



## Differential responses of two Iranian rice cultivars to arsenite toxicity

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### Abstract

Arsenic (As) is a toxic metalloid which is usually found in rice fields as a contamination and has negative effects on the plant growth and reduces the crop yield. Similar to heavy metals, the deleterious effects of As may be due to, at least in part, the amount of absorbed As and disturbance of the plant water status. In this connection, different varieties of the same species may show differential performances. Comparative responses to 75  $\mu\text{M}$   $\text{As}^{\text{III}}$  were evaluated in two Iranian rice cultivars adapted to moderate-humid vs. very warm and dry climates (Hashemi and Amber, respectively). Although both cultivars absorbed  $\text{As}^{\text{III}}$ , but its translocation factor was lower in Amber accompanied by accumulation of soluble sugars and glycine betaine and lower level of internal ABA. Moreover, adverse effect of  $\text{As}^{\text{III}}$  on relative water content and stomata closure was less prominent in Amber, compared to Hashemi. Results suggest that cultivars adapted to dry conditions are more capable to withstand against As and to control its entrance to human food chain.

**Keywords:** abscisic acid; arsenite; glycine betaine; rice; water content

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### Introduction

Arsenic (As) contamination of soil and groundwater has become an important environmental issue due to its worldwide distribution and its high toxicity to all organisms (Zhang et al., 2016). Soil contamination of As comes from anthropogenic activities like fossil combustion, mining, and the use of pesticides and herbicides containing As and natural resources which increase the concentration and mobilization of As in some countries (Zhao et al., 2010). Arsenic can occur in the environment predominantly in its

inorganic forms such as pentavalent arsenate ( $\text{As}^{\text{V}}$ ) and trivalent arsenite ( $\text{As}^{\text{III}}$ ) (Smedley and Kinniburgh 2013).

Rice (*Oryza sativa*) is the staff of life for over half the world's population. As rice is grown widely in areas impacted by arsenic contamination, it serves as a major dietary source of As (Banerjee et al., 2013; Fahad et al., 2016). Arsenic can enter plant cells by two main pathways. Arsenate ( $\text{As}^{\text{V}}$ ) as an analogue of phosphate can take up through high affinity phosphate transport channels, whereas aquaporin channels like silicon transporters have a critical role in  $\text{As}^{\text{III}}$  entrance into the roots (Abedin and Meharg, 2002; Ma et al., 2008; Wu et al., 2011). It is known that transfer of As to rice grains is greater

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than in other cereals, which might be due to differences in As species and dynamics in anaerobic and aerobic soils (Williams et al., 2007).

Arsenite is the dominant species of As in reducing environments such as flooded paddy soils and rice fields. Of additional concern is the evidence suggesting that paddy rice is particularly effective at accumulating high levels of As<sup>III</sup> when compared to other cereal species (Xu et al., 2008).

Availability of As can disturb normal functioning of plant biochemical and metabolic pathways, consequently leading to stunted growth and low crop productivity (Talano et al., 2013; Armendariz et al., 2016). Damage of cell membranes, lipid peroxidation and disruption of membrane integrity, electrolyte leakage, and reduction of transpiration intensity show the production of reactive oxygen species (ROS) at a level beyond cellular control, i.e., oxidative stress (Khan et al., 2009; Yadav, 2010). Generation of ROS occurs during the redox reactions between arsenate and arsenite or depletion of reduced glutathione in plants exposed to arsenic. Oxidative stress disrupts cellular redox state and interrupts the balance between oxidizing reactions and antioxidants in the cell. It leads to oxidation and modification of cellular amino acids and proteins and ultimately to cell death (Iqbal et al., 2010; Srivastava et al., 2011).

Uptake and distribution of metalloids within plant tissues depend on water flow (Wan et al., 2015). It has been reported that heavy metals disrupt plant water supply and consequently the water content. As a response, plants can trigger adaptive mechanisms, e.g. an increase may occur in compatible solutes that improve water retention by reducing water and osmotic potential (Yancey, 2005). In fact, greater tolerance to metals in plants has been attributed in part to a greater accumulation of compatible solutes (Khan et al., 2000; Anjum et al., 2016). Among them, glycine betaine and sugars have been shown to be involved in osmotic adjustment in several plants under different stress conditions (Khan et al., 2000; Yancey, 2005).

