III) movement while uncomplexed As(III) is highly mobile. Given that As(V) was the major As form in shoots, most As(III) might have been complexed and kept sequestered in root vacuoles (Wang et al., 2015). Rice plants have shown high As mobility through the xylem, the highest within non-hyperaccumulator species, which has been assumed to be a consequence of As(III) and As(V) sharing transporters with Si and P, respectively, resulting in enhanced accumulation in the shoots of rice plants (Panda et al., 2010; Zhao et al., 2009; Ma et al., 2008). Contrastingly, Zhao et al., (2012) carried out a hydroponics experiment where rice

Table 6. Aerial part PCA (Exp. 2).

	AP1	AP2	AP3	AP4
$[Tot\text{-}As]_{AP}$	-0.964			
$[As(III)]_{AP}$	-0.934			
$[Mg]_{AP}$	0.921			
$[Cu]_{AP}$	0.904			
$[As(V)]_{AP}$	-0.861			
$[Na]_{AP}$	-0.813			
$[P]_{AP}$	0.783			
$Length_{AP}$	0.768		-0.528	
$[N]_{AP}$	0.760			
FW_{AP}	0.725			
MDA	-0.652			
DW_{AP}	0.602		-0.533	
$[Zn]_{AP}$		0.881		
$[Fe]_{AP}$		0.856		
$[Ca]_{AP}$		0.827		
$[Mn]_{AP}$		0.706		
$[K]_{AP}$			0.857	
Carbonyl Prot.				0.923
% Variance	49.8	18.9	10.6	6.4

Table 7. Roots (+oxidative stress) PCA (Exp. 2).

	RT1	RT2	RT3	RT4
$[P]_{Root}$	0.930			
$[Cu]_{Root}$	0.914			
$[N]_{Root}$	0.903			
$[K]_{Root}$	0.867			
$[Mg]_{Root}$	0.858			
$[Tot.-As]_{Root}$	-0.850			
$[As(III)]_{Root}$	-0.841			
DW_{Root}	0.710	-0.599		
$[Ca]_{Root}$	-0.686	0.558		
FW_{Root}	0.680	-0.639		
$[Fe]_{Root}$	-0.590		-0.500	
Carbonyl Prot.		0.893		
$Length_{Root}$		-0.681		
MDA		0.670		
$[Mn]_{Root}$	-0.623	0.637		
$[Zn]_{Root}$			0.860	
$[Na]_{Root}$			0.607	
$[As(V)]_{Root}$				-0.914
% Variance	61.8	10.6	7.4	6.1

plants were provided with radioactive $^{73}As(III)$ for 2 days, and observed that only 10 % of the added As reached the shoot. This is in agreement with the results of Wang et al. (2010), who reported As concentrations in rice leaves ten times lower than in the roots when the plants were grown in the presence of 2.4 mg L^{-1} of As(III) or As(V) indistinctly.

3.2.2. Oxidative stress related parameters in the plants and principal component analyses

The concentration of MDA in rice leaves from the 10 mg As L^{-1} treatment showed significantly higher oxidative damage than those from the 0.5 mg As L^{-1} one (Table 5), although no significant differences among the control and the rest of the treatments were found. Higher values (1.75 μmol MDA g^{-1} FW) were found by Shri et al. (2009) in plants exposed to 37.5 mg As(V) L^{-1}. These authors reported higher lipid peroxidation at the highest As concentrations studied, which is in good agreement with the results obtained in the present experiment (significant Pearson correlation coefficient between As in the nutrient solution and MDA concentration in plants; Table 5). Contrastingly, protein carbonylation was not remarkably influenced by As dose, and even higher carbonyl protein concentration was observed in control (no As) treated plants than in those from the lowest As concentration treatment (Table 5). Protein carbonylation, which alters protein activity and predominantly increases their susceptibility to proteolysis, is widely used as a protein oxidation marker but, in our experiment, lipid peroxidation was more susceptible to the ROS-mediated oxidative damage.

