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Effects of ascorbic acid addition on the oxidative stress response of *Oryza sativa* L. plants to As(V) exposure

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ABSTRACT

Accumulation of noxious elements in the edible part of crops and its impact on food safety is of increasing concern. Rice is one of the major staple food crops worldwide, including arsenic (As)-polluted areas, in which dietary As exposure is becoming a widespread health threat. Plant chemical priming has been shown to be an effective strategy to enhance tolerance to environmental stresses, including metal(loid) exposure. The priming effect of ascorbic acid (AsA) was assessed in rice seedlings exposed to As(V) in a hydroponics experiment. AsA treatment (co-addition to the growing media concomitantly (t_0) or 24 h in advance (t_{24})) prevented an excessive accumulation of As in the roots (that decreased ~ 60%) and stimulated the activities of photosynthetic and antioxidant attributes (~1.2-fold) in the aerial part of the plants. The increase in proline levels in both shoots (~2.1-fold) and roots (~2.4-fold) was found to be the most sensitive stress parameter, and was able to reflect the AsA-induced reduction of As toxic effects (concentrations back to Control levels, both simultaneously added or added as a pretreatment) in the aerial part of the plants. However, the phytotoxic effects related to As exposure were not fully prevented by priming with AsA, and further research is needed to find alternative priming approaches.

1. Introduction

The non-essential metalloid arsenic (As) is becoming a global contaminant that entails serious hazard to human health, plants and animals (Nath et al., 2014). The accumulation of As in agricultural soil seriously increases the risk of As entry into the food chain, which threatens agricultural trade and increases demand of food safety (Bali and Sidhu, 2021). Among crops, rice cultivars (*Oryza sativa* L.) are facing many threats regarding As toxicity, mainly due to the use of As-polluted irrigation groundwater, especially in South Asian countries (Moulick et al., 2016; Nath et al., 2014). Rice has consequently been the subject of extended research, which, together with its small and well-organized genome, has turned this species into a model plant for As toxicity studies (Ahsan et al., 2008).

The mobility and bioavailability of As in soil solution strongly depend on its chemical forms and speciation, with arsenate (As(V)) being the most abundant in aerobic soils. However, under reducing conditions arsenite (As(III)) is predominant (Bali and Sidhu, 2021; Panda et al., 2010). In fact, arsenate has been found to be the dominating As species in the soil solution of strongly oxidized contaminated mine soils (Álvarez-Robles et al., 2022; Beesley et al., 2014). Both inorganic forms of As (As(V) and As(III)) are highly phytoavailable and more noxious than its major pentavalent methylated species, *i.e.,* monomethylarsonic acid (MMA) and dimethylarsinic acid (DMA) (Bali and Sidhu, 2021; Panda et al., 2010). In plants, As(V) and As(III) are mostly taken up by phosphate transporters and aquaporins, respectively (Tripathi et al., 2007). Inorganic As species are highly phytotoxic: As(V) can replace phosphate groups (Pi) due to their structural analogy, and disrupt energy flows in cells (Tripathi et al., 2007; Nath et al., 2014); and As(III) can interact with thiol groups of proteins altering their structure and functions (Panda et al., 2010; Singh et al., 2015).

Moreover, As exposure can enhance the production of reactive oxygen species (ROS) that can cause a series of damages to cellular structures and metabolic pathways (Finnegan and Chen, 2012; Singh et al., 2015). In plants, ROS homeostasis is controlled through a complex and redundant ROS-metabolizing system, which includes enzymatic and non-enzymatic reactions that remove and keep ROS at basal non-toxic levels (Noctor et al., 2018). The main redox-active metabolites involved in non-enzymatic ROS scavenge pathways are ascorbate, glutathione (GSH) and pyridine nucleotides NAD(P)H (Foyer and

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Noctor, 2011). Apart of its role in ROS homeostasis, ascorbic acid (AsA) is known to regulate plant growth and development as well as to determine the level of tolerance to several environmental constraints (Gallie, 2013; Akram et al., 2017). In fact, as an effective antioxidant, the exogenous application of AsA has proved to ameliorate As-induced oxidative stress in roots of eggplant (Alamri et al., 2021) and Cd toxicity in wheat (Zhou et al., 2021) and rice (Chao and Kao, 2010).

Also, it is well-established that an early sensing of stress and the induction of an appropriate defense response are vital for the successful adaptation of plants to stress conditions (Jakab et al., 2005). Interestingly, plants show a stronger and faster defense response if they have been previously undergone an acclimation process, a phenomenon known as priming (Conrath et al., 2015). In addition, different natural or synthetic compounds have the potential to act as priming agents, such as phytohormones (López-Orenes et al., 2020; Sytar et al., 2019), reactive oxygen-nitrogen-sulfur species (Antoniou et al., 2016), amino acids (Vijayakumari et al., 2016) and AsA (Akram et al., 2017; Elkelish et al., 2020), among others (Savvides et al., 2016; Siddiqui et al., 2020).

Our starting hypothesis was that priming with AsA would improve As tolerance in rice plants through the modulation of the stress response and the limitation of As accumulation. Therefore, the aim of this work was to analyze to which extent pretreatment (priming) or simultaneously treatment with AsA affect the response of rice plants to As(V) exposure. To prove this, a hydroponics experiment was carried out to examine the effectiveness of AsA feeding treatments in reducing the phytotoxicity of As on the growth, photosynthetic activity, antioxidant compounds and oxidative stress response in rice plants.

2. Materials and methods

2.1. Experimental design, growing conditions, and treatments

Rice seeds (var. J. Sendra; provided by Instituto Valenciano de Investigaciones Agrarias, Moncada, Spain) were wrapped in moistened paper and kept in the darkness during five days for germination. After that, the rice seedlings were shifted to vermiculite for 7 days, and then transferred to hydroponic pots where they were allowed to grow in nutrient solution for 20 days more before the treatments were applied. The nutrient solution used was a modified version of the Hoagland solution (1.50 mM KNO_3, 1.28 mM $Ca(NO_3)_2$; 0.37 mM $MgSO_4$, 0.17 mM KH_2PO_4, 0.15 mM NaCl, 24.71 μM Fe-EDDHA, 16.65 μM H_3BO_3, 2.37 μM $MnSO_4$, 0.92 μM $ZnSO_4$, 0.63 μM $CuSO_4$ and 0.63 μM $(NH_4)_6Mo_7O_{24}$; (Álvarez-Robles et al., 2020)). The solution was renewed weekly and the pH was adjusted to 5.5 (using either NaOH or HCl) every three days. Aliquots of a water extract (1:10 w/v) of a mature olive-mill waste compost were added as a source of dissolved organic carbon (to a final concentration of 100 mg DOC L^{-1}), as described in Álvarez-Robles et al. (2020). Arsenic was added as $Na_2HAsO_4{\cdot}7H_2O$ (Sigma-Aldrich) in the corresponding As treatments. The different treatments were applied when rice plants (20 per pot) were of uniform size (25 cm height on average, 20 days after transplanting to the pots). The corresponding solutions were renewed three days after the start of the experiment, which lasted one week. The experiment was run in a growth chamber with a 12 h day/night cycle of 25/18 °C temperature and 58/70% of relative humidity. The five treatments applied were the following: i) control: nutrient solution with no added AsA or As; ii) AsA: nutrient solution + 2 mM AsA; iii) As: nutrient solution + 50 μM As(V); iv) AsA + As t_0: nutrient solution + 2 mM AsA + 50 μM As(V), added simultaneously, and v) AsA + As t_{24}: nutrient solution + 2 mM AsA + 50 μM As (V) added 24 h after AsA addition. After 7 days of treatment, the plants were harvested and divided into the aerial part and the roots. The concentrations of AsA (2 mM) and As (50 μM) were selected in view of previously published results regarding priming with AsA (Jung et al., 2018) and As-rice interaction in similar conditions (Álvarez-Robles et al., 2020).