Plant hormones are ubiquitously present in plants and play central roles in regulating and coordinating growth, and are involved in all developmental processes and coordinate various signal transduction pathways (Spoel and Ong,

2008). Emerging evidence has demonstrated that synergistic and antagonistic effect of plant hormones regulate plant's response against metalloids by changing their endogenous levels and distribution (Peleg and Blumwald, 2011; Kazan, 2015). It has been reported that modulation of plant hormones can induce antioxidant response and accumulation of antioxidants in rice (Vassilev et al., 2004).

In the present study the effect of As<sup>III</sup> treatment on the plant water status, osmolytes, and endogenous levels of abscisic acid (ABA), in connection with the stomatal functions were evaluated in rice plants. In order to evaluate the impact of genetic/environment on As<sup>III</sup> effects, the parameters were compared in two Iranian rice cultivars from two different ecological areas.

## Material and Methods

### Plant materials and treatments

Seeds of two rice cultivars (*Oryza sativa* L.) Hashemi and Amber were used. Hashemi is a popular local rice variety characterized by high yield potential and long kernels, cultivated in the north of Iran with a moderate-humid climate and Amber which is cultivated in southern-west of Iran with a very warm, dry, and long summers and moderate winters (Tayefeh et al., 2018). The seeds were surface sterilized by immersing in 1% sodium hypochlorite solution for 5 min followed by rinsing with deionized water three times. The seeds were then allowed to germinate between two layers of moistened Watman paper for seven days. Uniform seedlings were selected and transferred to aerated hydroponic cultures containing Kamachi nutrient solution (Kamachi et al. 1991), pH 5.5. Plants were grown in a growth chamber with a 16 h light/8 h dark photoperiod, 24±2 °C, relative humidity 60% and 107 mmol s<sup>-1</sup>m<sup>-2</sup> PPFD (photosynthetic photon flux density). Nutrient solutions were renewed every 3 days. Arsenite was supplied in the form of sodium arsenite (Na<sub>2</sub>HAsO<sub>2</sub>). A preliminary study was conducted applying different concentrations from 0 to 250 μM of As<sup>III</sup>, and the plant growth was monitored in a time course manner. Based on the results of this study, 75 μM was selected as a concentration which inhibited the plants growth while the other desired parameters were measurable. Higher

concentrations were severely detrimental leading to death of the plants after 12 h. Therefore, twenty-day-old plants were treated with 75  $\mu\text{M}$   $\text{As}^{\text{III}}$  for 6 hours. Plants were harvested at different intervals, thoroughly washed with deionized water, gently blotted, frozen with liquid  $\text{N}_2$ , and stored at  $-80^\circ\text{C}$  until used for biochemical analysis.

### Determination of $\text{As}^{\text{III}}$ content

Total arsenic was determined according to Meharg and Jardine (2003) with a few modifications. In brief, the plants were digested in 5 mL of  $\text{HNO}_3$  (65%) and 1 mL  $\text{H}_2\text{O}_2$  (30%) at  $110^\circ\text{C}$ . The digest was diluted with a solution containing 10%  $\text{HCl}$ , 5% ascorbic acid, and 10%  $\text{KI}$ , and then analyzed using hydride generation–atomic absorption spectrometry (Shimadzu AA-6200, Japan) and inductively coupled plasma mass spectrometry (ICP-MS, Agilent 7500, Carnation, Washington, USA). Standard solutions containing different concentrations of  $\text{As}^{\text{III}}$  (Beach leaves material FD8, Commission of the European Communities, Joint Research Centre ISPRA) were used for calibration. As translocation factors were calculated as the shoot/root As concentration ratio (Duan et al., 2012).