Arsenic-derived ROS formation may occur through the electron leakage during the conversion of As(V) to As(III), causing lipid peroxidation, enzyme inactivation and protein or nucleic acids oxidation. The affected proteins are normally chaperons, Krebs cycle enzymes and ROS detoxifiers (Srivastava et al., 2014). In this sense, antioxidant enzymes like SOD and CAT play a role in ROS scavenging and inactivation and, therefore, their formation is normally promoted in the presence of trace element induced oxidative stress (Pandey and Bhatt, 2016; Pramanik et al., 2017, 2018; Sharma et al., 2012). Nevertheless, thiol rich peptides are major targets of ROS attack. Even though As(III) is detoxified by forming complexes with GSH and PC, it also binds to sulfhydryl groups of the mentioned proteins and interferes with their functions (Panda et al., 2010; Dat et al., 2000; Srivastava et al., 2014). However, in rice plants from Exp. 2, MDA (lipid peroxidation) acted as a better oxidative stress indicator of As toxicity than protein oxidation (no significant correlation between As dose and carbonyl proteins concentration; Table 5). This agrees with the results of Clemente et al. (2019), who reported that MDA acted as a better oxidative stress indicator in *Silybum marianum* (L.) plants than carbonyl proteins.

Two PCA were performed in order to integrate whole data regarding either aerial part or roots information and the MDA and carbonyl proteins concentration in plant leaves. In the case of the aerial part, four different components were obtained that explained more than 85 % of the variance (Table 6). The first one (AP1) related negatively plant growth and nutrients with total and major species concentrations of As in the shoots and MDA (Figure 5), showing how increasing As concentrations limited plant growth and caused lipid peroxidation in the plants. The second component (AP2) related micronutrients (Fe, Mn and Zn) and Ca concentrations among them (Figure 5), which indicated a concomitant increase of these elements in the plants that did not affect plant growth or the analyzed oxidative stress related parameters. The third component (AP3) associated negatively plant growth with K concentrations (Table 6), which can be considered to be in agreement with the lower K concentrations observed in plant roots at the higher As doses (Table 4) that impaired the correct development of the plants. Whereas, the last component (AP4) included only carbonyl proteins concentration (Table 6), which indicates that this parameter was not affected by As toxicity and did not affect the development of the aerial part of the plants.

Regarding roots PCA, four components were again obtained that explained >85 % of the variance (Table 7). The first component (RT1) was similar to AP1 and associated plant yield and nutrient (N, P, K, Mg, Cu) concentrations in the roots negatively with those of total-As, As(III), Ca, Fe and Mn (Figure 5). MDA and carbonyl proteins in the leaves were, in this case, related positively to Ca and Mn concentrations in the roots, and negatively to root yield and length increase (RT2, Figure 5), indicating that oxidative stress in the plants limited root growth and that this can be associated to Mn accumulation in the roots. Interestingly, Mn concentration in the roots increased significantly with increasing As dose (Table 4), which could be indirectly indicating a toxic effect of As in the plants. Components 3 and 4 (Table 7) were less informative and simply associated Zn and Na concentrations negatively with those of Fe (RT3), while As(V) concentrations in the roots (RT4) did not seem to be involved in any oxidative stress or plant growth effect. This is in agreement with

the predominance of As(III) over As(V) in the roots of rice plants previously commented.

4. Conclusions

Rice plants showed clear symptoms of acute toxicity at elevated As(V) concentrations and strong negative effects were observed in the plants above 2.5 mg As L^{-1} in the growing medium. These results may contribute to the establishment of As toxicity thresholds for rice plants (between 0.5 and 1.25 mg L^{-1} As(V) according to the results obtained in the present experiment). Arsenic(V) was largely transformed to As(III) in plant roots, while As(V) forms were dominant in the aerial part of the plants. Root to shoot translocation was scarce at low As concentrations, but was apparently not regulated at the highest As doses (>5 mg L^{-1}). This can be considered to be not only an original but also a relevant finding as this implied a serious plant growth impairment. The MDA concentration was found to be a useful oxidative stress indicator of As(V) induced toxicity and damage in rice plants. The PCA performed allowed relating for the first time increasing MDA concentrations with increasing total and inorganic species As concentrations in the leaves, as well as with plant growth related parameters. These results highlight the relevance of the determination of major As chemical forms within the plant for the study of As accumulation and toxicity (plant growth and oxidative stress status) in this species. Further research is needed to elucidate the mechanisms of transport and translocation of the different As forms within the plant as well as the extrapolation of these results to real soil/cultivars crop experiments.

Declarations

Author contribution statement

M. José Álvarez-Robles: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

M. Pilar Bernal, Rafael Clemente: Conceived and designed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Antonio Sánchez-Guerrero: Analyzed and interpreted the data.

Francisca Sevilla: Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Funding statement

This work was supported by Fundación Séneca (Murcia Region) through the projects 19460/PI/14 and 19876/GERM/15-Excellence Project, and the Intramural-CSIC project 201840E107.

Competing interest statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

Acknowledgements

The authors would like to thank Sandra Correa and Dr. E. Arco-Lázaro for their assistance in the chemical analyses.

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