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Arsenic stress effects on root water absorption in soybean plants: Physiological and morphological aspects

Mariana E. Vezza, Analía Llanes, Claudia Travaglia, Elizabeth Agostini, Melina A. Talano



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1 **Title**

2 **Arsenic stress effects on root water absorption in soybean plants: physiological and**
3 **morphological aspects**

4

5 **Mariana E. Vezza**^{a,c,*}, **Analía Llanes**^{b,c}, **Claudia Travaglia**^{b,c}, **Elizabeth Agostini**^{a,c}, **Melina A. Talano**
6 ^{a,c}

7 ^a Departamento de Biología Molecular, FCEFQyN, Universidad Nacional de Río Cuarto, Ruta
8 Nacional 36 Km 601, CP 5800 Río Cuarto, Córdoba, Argentina.

9 ^b Departamento de Ciencias Naturales, FCEFQyN, Universidad Nacional de Río Cuarto, Ruta
10 Nacional 36 Km 601, CP 5800 Río Cuarto, Córdoba, Argentina.

11 ^c Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Argentina.

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13 * Corresponding author. Tel.: +54 358 4676537; fax: +54 358 4676232.

14

15 E-mail addresses: marianavezza@gmail.com (M.E. Vezza), allanes@exa.unrc.edu.ar (A. Llanes),
16 ctravaglia@exa.unrc.edu.ar (C. Travaglia), eagostini@exa.unrc.edu.ar (E. Agostini),
17 mtalano@exa.unrc.edu.ar (M.A. Talano).

18 **ABSTRACT**

19 Soybean (*Glycine max* L.) is often cultivated in areas contaminated with arsenic (As), which
20 negatively affects plant growth and reduces crop yield. The deleterious effects may be due, at
21 least in part, to disturbances in the water status, as was reported for some plants exposed to
22 heavy metals. However, to our knowledge, these mechanisms have not been studied in depth in
23 soybean plants exposed to As. The aim of the present work was to analyze possible changes in
24 water relations and the responses developed in soybean plants under arsenate (AsV) and arsenite
25 (AsIII) stress. We discuss physiological and morphological aspects of the As stress response, such
26 as root absorption rate, water content, stomatal conductance, water and osmotic potential,
27 accumulation of compatible solutes, leaf conducting tissues and stomata characteristics. AsV and
28 AsIII caused a significant decrease in root absorption rate, which could reduce metalloid uptake.
29 On the other hand, water content decreased at the beginning of the treatment but was re-
30 established after 4 and 8 d. This was correlated with a decrease in stomatal conductance and a
31 reduction in leaf water and osmotic potential due to the accumulation of proline and soluble
32 sugars. Besides, smaller leaf xylem vessels and abnormal stomata were observed in plants under
33 As treatment. These mechanisms increased the plant's ability to retain water and therefore to
34 avoid dehydration. Thus, the results of the present work contribute to the understanding of how
35 soybean responds to As, by describing key tolerance strategies to the metalloid.

36

37 **Keywords**

38 Soybean; arsenic; water relations; stomata; leaf anatomy.

39 1. INTRODUCTION

40 Arsenic (As) contamination is an important environmental problem due to its worldwide
41 distribution and its high toxicity to all organisms (Zhao et al., 2009; Zhang et al., 2016). Presence
42 and mobilization of As occur due to natural biogeochemical reactions, but anthropogenic activities
43 like fossil combustion, mining and the use of pesticides and herbicides containing As increase the
44 concentration and mobilization of As in the environment. This metalloid can occur in soil and
45 water predominantly in its inorganic forms, such as arsenate (AsV) and arsenite (AsIII) (Smedley
46 and Kinniburgh, 2002; 2013). Arsenic can enter plant cells by channels of essential elements and
47 cause adverse effects in several metabolic processes, resulting in reduced germination, growth
48 and yield of some crops (Abedin and Meharg, 2002; Rahman and Naidu, 2009; Zhao et al., 2009).

49 Soybean (*Glycine max* L.) is one of the most important crops around the world due to its
50 nutritional value and its use for biofuel production. It is often cultivated under stressful conditions,
51 including soils with a high As concentration (Masuda and Goldsmith, 2009; Bundschuh et al., 2012;
52 Miransari, 2015). This situation is particularly relevant in Argentina, where a wide geographical
53 area is affected by high As levels in groundwater used for irrigation, as a result of the expansion of
54 agricultural land into arid regions (Nicolli et al., 2012; Sigrist et al., 2013).

55 Severe damage has been detected in the growth of soybean plants exposed to As: root and shoot
56 biomass is significantly lower, as is crop yield (Talano et al., 2013; Reichman, 2014; Armendariz et
57 al., 2016a). Bustingorri and Lavado (2014) observed a decrease of up to 45 and 30% in the biomass
58 and number of pods and beans, respectively, when plants were exposed to irrigation water
59 containing As from 10 mg/L upwards. These deleterious effects could be due to oxidative damage
60 and a decrease in photosynthetic pigments, as has been suggested by several authors (Armendariz
61 et al., 2016a; Spagnoletti et al., 2016). However, soybean plants induce detoxification pathways in
62 response to As. The activation of antioxidant enzymes to scavenge reactive oxygen species (ROS) is
63 one of these pathways (Armendariz et al., 2016a; Spagnoletti et al., 2016). Moreover, soybean
64 plants accumulate this metalloid mainly in the roots (Bustingorri and Lavado, 2014), possibly
65 through the formation of complexes with thiol compounds, particularly homo-glutathione (hGSH)
66 and homo-phytochelatin (hPC), and then through compartmentalization in the vacuole (Vazquez
67 et al., 2009). Despite the major accumulation of As in soybean roots, a small fraction of the
68 metalloid reaches the grains, which could pose a risk for human and animal health through the
69 food chain.