### Relative water content (RWC)

Rice leaves were kept in plastic bags that were weighed previously to avoid weight loss. The sealed plastic bags with the leaf were weighed, and the weight of the bag was subtracted from the total weight to get the fresh weight. Two milliliters of 5 mM  $\text{CaCl}_2$  was added to each plastic bag, and the leaves were left to soak in the dark at room temperature for 7 to 9 h. Then, the leaves were gently removed from the bags and placed between two paper towels to absorb excess water, and the turgid weight was recorded. The leaves' total dry weight (DW) was measured after they had been dried in an oven at  $60^\circ\text{C}$  for 72 to 84 h. Leaf RWC was calculated as percentage =  $(\text{fresh weight} - \text{DW} / \text{turgid weight} - \text{DW}) \times 100$  (Sade et al., 2014).

### Total sugars

For determination of total soluble sugars, 1 g of rice shoots were dried in an oven (48 h at  $60^\circ\text{C}$ ) and dried tissues were boiled in 80% (v/v) ethanol for 2 min. Extracts were shaken for 20 min and centrifuged, the supernatant decanted, and the pellet extracted twice. The supernatants were combined, and final volumes were measured. Sample pigment was removed by adding 35 mg activated charcoal, and soluble sugars were assayed using the phenol–sulfuric acid method (Dubois et al., 1956).

### Determination of glycine betaine

Quaternary ammonium compounds (QACs) were determined as glycine betaine (GB) equivalents, as described by Rieve and Grattan (1983). Rice leaves were shaken with deionized water (24 h at  $25^\circ\text{C}$ ) and centrifuged at 10,000 rpm for 10 min. The supernatants were diluted (1:1) with 2N  $\text{H}_2\text{SO}_4$  and cooled in ice for 1 h. Then, 0.4 mL of cold  $\text{KI-I}_2$  reagent (15.7 g of iodine and 20 g of potassium iodide dissolved in 100 mL of distilled water) was added and gently stirred. The tubes were stored at  $4^\circ\text{C}$  for 16 h and then centrifuged at 10,000 rpm for 15 min at  $0^\circ\text{C}$ . The supernatant was discarded and periodide crystals were dissolved in 9 mL of 1,2-dichloroethane. After 2 h, absorbance was measured at 365 nm. A calibration curve was obtained using GB reference standards from 30 to 75  $\mu\text{g mL}^{-1}$  dissolved in 1N  $\text{H}_2\text{SO}_4$ .

### Stomata Statues

Stomata statue was studied using nail polish imprint of the widest part of the rice leaf blades at different treatment intervals. Five images from each replicate were captured using a light microscope (Olympus, BH2, Japan) and the stomatal opening were measured according to Volenikova and Ticha (2001).

## Measurement of ABA

Frozen shoots were extracted with 10 mL of methanol with mortar and pestle followed by sonication for 2 h at 4 °C and was left overnight at 0 °C. The methanolic extracts were centrifuged at  $16,000 \times g$ . The supernatant was filtered and concentrated by evaporation under dark conditions, and then filtered through 0.22- $\mu\text{m}$  BioFil filters. Aliquots (20 mL) of the filtrates were analyzed by high-performance liquid chromatography (HPLC) (Waters, New Hampshire, United States). The system was equipped with a C18 column (Perfectsil Target ODS3, 5  $\mu\text{m}$ , 250  $\times$  4.6 mm, MZ-Analysentechnik, Mainz, Germany). Phytohormones were eluted at a flow rate of 0.6 mL  $\text{min}^{-1}$  with a concave gradient of methanol and deionized water containing 0.67% acetic acid, pH 3. The gradient was as follows: from 80:20 to 70:30 (6 min) and then to 60:40 (15 min). Abscisic acid (ABA) was detected at 220 nm using an ultraviolet detector (PDA, Berlin, Germany) and was quantified by comparison of its retention times and peak area with genuine standards (Sigma) (Delavar et al., 2017).

## Statistical Analysis

All of the experiments were carried out with at least three independent repetition using three samples, and all of the data are expressed as the mean values  $\pm$  the standard deviation (SD). Statistical analysis was performed using LSD (least significant difference), and the differences between the treatments were expressed as significant at a level of  $p \leq 0.05$ .