2.2. Plant measurements and analytical procedures

The plant height was measured at the beginning (day 0) and the end of treatment exposure (day 7). Similarly, roots were marked with a permanent marker 1 cm above the tip at day 0 and the length increase was measured at day 7 of the experiment. Half of the separated plant parts were rapidly frozen in liquid N_2, while the rest of the samples were oven-dried (65 °C) until constant weight and ground to a fine powder in an electric mill (A10 IKA-Labortechnik, Staufen, Germany) for analysis.

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 6500DUO ONE FAST, Thermo Scientific, Waltham, MA USA) after microwave assisted acid digestion (UltraClave, Milestone, Shelton, CT USA). The analytical accuracy was checked with a certified reference material (NCS DC 73349). Frozen shoot and root samples 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), MMA and 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).

2.3. Physiological, antioxidant and oxidative stress status parameters

The evaluation of the physiological status in rice plants was performed by measuring the content of photosynthetic pigments, total soluble sugars and proteins as previously described (López-Orenes et al., 2018a). In short, shoot and root samples (~0.1 g N_2-powdered tissue) were extracted with 80% ethanol (1 mL) by sonication at 40 °C for 30 min and centrifuged at 15,000×*g* for 15 min at 4 °C. Shoot ethanolic supernatants were used for chlorophyll *a* (Chl-a), chlorophyll *b* (Chl-b) and total carotenoids determinations and their levels were estimated using the extinction coefficients and the equations reported by Lichtenthaler and Wellburn (1983).

The total soluble sugars (TSS) were determined by the anthrone-sulfuric acid method using the ethanolic extracts and glucose (25–500 μg/mL) as standard. The total soluble protein (TSP) concentrations were measured by the Bradford method, using bovine serum albumin (BSA) as the standard.

The total antioxidant capacity was estimated by the FRAP (ferric reducing/antioxidant power) and DPPH (2,2-diphenyl-1-picrylhydrazyl radical scavenging activity) assays using both shoot and root ethanolic extracts as described by Pérez-Tortosa et al. (2012). The reducing power was expressed as μmol Fe(II) per gram (fresh weight) and a standard curve in the range 0–3 mM of $FeSO_4{\cdot}7H_2O$ was used for calibration. DPPH activity was expressed as μmol of gallic acid equivalents (GAE) per gram of fresh weight. The total phenol concentration (TPC) was determined by the Folin-Ciocalteu method using gallic acid (25–2000 μM) as a standard (Everette et al., 2010). The pellets from ethanol extractions, after thoroughly washing with ethanol, were used to estimate the content of cell wall-associated proanthocyanidins (PAs) by measuring the absorbance at 545 nm of the supernatants obtained after an acid attack (butanol-HCl). Results were expressed as cyanidin equivalents by using an $\varepsilon_{545} = 34.7\ mM^{-1}\ cm^{-1}$ (Vermerris and Nicholson, 2007).

The determination of hydrogen peroxide was carried out by the ferrous ion oxidation–xylenol orange (FOX) method, as described by Cheeseman (2006). Briefly, frozen samples (0.1 g) were homogenized in 1 mL of 6% trichloroacetic acid (TCA) and centrifuged (15,000 g, 10 min, 4 °C). Then, 50 μl of supernatant were mixed with 200 μL of FOX1 medium (0.25 mM $Fe(NH_4)_2(SO_4)_2$, 25 mM H_2SO_4, 0.1 mM xylenol

orange, 100 mM sorbitol, and 1% (v/v) ethanol). After 30 min of incubation in the dark, the concentration of H_2O_2 was determined based on the difference in absorption at 560 nm, using a H_2O_2 standard curve covering the range of 0.1–10 μM.

The concentration of proline (Pro) was determined spectrophotometrically in a sulfosalicylic acid extract, using acid ninhydrin reagent (López-Orenes et al., 2013). Absorbance of the proline-ninhydrin complex was recorded at 518 nm. The proline concentration was determined from a calibration curve with known concentrations of proline in the range 10–1000 μM.

The degree of lipid peroxidation was determined as the concentration of malondialdehyde (MDA) by measuring thiobarbituric acid-reacting substances (TBARS) at 532 nm, with a correction for non-specific absorbance at 440 and 600 nm (Hodges et al., 1999), using the same supernatants as in the FOX1 assay. The concentration of MDA was finally calculated using an extinction coefficient of 155 mM^{-1} cm^{-1}. Oxidized and reduced forms of ascorbate were measured using the α-α′-bipyridyl method (Gillespie and Ainsworth, 2007).

Oxidized proteins were estimated through the reaction of (2,4-dinitrophenyl)hydrazine (DNPH) with protein carbonyls after inhibition of proteases in plant extracts obtained with cOmplete® (Roche) and phenylmethylsulfonyl fluoride (PMSF) (Levine et al., 1994). Carbonyl proteins were referred to total proteins content calculated using the Bradford method and bovine serum albumin (BSA) as standard.

For ascorbate (AsA), GSH and non-protein thiol (NPT) determinations, about 0.2 g N_2-powdered tissue were homogenized with ice-cold 5% (w/v) metaphosphoric acid. The homogenates were centrifuged at 15,000 g for 15 min at 4 °C, and the supernatants were used for the analysis of AsA, GSH and NPT using the α-α′-bipyridyl method (Gillespie and Ainsworth, 2007), the recycling assay (Queval and Noctor, 2007) and Ellman's reagent (López-Orenes et al., 2018a), respectively. The concentration of total phytochelatins (PCs) was estimated from the difference between NPT and GSH as previously described (Hartley-Whitaker et al., 2001). All the spectrophotometric assays were performed with a microplate UV–Vis spectrophotometer reader (Multiskan GO, Thermo Scientific).