## Results

Arsenite content of control rice plants was negligible before treatment (0.03 and 0.02  $\mu\text{g} \cdot \text{g}^{-1}$  FW $^{-1}$  in Hashemi and Amber, respectively). Upon exposure to As<sup>III</sup>, it was easily and rapidly absorbed by both cultivars and translocated to shoots, so that its content in the plant at 6 h of the treatment was detected as 34.28 and 21.04  $\mu\text{g} \cdot \text{g}^{-1}$  FW $^{-1}$ , in Hashemi and Amber, respectively (Fig. I). Transfer factor of As<sup>III</sup> from root to shoot of Hashemi was more than Amber and accumulated a high amount of absorbed As<sup>III</sup> in the shoots (Fig. II).

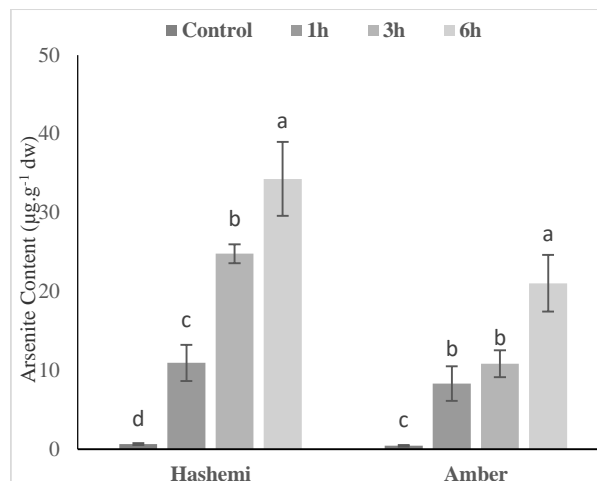


Fig. I. Arsenite uptake by two rice cultivars, Hashemi and Amber upon exposure to 75  $\mu\text{M}$  As<sup>III</sup> for 0, 1, 3, and 6 h; data are presented as mean  $\pm$  SD,  $n=3$ . Bars with different letters in each cultivar are significantly different at  $p \leq 0.05$  according to LSD test.

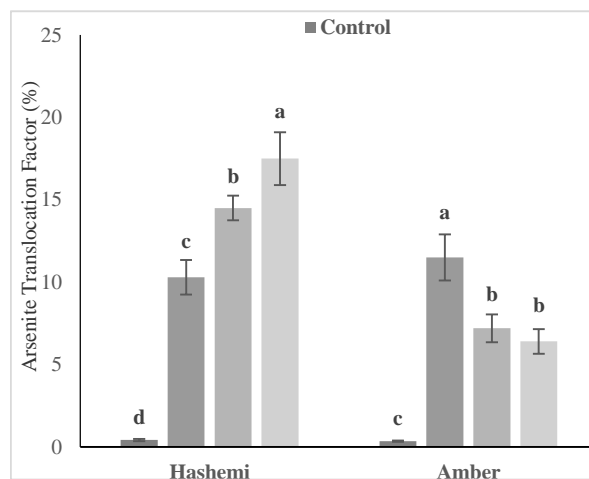


Fig. II. Arsenite translocation factor in two rice cultivars, Hashemi and Amber upon exposure to 75  $\mu\text{M}$  As<sup>III</sup> for 0, 1, 3, and 6 h; data are presented as mean  $\pm$  SD,  $n = 3$ . Bars with different letters in each cultivar are significantly different at  $p \leq 0.05$  according to LSD test.

The RWC of both rice cultivars were decreased in As<sup>III</sup>-exposed plants significantly. The rate of decrease of RWC was lower in Amber than Hashemi (25% vs 44%) (Fig. III).

As shown in Fig. IV, total soluble sugar content increased in the leaves of As-treated rice plants. The rate of increase of soluble sugar was more prominent in Amber (90%) than Hashemi (39%).