2.4. Statistical analysis

The statistical data analysis was performed using IBM SPSS Statistics Version 26.0 software (IBM Corporation, New York, USA). The one-way analysis of variance (ANOVA), followed by Tukey's HSD test, was carried out to assess the significant differences among treatments ($P < 0.05$). Two-way ANOVA (plant part × treatment) was also performed to test significant differences among plant parts data for the different treatments. A Principal Component Analysis (PCA) was run (Varimax rotation) considering all the determined parameters to reveal general tendencies. All determinations were conducted at least in duplicate, and all results are shown as mean ± standard error (SE).

3. Results

3.1. Effects of AsA feeding treatments on plant growth and nutrient and As (total and major chemical species) accumulation

No significant differences between the yield of rice plants in the different As treatments as compared to controls were observed (Fig. 1). However, the roots of As-treated plants showed a marked reduction in both root biomass (~30%) and growth; the addition of AsA to the nutrient solution did not prevent the negative effects observed in the As treatment.

The analysis of macronutrients revealed that As-alone treatment did not provoke significant changes in the accumulation of N, P, K, Ca, and Mg in both shoots and roots (Table 1). However, the addition of AsA to the growing medium resulted in a general decrease of macronutrient concentration (from 22 to 32%) in shoots. In AsA + As-treated plants, the AsA-induced changes were mostly unaffected in shoots, whereas a decrease in K levels (~35%) was found in roots.

Contrastingly, the analysis of micronutrients showed that As-alone treatment markedly reduced the shoot Fe levels (>50%) and to a lower extent the concentrations of Cu and Zn (~40%), whereas in roots the micronutrient concentrations were indistinguishable among the treatments (Table 1). AsA-alone pretreatments also provoked a reduction in the concentrations of Cu, Mn and Zn (~40%) (Table 1). Interestingly, the AsA pretreatments (AsA + As t_{24}) alleviated the reduction in shoot micronutrient concentrations, particularly the levels of Fe.

As expected, the concentrations of As in shoot and root tissues were below the detection limit in the non-As treatments (Table 2). In the shoots of all As-treated rice plants, the levels of As found were slightly above the toxicity limits stated for plants (5–20 mg kg^{-1}; Kabata--Pendias, 2011) and were unaffected by AsA exposure. In roots, however, the concentrations of As were much higher (~600 mg kg^{-1}), but significantly dropped to ~ 60% in AsA + As treated plants, either with simultaneous application or with AsA priming (Table 2).

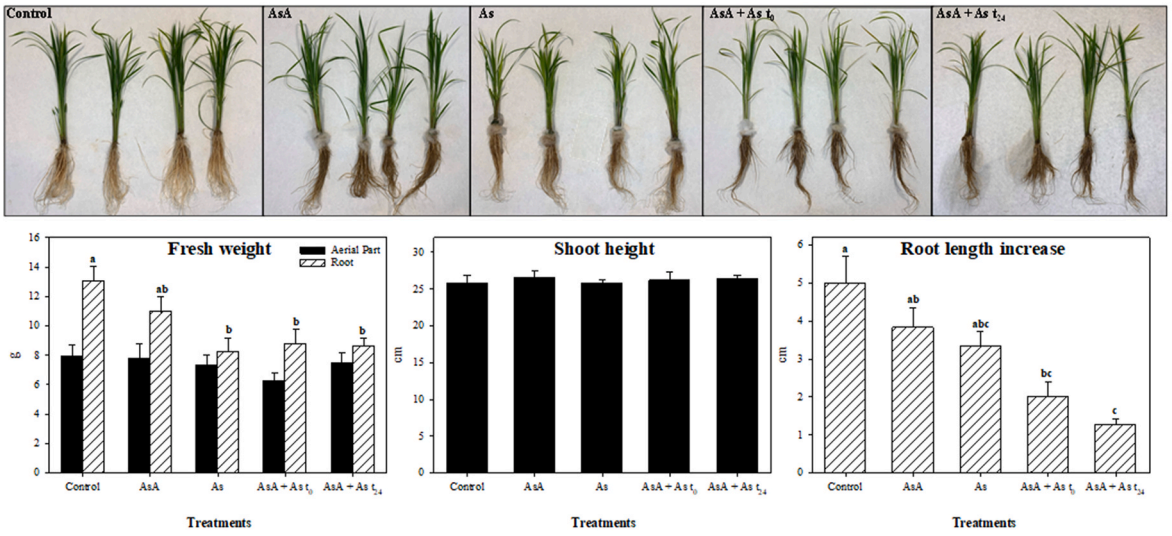

Fig. 1. Effect of AsA addition on the biomass production (grams of fresh weight per pot) and growth (shoot height (final) and length increase (days 0–7 after treatment) in the roots, cm) in 20 days old rice plants (N = 4) after 7 days of As exposure. Non-marked bars and bars marked with the same letter for each parameter do not differ significantly according to Tukey's test at $P < 0.05$.

Table 1
Effect of AsA addition on the macro- (N, P, K, Ca and Mg) and micronutrients (Cu, Fe, Mn and Zn) concentration (g kg^{-1} and mg kg^{-1}, respectively) in 20 days old rice plants (N = 4) after 7 days of As exposure.