A significant increase in glycine betaine content was observed in As-treated plants after 6 h of treatment. Arsenite treatments led to about

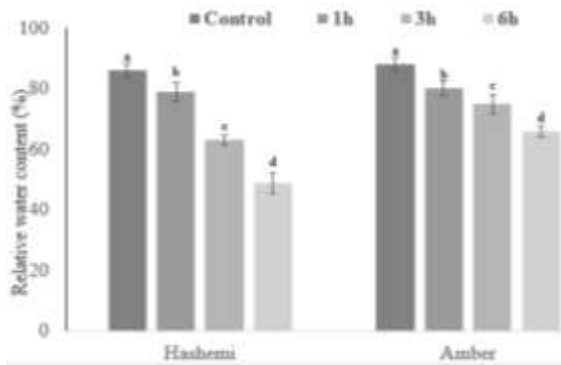


Fig. III. Alteration in Relative Water Content (RWC) of two rice cultivars Hashemi and Amber after exposure to 75  $\mu\text{M}$   $\text{As}^{\text{III}}$  for 0, 1, 3, and 6h. Data are presented as mean  $\pm$ SD, n=3. Bars with different letters in each cultivar are significantly different at  $p \leq 0.05$  according to LSD test.

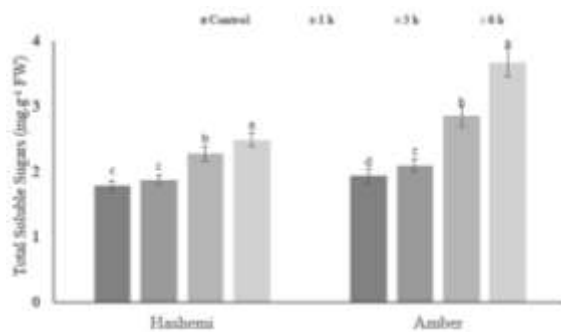


Fig. IV. Alteration in Total Soluble Sugars of two rice cultivars Hashemi and Amber after exposure to 75  $\mu\text{M}$   $\text{As}^{\text{III}}$  for 0, 1, 3, and 6h. Data are presented as mean  $\pm$ SD, n=3. Bars with different letters in each cultivar are significantly different at  $p \leq 0.05$  according to LSD test.

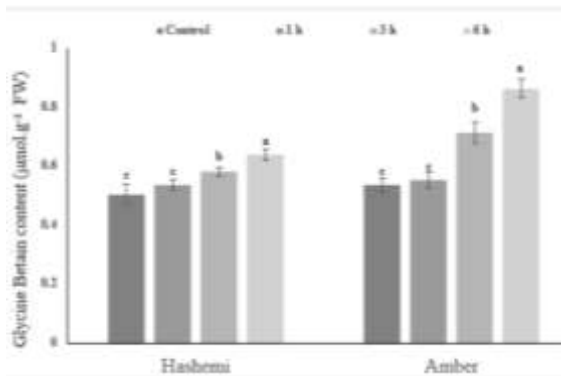


Fig. V. Alteration in glycine betaine content of two rice cultivars Hashemi and Amber after exposure to 75  $\mu\text{M}$   $\text{As}^{\text{III}}$  for 0, 1, 3, and 6 h; data are presented as mean  $\pm$  SD, n=3. Bars with different letters in each cultivar are significantly different at  $p \leq 0.05$  according to LSD test.

26% and 61% increment of glycine betaine content in Hashemi and Amber, respectively (Fig. V).

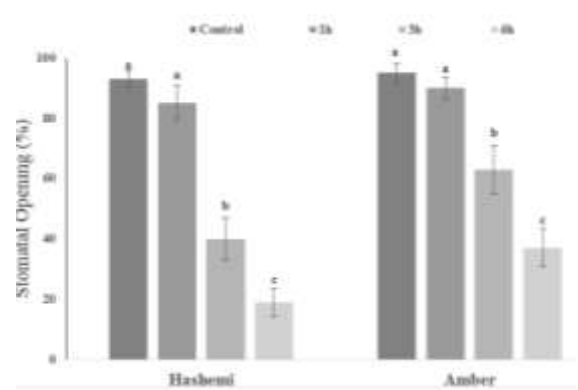


Fig. VI. Effect of  $\text{As}^{\text{III}}$  on stomatal opening of two rice cultivars Hashemi and Amber after exposure to 75  $\mu\text{M}$   $\text{As}^{\text{III}}$  for 0, 1, 3, and 6h. Data are presented as mean  $\pm$ SD, n=3. Bars with different letters in each cultivar are significantly different at  $p \leq 0.05$  according to LSD test.