		N	P	K	Ca	Mg	Cu	Fe	Mn	Zn
Aerial part	**Control**	28.00 ± 1.04 a	4.17 ± 0.15 a	27.85 ± 1.81 a	4.17 ± 0.27 a	3.73 ± 0.15 a	15.54 ± 1.12 a	175 ± 27 a	350 ± 25 a	120 ± 6 a
	AsA	18.89 ± 1.37 b	2.82 ± 0.16 bc	20.46 ± 1.10 b	3.22 ± 0.12 ab	2.62 ± 0.10 b	9.31 ± 0.53 b	130 ± 11 ab	225 ± 18 b	71.5 ± 5.2 b
	As	25.39 ± 1.37 a	3.46 ± 0.27 ab	25.92 ± 1.45 a	3.66 ± 0.18 ab	3.38 ± 0.08 a	9.99 ± 0.69 b	82.4 ± 5.7 b	307 ± 10 ab	74.7 ± 5.3 b
	AsA + As t_0	17.65 ± 2.25 b	2.46 ± 0.16 c	15.10 ± 0.60 b	2.69 ± 0.31 b	2.31 ± 0.13 b	7.00 ± 0.55 b	92.7 ± 8.9 b	243 ± 30 b	55.6 ± 3.4 b
	AsA + As t_{24}	17.13 ± 0.68 b	2.54 ± 0.05 c	16.71 ± 0.38 b	3.03 ± 0.20 b	2.62 ± 0.10 b	8.57 ± 0.62 b	133 ± 22 ab	235 ± 6 b	63.4 ± 7.9 b
	ANOVA	***	***	***	**	**	***	*	**	***
Roots	**Control**	23.05 ± 0.99	2.11 ± 0.06	21.25 ± 1.29 a	2.02 ± 0.13	5.85 ± 0.92	38.2 ± 3.82	2048 ± 376	94.9 ± 12.0	60.6 ± 9.0
	AsA	26.90 ± 2.13	2.35 ± 0.26	18.27 ± 2.24 ab	2.44 ± 0.11	4.43 ± 0.38	27.8 ± 2.53	1539 ± 224	86.8 ± 12.6	51.8 ± 10.8
	As	25.90 ± 0.68	2.24 ± 0.06	22.69 ± 1.28 a	2.11 ± 0.15	4.71 ± 0.68	29.0 ± 2.47	1603 ± 303	85.9 ± 9.6	46.4 ± 1.3
	AsA + As t_0	26.92 ± 2.76	2.14 ± 0.34	13.84 ± 1.22 b	2.47 ± 0.47	4.40 ± 0.51	26.8 ± 2.18	1548 ± 210	79.4 ± 9.8	49.5 ± 4.9
	AsA + As t_{24}	23.74 ± 1.77	1.82 ± 0.18	13.82 ± 0.88 b	2.59 ± 0.36	4.71 ± 0.66	29.0 ± 2.86	1834 ± 267	84.3 ± 8.3	48.1 ± 7.5
	ANOVA	NS	NS	**	NS	NS	NS	NS	NS	NS
ANOVA	**Plant Part**	**	***	**	***	***	***	***	***	***
	Treatment	*	***	***	NS	*	***	NS	**	***
	PxT	***	**	NS	**	NS	NS	NS	**	**

NS: not significant. ***, ** and *: significant at P < 0.001, 0.01 and 0.05, respectively. Values with no letter or followed by the same letter in each column for each plant part do not differ significantly according to Tukey's test at P < 0.05.

Table 2
Effect of AsA addition on the total As and As species concentration (mg kg^{-1} DW) in 20 days old rice plants (N = 4) after 7 days of As exposure.

		Total-As	As(III)	As(V)
Aerial part	**Control**	bdl	bdl	bdl
	AsA	bdl	bdl	bdl
	As	36.2 ± 6.64	7.52 ± 0.89 a	4.70 ± 1.33 a
	AsA + As t_0	33.7 ± 6.41	4.56 ± 0.96 ab	1.80 ± 0.55 ab
	AsA + As t_{24}	46.0 ± 5.85	3.48 ± 0.95 b	1.39 ± 0.14 b
	ANOVA	NS	*	*
Roots	**Control**	bdl	bdl	bdl
	AsA	bdl	bdl	bdl
	As	605 ± 24.7 a	459 ± 106 a	12.8 ± 6.69
	AsA + As t_0	256 ± 40.1 b	135 ± 23.0 b	28.0 ± 5.07
	AsA + As t_{24}	232 ± 3.29 b	158 ± 33.2 b	32.7 ± 3.47
	ANOVA	***	**	NS
ANOVA	**Plant Part**	***	***	***
	Treatment	***	***	**
	PxT	***	***	***

bdl: below detection level (1 mg kg^{-1} DW). NS: not significant. ***, ** and *: significant at P < 0.001, 0.01 and 0.05, respectively. Values with no letter or followed by the same letter in each column for each plant part do not differ significantly according to Tukey's test at P < 0.05.

When major As chemical species were analyzed in the plants, only As (III) and As(V) were found in detectable amounts. The concentrations of these species were slightly lower than those previously reported for rice plants exposed to similar concentrations of As(V) in the growing medium (Álvarez-Robles et al., 2020). The concentrations of As(III) and As (V) extracted from the aerial parts of the plants were particularly low (~8 and 5 mg kg^{-1}, respectively), and the addition of AsA 24 h before As treatment resulted in a significant decrease of their levels (Table 2). Higher As(V) and, especially As(III) concentrations were found in the roots, where AsA feeding treatments provoked a drastic decrease in As (III) concentration (~70%) (Table 2).

3.2. Effects of AsA feeding treatments on physiological and oxidative stress parameters in rice plants under arsenate exposure

The biochemical parameters such as photosynthetic pigments, soluble sugars, and proteins were differently affected by As exposure (Table 3): As-alone treatment led to a reduction in the content of Chl-b (>20%) in the plants compared to AsA treatment, while no major changes occurred in the content of sugars and proteins in shoots.

With respect to oxidative stress, no pronounced changes were observed in the H_2O_2 contents either in shoot or root tissues among the different treatments (Fig. 2). In contrast, the accumulation of the stress amino-acid proline showed a marked increase (>2-fold) in both shoots and roots in As-treated plants compared to control treatment. Interestingly, proline concentration in AsA + As treatments was similar to that in the controls and AsA-alone treatment, although this effect was not observed in plant roots (Fig. 2). The concentrations of MDA and carbonyl proteins in both the aerial part and the roots of the plants from the different treatments did not show significant differences (data not shown), in agreement with previous results that did not find these parameters to be good indicators of As induced oxidative stress in rice plants (Álvarez-Robles et al., 2020).

The As-alone treatment did not provoke significant changes in the values of the antioxidant properties FRAP and DPPH concentrations in plant shoots or roots (Fig. 3). Only AsA + As t_{24} treatment showed significantly lower FRAP concentrations in plant shoot compared to AsA-alone treatment, and higher FRAP and DPPH ones in plant roots compared to control treatment.

The analysis of total phenolics showed a similar trend as in the FRAP and DPPH tests in both shoot and root tissues, with higher levels in shoots than in roots and no significant differences in the aerial part and higher concentrations in all AsA and As treatments in the roots (Fig. 3). Moreover, strong correlations between TPC and FRAP or DPPH values were found ($r > 0.7$, $P < 0.01$ in shoots and $r > 0.9$, $P < 0.01$ in roots). Interestingly, As(V) exposure caused an increase in the accumulation of proanthocyanidins in root tissues (~2-fold), and this increase was even more prominent in both AsA feeding treatments (>3-fold) as compared

Table 3
Effect of AsA addition on the physiological parameters in 20 days old rice plants (N = 4) after 7 days of As exposure.

		Chl-a (μg Chl-a g^{-1} FW)	Chl-b (μg Chl-b g^{-1} FW)	Carotenoids (μg carotenoids g^{-1} FW)	TSS (mg glucose g^{-1} FW)	TSP (mg protein g^{-1} FW)
Aerial part	**Control**	792 ± 66	353 ± 26 ab	240 ± 22	23.3 ± 1.8	9.14 ± 0.36
	AsA	990 ± 128	450 ± 61 a	291 ± 38	26.3 ± 2.9	9.67 ± 1.30
	As	593 ± 81	271 ± 34 b	184 ± 20	23.6 ± 1.9	9.52 ± 1.25
	AsA + As t_0	702 ± 49	314 ± 21 ab	214 ± 7	26.4 ± 2.9	8.80 ± 0.91
	AsA + As t_{24}	792 ± 80	367 ± 35 ab	233 ± 19	25.8 ± 0.9	7.74 ± 0.39
	ANOVA	NS	*	NS	NS	NS
Roots	**Control**	–	–	6.53 ± 2.34	4.15 ± 0.57	2.17 ± 0.21
	AsA	–	–	6.30 ± 0.70	3.26 ± 0.29	2.67 ± 0.32
	As	–	–	8.64 ± 1.18	3.01 ± 0.36	2.21 ± 0.10
	AsA + As t_0	–	–	9.16 ± 1.53	3.57 ± 0.54	2.70 ± 0.26
	AsA + As t_{24}	–	–	9.37 ± 1.98	3.38 ± 0.30	2.38 ± 0.10
	ANOVA	–	–	NS	NS	NS
ANOVA	**Plant Part**	–	–	***	***	***
	Treatment	–	–	**	NS	NS
	PxT	–	–	*	NS	NS

Chl-a: chlorophyll *a*. Chl-b: chlorophyll *b*. TSS: total soluble sugars. TSP: total soluble proteins. NS: not significant. ***, ** and *: significant at $P < 0.001$, 0.01 and 0.05, respectively. Values with no letter or followed by the same letter in each column for each plant part do not differ significantly according to Tukey's test at $P < 0.05$.

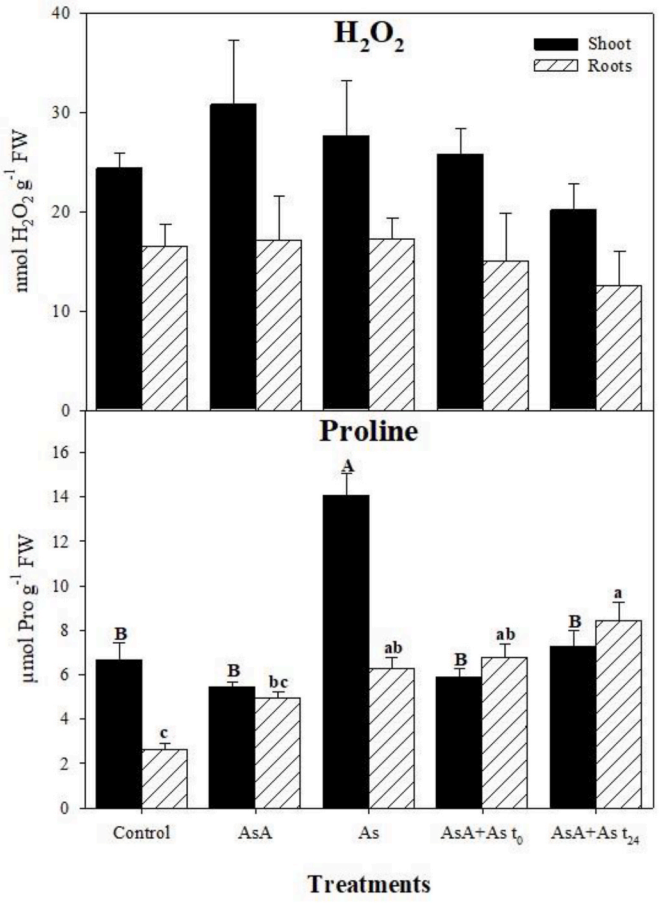

Fig. 2. Effect of AsA addition on H_2O_2 (nmol H_2O_2 g^{-1} FW) and proline (μmol Pro g^{-1} FW) concentration in 20 days old rice plants (N = 4) after 7 days of As exposure. Non-marked bars and bars marked with the same letter (uppercase for shoot and lowercase for roots) for each parameter do not differ significantly according to Tukey's test at $P < 0.05$.

to controls (Fig. 3).

The analysis of AsA concentrations in the plants showed that neither As-alone nor AsA treatments provoked any significant effect compared to controls in both shoots and roots (Fig. 4). Similarly, no significant differences were observed between GSH, non-protein thiol and phytochelatin concentrations in all assayed conditions in shoot tissues. However, As(V) exposure provoked a marked increase in the content of NPT and PC (~2.8-fold) in the roots, which decreased to normal (control) values in the combined AsA + As treatments. In the roots of AsA-As-exposed plants, the concentrations of GSH were significantly lower than in control and AsA-alone treatments (Fig. 4).

3.3. *Relationships between plant growth and nutritional parameters and antioxidant/oxidative stress markers*

Two PCAs were performed, one with data corresponding to the aerial part of the plants and another one with data from the roots, in an attempt to elucidate interrelationships and possible dependencies between the plant stress related parameters and the concentrations of As (total and major species) in the plants. The first PCA (aerial part) resulted in six different components, from which the first three accounted for more than 65% of the variance (Fig. 5a, Table S1; SI). The first component grouped together most of the stress and antioxidant activity related parameters and the photosynthetic pigments (Fig. 5a). The second component associated the concentrations of the different As forms determined in the plants (total, As(III) and As(V)) positively among them and with proline content, and negatively with Fe concentration in the aerial part of the plants. Proline was one of the few parameters that showed a significant response (increased concentration) to the presence of As in the growing media that was then alleviated by AsA addition. This fact appears to be related to the accumulation of As (both As(III), As(V) and total) in the plants (Abbas et al., 2018). This can be considered to be an interesting finding, as this parameter is determined quite easily in the plants, and may act as an early marker of As toxicity in the plants. The third component related plant nutrients (N, P) concentrations in the plants and the rest of the components did not provide any relevant relationships (Table S1; SI).

The PCA performed with roots data resulted also in 6 different components, the first three again justifying more than 64% of the variance (Fig. 5c, Table S2; SI). The first component associated some of the oxidative stress (MDA) and antioxidant activity (PAs, TPC, FRAP, proline and DPPH) parameters positively with As(V) concentration in the roots, and negatively with roots length, fresh weight and GSH concentrations. This indicates that As(V) accumulation in the roots of the plants may affect their normal growth and increase the antioxidant and stress response of this part of the plants. The second factor related positively total As and As(III) concentrations to phytochelatins and NPT concentrations in the roots. This points out that, in the roots, the formation of NPT and phytochelatins was the response to the presence of As(III) and the accumulation of total As in these tissues, while the addition of AsA to the growing media prevented this from happening. The third component related negatively total proteins and AsA concentrations with carbonyl