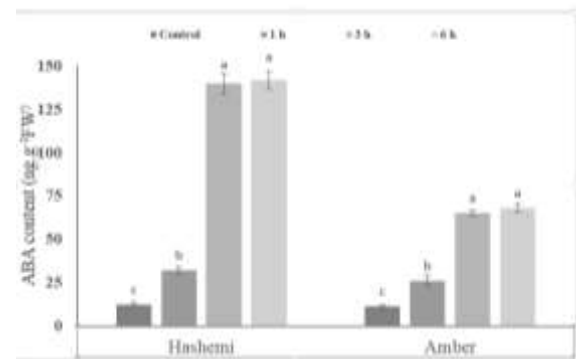


Fig. VII. The contents of Abscisic acid (ABA) along with the period of treatment with 75  $\mu\text{M}$   $\text{As}^{\text{III}}$  in two rice cultivars Hashemi and Amber; data are presented as mean  $\pm$  S D, n=3. Bars with different letters in each cultivar are significantly different at  $p \leq 0.05$  according to LSD test.

Arsenite treatment also decreased the stomatal opening percentage of both rice cultivars (Fig. VI). Interestingly, in Amber cultivar stomatal opening percentage (37%) and the possibility of water loss through respiration was higher than Hashemi (19%).

The content of ABA rapidly and significantly increased in  $\text{As}^{\text{III}}$ -exposed plant of both cultivars. Remarkable induction was observed in ABA content of both cultivars after 6 h  $\text{As}^{\text{III}}$  treatment, albeit with a much moderate tendency in Hashemi (12 and 6 fold of their controls in Hashemi than Amber, respectively) (Fig. VII).

**Discussion**

As described above, arsenite was rapidly absorbed by both examined rice cultivars during the treatment period and continuously translocated to the shoots (Fig. I). It is accepted that rice plant accumulates As higher than other cereals (Su et al., 2010) and the rate of uptake is associated with environmental As concentrations (Williams et al., 2006). There are also reports denoting that rice plant can accumulate relatively large amounts of As even from the soils with low concentrations of As (Ahmed et al., 2011). It has been revealed that organic forms transferred to the shoots with a larger magnitude than inorganic species and As<sup>III</sup> is dominant transportable form in both the phloem and the xylem (Carey et al., 2010).

In our study, exposure to As<sup>III</sup> significantly decreased RWC compared to control plants of both rice cultivars mainly in Hashemi (Fig. III). Rucinska-Sobkowiak (2016) reported that metals such as Cd, Ni, Pb, Cu, Co, Cr, and Zn induced a reduction in water absorption rate by roots and causes leaf dehydration in several plant species. Decrease of water absorption was reported in soybean plants treated with As<sup>III</sup> (Veza et al., 2018).

It has been widely accepted that As<sup>III</sup> uptake by rice plant, shares the highly efficient water pathway of entry to root cells and efflux into xylem through aquaporins (Ma et al., 2008; Bienert et al., 2008; Zhao et al., 2009). Investigating on the role of PIPs (plasma membrane located aquaporins) in As<sup>III</sup> toxicity in *Brassica juncea*, Sirvastava et al., (2013) observed the decrease of transcripts of PIP upon treatment of the plants with As<sup>III</sup>. They stated that decrease in water uptake and its flow within the plants is associated with the effects of As<sup>III</sup> on aquaporins. There is also possibility that functions of aquaporins are inhibited due to the binding of arsenite to sulfhydryl groups in their structures (Zhao et al., 2010). Although clarification of cross-talk between aquaporins and As<sup>III</sup> was out of the present study goals, the results presented here support a relationship between As<sup>III</sup> uptake and water content of rice plants. The fact that water uptake in Amber cultivar was not severely influenced by As<sup>III</sup> can be attributed to lower content of As<sup>III</sup> resulting in lower damage of water channels.