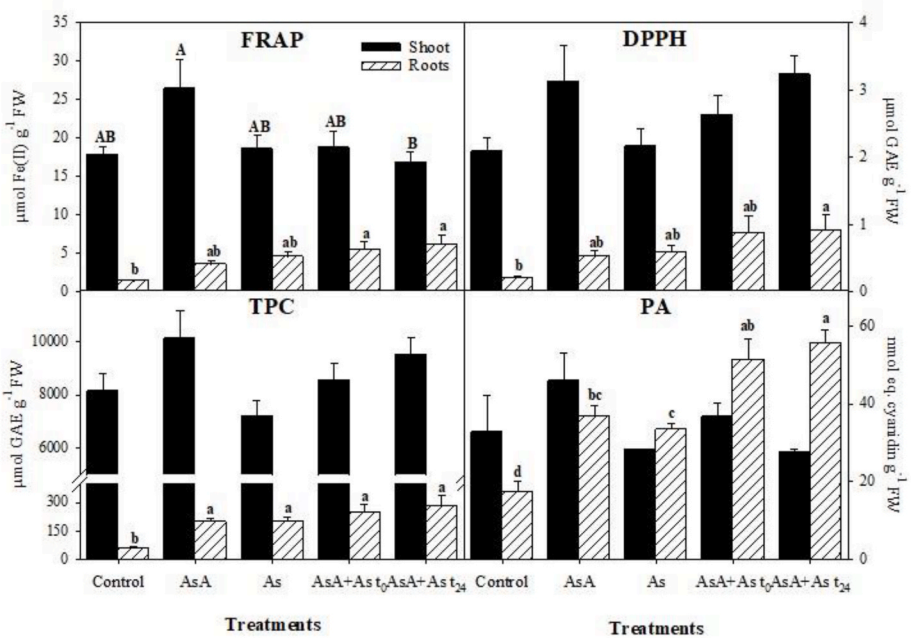

Fig. 3. Effect of AsA addition on the total antioxidant activity (FRAP and DPPH assays; µmol Fe(II) g^{-1} FW, µmol gallic acid equivalent (GAE) g^{-1} FW, respectively), total phenolics (TPC; µmol GAE g^{-1} FW) and proantocyanidins (PA; nmol eq. cyaniding g^{-1} FW) concentration in 20 days old rice plants (N = 4) after 7 days of As exposure. Non-marked bars and bars marked with the same letter (uppercase for shoot and lowercase for roots) for each parameter do not differ significantly according to Tukey's test at $P < 0.05$.

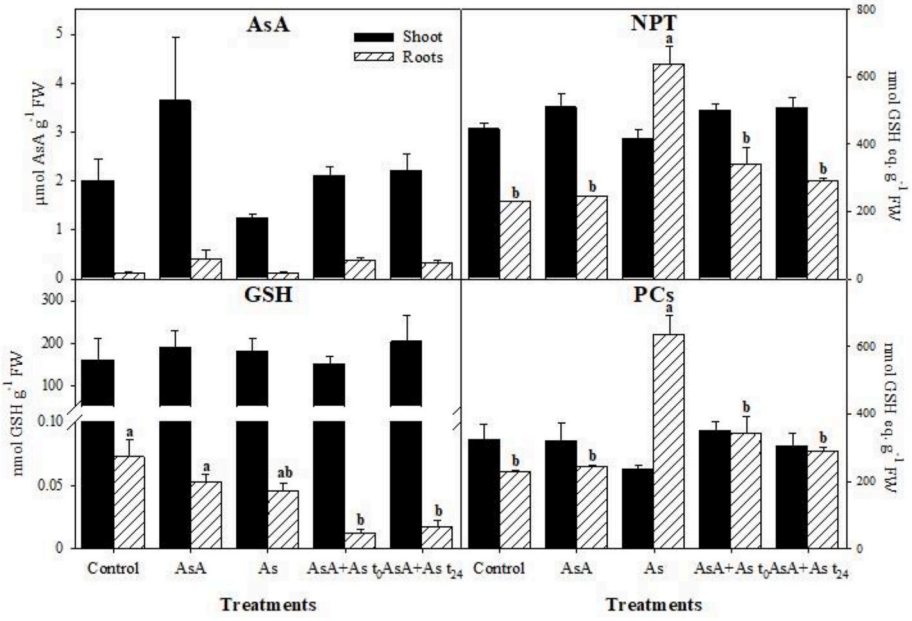

Fig. 4. Effect of AsA addition on total ascorbic acid (AsA, µmol g^{-1} FW), total glutathione (GSH, nmol g^{-1} FW), non-protein thiols (NPT, nmol eq. GSH g^{-1} FW) and phytochelatins (PCs, nmol GSH g^{-1} FW) concentrations in 20 days old rice plants (N = 4) after 7 days of As exposure. Non-marked bars and bars marked with the same letter for each parameter do not differ significantly according to Tukey's test at $P < 0.05$.

proteins in plant roots, and the fourth related again N and P concentrations (Table S1; SI).

A clear separation between As only and control treatments was observed in the aerial part PCA, mainly along PC2 (As, proline and Fe concentrations), whilst AsA and combined AsA-As treatments were not so clearly separated among them (Fig. 5b). The factors obtained for treatments in the roots PCA (Fig. 5d) separated As only from the rest of the treatments along PC2 (As(III), NPT and PCs concentrations), which were not evidently differentiated in any component. This suggests again the mitigation of As toxicity by AsA addition.

Therefore, in the aerial part of the plants, proline was the compound whose concentration significantly increased in As-alone treatments and reflected the positive effect on the plants of AsA addition. A different situation was found in the roots, where As(V) was responsible of the reduced plant growth and the antioxidant response of the plants, while As(III), which was the major form in this part of the plants (Table 3), provoked the formation of NPT and PCs, which were significantly decreased when AsA was added to the growing medium.

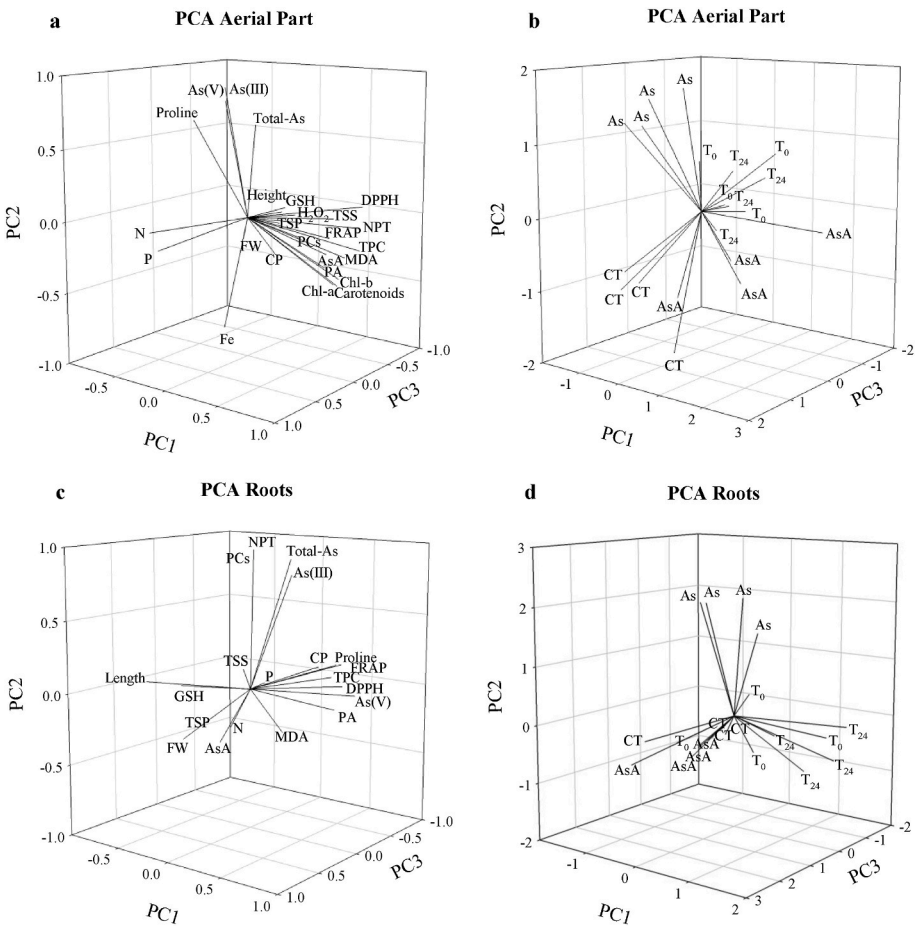

Fig. 5. PCA combined plot for rice plant parameters (a,c) and treatments (b,d) in the aerial part and in the roots. CT: Control; T_0: AsA + As t_0; T_{24}: AsA + As t_{24}; AsA: ascorbic acid; FW: fresh weight; MDA: malondialdehyde; CP: carbonyl proteins; TPC: total phenolics; GSH: total glutathione; PA: proantocyanidins; FRAP and DPPH: total antioxidant activity; NPT: non-protein thiols; PCs: phytochelatins; Chl-a: chlorophyll *a*; Chl-b: chlorophyll *b*; TSS: total soluble sugars. TSP: total soluble proteins.

4. Discussion

4.1. AsA feeding alleviation of the as-induced negative effects on rice plants

The As-dependent reduction of root growth was mainly attributed to the higher accumulation of As(III) in these tissues (Table 2), since no differences in macro- and micronutrients were noticed in comparison to untreated root controls (Table 1). It is well-established that As(V) is uptaken by phosphate transporters (Zhao et al., 2009). In the experiment, phosphate concentration in the nutrient solution was 0.17 mM, thus, the high phosphate/As molar ratio can explain that phosphate intake in As-treated plants was the same as did untreated control plants.

Moreover, the addition of AsA to the nutrient media lead to a marked decrease in accumulation of As(III) in both shoots and roots (Table 2). AsA reduction of As(V) uptake has also been reported in eggplant plants (Alamri et al., 2021). Arsenic is well known to be accumulated in the roots mainly as As(III) (Tripathi et al., 2007) and the efflux of As(III) to the nutrient solution due to the intracellular reduction of As(V) in rice plants exposed to arsenate has been also reported (Awasthi et al., 2017; Su et al., 2010; Xu et al., 2007). In fact, the reduction of As(V) to As(III) is considered the first step of As detoxification, and the resulting As(III) can be either extruded outside the cells or complexed with thiol-rich peptides (Bali and Sidhu, 2021). Here, the results suggest that AsA is limiting As accumulation in the roots (mainly As(III)) as well as reducing the translocation of As(III) and As(V) to the aerial part of the plants. Then, AsA feeding appears as a promising strategy to reduce As content in rice from As rich soil/water areas.

AsA feeding treatments also alleviated the As-induced reduction of Fe in shoots (Table 1). These results are in accordance with those reported by (Ghorbani et al., 2021) where the inoculation of As-stressed rice plants with the endophytic fungus *Piriformospora indica* increased both Fe translocation to shoots by upregulating the expression of Fe transporters and AsA concentration in shoots. Taken together, these results suggest that As exposure provoked a downregulation of nutrient transporter genes and AsA reduced As toxicity by enhancing Fe uptake. In fact, AsA is known to play a role in the chemical reduction and transport of Fe(II) in plants (Grillet et al., 2014).

The observed reduction in the concentration of major macro- and micro-nutrients in the aerial parts of the plants (Table 1) in the AsA only treatment could be consequence of stomatal closure, which is known to be strongly induced by high AsA concentrations (Castro et al., 2018) and results in low transpiration, reducing the uptake and transportation of nutrients (Arve et al., 2011).

The photosynthetic pigment levels showed a (non-significant) trend to increase in AsA-As-exposed plants compared to As-alone ones (Table 3). In addition, AsA feeding treatments had a positive effect on root performance under As exposure. Roots are the first line of defense against metal(loid) toxicity as well as the first organ to respond and to adapt to metal(loid) stress (Kul et al., 2021; Hasanuzzaman et al., 2018). Thus, the high content of soluble sugars in roots could contribute to an adequate root performance and to tackle As phytotoxicity (positive significant correlations were found between TSS concentration in the roots and fresh and dry root weight; $r = 0.484$ and 0.640, $P < 0.05$ and 0.01, respectively). In fact, the low root biomass found in AsA + As treatments seemed to point towards a trade-off between root growth and As stress acclimation.

No significant effects on the content of soluble sugars and proteins were found in shoots upon As exposure, although the content of photosynthetic pigments tended to decrease, particularly the levels of

carotenoids. Similar results have been described in rice plants exposed to 25 μM As(III) (Chauhan et al., 2017) and in fava beans exposed to 5 μM As(V) (Siddiqui et al., 2020), which seemed to indicate that photosynthetic pigments are very sensitive to As stress even at low doses (Finnegan and Chen, 2012). Here, the beneficial effects of AsA supplementation upon As stress in shoots could be attributed mainly to the enhancement of carotenoids levels, as regard the results of the two-way ANOVA (Plant part $P < 0.001$; Treatment $P < 0.01$; PxT $P < 0.05$; Table 3). Carotenoids are known to play a key role in the protection of the photosynthetic apparatus from oxidative stress generated by ROS (Niyogi, 2000; Song et al., 2006). These results confirmed that the maintenance of photosynthesis is vital for plant growth and survival under stress conditions (Allakhverdiev, 2020).