Arsenite treatment decreased the stomatal opening percentage of both rice cultivars. Interestingly, in Amber cultivar stomatal opening percentage and the possibility of water loss through respiration was higher than in Hashemi (Fig. IV). Similar adverse effects of As on RWC and stomatal opening were observed in *Vigna radiate* and soybean (Gupta and Bhatnagar, 2015). Stomatal closure by As<sup>III</sup> has been suggested as an adaptive response to the metalloid to reduce water loss by transpiration and avoid dehydration (Armendariz et al., 2016).

Results showed that total soluble sugars (Fig. IV) increased in the leaves of rice plants during 6 h of the As<sup>III</sup> treatment. We also observed the same results in glycine betaine concentration (Fig. V). Increase in soluble sugars may be due to alterations in enzyme activity involved in carbohydrate metabolism, a decrease in the utilization of carbohydrates for growth or a reduction in their translocation (Mishra and Dubey, 2013). Increase in osmolytes like soluble sugars and glycine betaine concentration may provide an adaptive mechanism to induce the ability of plant cells to maintain the favorable osmotic potential and retain water without disturbing cellular functions and it is a frequent acclimatization strategy under abiotic stresses (Yancey, 2005). It was observed that the greater tolerance to heavy metal stress is due to a greater osmolyte accumulation in plant tissues (Anjum et al., 2016). It has been well documented that this compounds stabilizes the structures and activities of enzymes and protein complexes and maintains the integrity of membranes against the damaging effects of stress conditions. The type and concentration of accumulated osmolyte depends on the plant species and the stressful situation (Chen et al., 2013; Wu et al., 2014).

Percentage of stomatal opening was significantly lower in As-treated rice plants at all times evaluated, reaching a reduction of between 63% and 80% in respect to control plants (Fig. VI). Similar results were observed in soybean (*Glycine max L.*) plants (Armendariz et al., 2016) and *Vigna radiate* treated with As (Gupta and Bhatnagar, 2015). Stomata closure is induced through three mechanisms, ABA pathway (hydroactive), leaf cell turgor reduction, and xylem sap pH change (hydropassive) (Franks, 2013).

Plant water content is regulated by ABA which promotes stomatal pore closure thereby helps the plant to water saving (Wilkinson et al., 2012). The role of ABA in maintenance of RWC and avoidance of dehydration under metalloids stress has been previously explained (Marchiol et al., 1996). The content of ABA significantly and continuously increased after exposure of rice plants to As<sup>III</sup> (Fig. VII). Arsenite-induced increase of ABA content was more remarkable in Hashemi than in Amber (respectively, 12 and 6 fold of their controls) and was in line with its lower stomatal opening percentage.

Although water is essential for plant growth and development, rice plants showed a reduction in water absorption rate when they were treated with solutions containing As<sup>III</sup>. Reduction in water content in the leaves of As-treated plants was identified as an early stress response. However, rice plants trigger a set of adaptive events that could be responsible for restoring the water status, including changes in osmolytes, hormones, and stomatal conductance. Accumulation of osmolytes such as glycine betaine and soluble sugars observed in leaves of As-treated plants reduced the water potential and increased the ability to retain water within the plant and could also be a consequence of osmotic potential reduction. Also, the rapid and significant increase in ABA content observed in As-treated plants, resulted in reduction of stomatal opening and it could contribute to avoiding water loss through the leaves. This suggests that both physiological and morphological changes could be involved in the complex network of responses that allow a restoration of the water status, in order to avoid dehydration in rice plants exposed to As.

Our results provide relevant knowledge on water relations in rice plants exposed to As<sup>III</sup>, contributing to the understanding of the response mechanisms developed by this crop in an As-contaminated environment. Regarding the ever-increasing semiarid and arid lands in the world it may be reasonable to suggest Amber and similar cultivars for agricultural sustainable development programs in As-contaminated soils.

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