4.2. AsA feeding effects on oxidative stress and antioxidant parameters in the plants

A common hallmark of environmental stress factors is the generation of ROS in plant cells (Gill and Tuteja, 2010). The perception of the stress stimulus is followed by an increase of diverse signaling molecules such as free Ca^{+2}, reactive nitrogen species (RNS) and ROS within the first minutes. These signaling molecules, in turn, trigger the appropriate defense response through transcriptomic and metabolic changes (Garcia-Brugger et al., 2006). Here, H_2O_2 levels were determined in plants exposed to As(V) during 1 week, and no major changes were noticed either in shoot or root tissues (Fig. 2). These results suggest that rice plants have induced acclimation mechanisms to withstand As(V) stress. In fact, the results clearly revealed that As exposure provoked a sharp rise in proline contents in both shoot and root tissues in As-challenged rice plants (Fig. 2). AsA feeding treatments provoked an even further increased proline levels in roots, whereas in shoots proline content remained unaffected, compared to control treatment, upon AsA + As(V) exposure. Accumulation of proline in plants subjected to both abiotic and biotic stresses is a known response (Szabados and Savouré, 2010; Verslues and Sharma, 2010), including As exposure (Siddiqui et al., 2020). Proline is known to act as an osmoprotectant, as well as to protect and stabilize macromolecules and to maintain cellular ROS balance (Szabados and Savouré, 2010; Verslues and Sharma, 2010).

The present results also suggest an association between proline and phenol compounds under As exposure, and their upregulation particularly in roots by AsA feeding treatments (a positive correlation between proline and TPC in plant roots was found; $r = 0.903$, $P < 0.001$). Phenol compounds, particularly flavonoids, are known to possess metal-chelation, ROS-scavenging and antioxidant properties (Agati et al., 2012; Rice-Evans et al., 1997). In fact, phenolics are considered to act as key mediators of plant defense response to environmental constraints (Cheynier et al., 2013) including metal stress (López-Orenes et al., 2018a, 2018b; Michalak, 2006) and As exposure (Chauhan et al., 2017). Proanthocyanidins (the polymeric condensation products of flavan-3-ols) have been also reported to be effective scavengers of ROS, peroxyl radicals and the powerful oxidant peroxynitrite ($ONOO^-$) (Gould et al., 2002). In the present study, strong correlations between TPC and total antioxidant activities as well as between PAs and FRAP (shoots and roots) and DPPH (roots) were found ($r > 0.6$, $P < 0.01$), indicating that these compounds could provide a robust line of defense to control ROS homeostasis. Genes involved in PAs production have also been reported to be up-regulated upon As treatment in *Salix purpurea* plants (Yanitch et al., 2017).

Taken together, these results revealed that both proline and phenolic compounds were relevant in the acclimation response to counteract As toxicity in rice plants. The beneficial effects of AsA application can be related to the higher induction of PAs and proline in roots that boost the tolerance of rice plants challenged with As(V).

As mentioned above, As(III) in roots can be detoxified by either efflux outside the cells or chelation with thiol(SH)-rich compounds (Bali and Sidhu, 2021). Phytochelatins are considered the main chelators of As(III) in plants (Bali and Sidhu, 2021). Here, a marked increase in PCs and NPTs was observed in roots of As-treated plants (Fig. 4). These results are also in line with previous results reported in the same plant species (Zhang et al., 2011), confirming the role of these thiolic ligands to withstand As toxicity in rice. However, PC and NPT contents hardly increased in the roots of AsA-As-treated plants; this can be explained by the lower As(III) accumulation found in these tissues. The biosynthesis of PCs represents a high energy-cost to the cell associated with sulfate reduction, GSH metabolism and PC biosynthesis itself (Cobbett and Goldsbrough, 2002). Therefore, another beneficial effect of AsA application under chronic As exposure can be related to the use of alternative defense mechanisms with a lower energy demand for the plant (Maestri et al., 2010).

Reduced glutathione is the precursor for the synthesis of PCs (Cobbett and Goldsbrough, 2002), and also serves as an electron donor for the reduction of As(V) to As(III) (Duan et al., 2005). Here, a reduction of GSH levels was found in roots of AsA-As-treated plants in comparison to control and AsA-alone treated plants (Fig. 4).

These results contrast with those found in shoots where no changes in the levels of GSH were found irrespective of the treatment applied. This differential organ response can be clearly observed in Fig. 5 and would support the view that there exist organ specific mechanisms to counteract As toxicity. Moreover, these responses seem to be influenced by many factors, such as genotype, developmental stage, As levels, etc. (Zulfiqar and Ashraf, 2022). AsA and GSH are the main redox buffer systems present in plant cells, and there is a close relationship between these multifaceted molecules (Foyer and Noctor, 2011). Changes in their levels in response to developmental and environmental stimuli are well reported in the literature (Ferrer et al., 2018; Foyer and Noctor, 2011; Gill and Tuteja, 2010; Hasanuzzaman et al., 2019), but there are conflicting reports regarding the way in which AsA and GSH levels change in the roots and shoots of rice plants under As exposure (Zulfiqar and Ashraf, 2022). In the present study, results obtained point to exogenously added AsA as a key cue that provokes a metabolic switch in relation with the response of rice plants to As. Roots are the organs in which these induced changes seem to be more prominent. Arsenic tolerance mechanisms based on S-compounds (PCs and NPT) are reinforced with the accumulation of C- and N-based antioxidants (PAs and proline, respectively) in root tissues, which could contribute to energy balance of challenged plants.

5. Conclusions

Rice plants exposed to moderate As concentrations in the growing media did not show any major or evident sign of toxicity. However, certain oxidative related parameters, mainly proline concentration in the aerial part of the plants and NPT and PCs concentrations in the roots, were significantly increased by the presence of As in the nutrient solution. These effects disappeared when ascorbic acid was added to the growing media, either concomitantly or as a pretreatment. The concentration of As in the roots of the plants, retained mostly as As(III), was also significantly lowered in the presence of AsA, which accounted for the alleviation of the toxic effects that appeared in the plants according to the PCAs performed. These findings reinforce the previously observed compartmentalization of As as As(III) in the roots of rice plants and points out to proline concentration in the shoots, which can be easily determined, as a useful marker of As presence and toxicity in rice plants. This may be of relevance for the use of rice as a test plant in future As toxicity experiments or even as a parameter to be determined in cultivated rice as an indicator of As toxicity. The priming effect of AsA on As toxicity was also found to be useful in alleviating As toxicity to rice plants, although it may have to be further evaluated in plants suffering from more severe toxic symptoms.

Author individual contributions to the paper (CRediT roles)

M.J. Álvarez-Robles - Conceptualization; Investigation; Methodology; Formal analysis; Writing – original draft. **R. Clemente** - Conceptualization; Formal analysis; Funding acquisition; Supervision; Writing - original draft. **M.A. Ferrer** - Conceptualization; Formal analysis; Funding acquisition; Supervision; Writing - review & editing. **A. Calderón** - Conceptualization; Funding acquisition; Supervision; Writing - review & editing. **M.P. Bernal** - Conceptualization; Funding acquisition; Supervision; Writing - original draft.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.plaphy.2022.07.013.

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