70 On the other hand, it has been reported that different heavy metals affect the water flow in
71 several plants, disturbing the water status (Stoeva et al., 2005; Vernay et al., 2007; Kholodova et
72 al., 2011; Rucińska-Sobkowiak et al., 2013). Water flow is important not only for the uptake of
73 mineral nutrients but also for the entry of metals/metalloids and their distribution within plant
74 tissues (Wan et al., 2015; Sultana et al., 2016). Thus, changes in the water flow dynamic could be
75 considered an adaptive strategy to regulate the uptake and translocation of metals, avoiding their
76 accumulation and toxicity. Nevertheless, a reduction of water flow in response to metals has been
77 associated with a reduction in radical growth, changes in aquaporins and plasmodesmata,
78 obstruction and size/number reduction of xylem vessels, as well as a decrease in stomatal density
79 and opening: all of them factors which reduce root absorption efficiency and the movement of
80 water within the plant (Rucińska-Sobkowiak, 2016 and references therein). This can affect the
81 water supply and consequently the water content, which puts plant survival at risk. As a response,
82 plants can trigger adaptive mechanisms, e.g. there may occur an increase in compatible solutes
83 that improve water retention by reducing water and osmotic potential (Yancey, 2005; Blum,
84 2011). In fact, greater tolerance to metals in plants has been attributed in part to a greater
85 accumulation of compatible solutes (Khan et al., 2000; Anjum et al., 2016). Among them, glycine
86 betaine, proline and sugars have been shown to be involved in osmotic adjustment in several
87 plants under different stress conditions (Khan et al., 2000; Yancey, 2005). However, their
88 participation in soybean response to As is unknown.

89 As previously mentioned, some important advances have been made in the understanding of how
90 soybean plants respond to As stress, mainly of those aspects related with the toxicity of the
91 metalloid (Bustingorri and Lavado, 2014; Armendariz et al., 2016a; Spagnoletti et al., 2016). Metal-
92 induced water deficiency, on the other hand, is a less explored process. In previous studies
93 performed in our laboratory we obtained some interesting evidence of radical biomass reduction,
94 dark deposits obstructing the xylem vessels and decreased stomatal opening in As-treated
95 soybean plants. All of these events could affect water flow (Armendariz et al., 2016a,b), but
96 complementary studies are necessary.

97 Based on these facts, the aim of the present work was to analyze possible effects of AsV and AsIII
98 on water relations and the responses developed to maintain plant water status. In particular,
99 different physiological parameters were evaluated: root absorption rate, water content, stomatal
100 conductance, osmotic and water potential, as well as accumulation of compatible solutes (proline,

101 glycine betaine and soluble sugars). Morphological alterations of leaves, including characteristics
102 of stomata and conducting tissue, were also evaluated.

103 To our knowledge, this is the first work focused on the study and discussion of several parameters
104 that contribute to the water status in soybean plants exposed to AsV and AsIII.

105

106 **2. MATERIALS AND METHODS**

107

108 **2.1 PLANT MATERIAL AND GROWTH CONDITIONS**

109 Soybean seeds (*Glycine max* L.) cv. DM 4670 were used. Seeds were surface-disinfected using 70%
110 (v/v) ethanol for 1 min and then 30% (v/v) sodium hypochlorite for 8 min. They were then washed
111 thoroughly with sterile distilled water, and germinated in Petri dishes with moistened filter paper
112 at 28°C in the dark for 3 d.

113 Germinated seeds were transferred to pots containing sterile perlite, irrigated by capillarity with ½
114 Hoagland and kept in a growth chamber (model E15, Conviron, Winnipeg, Canada) under the
115 following conditions: 14h light/10h dark cycle, 200 $\mu\text{mol m}^{-2} \text{s}^{-1}$ light intensity, 28°C and 80%
116 relative humidity.

117 Fourteen days later, when the plants reached V2 phenological stage, they were exposed to
118 solutions containing 25 μM sodium arsenate ($\text{AsHNa}_2\text{O}_4\cdot 7\text{H}_2\text{O}$) (SIGMA) (AsV), 25 μM sodium
119 arsenite (NaAsO_2) (SIGMA) (AsIII) or distilled water for control plants. After 1, 4 and 8 d several
120 water parameters were evaluated, as described below.

121

122 **2.2 EFFECT OF AS ON WATER RELATIONS**

123

124 **2.2.1 Root absorption rate**

125 To determine root absorption rate (AR), pots with plants were placed inside trays. A known
126 volume of irrigation solution (distilled water, AsV or AsIII) was added to the tray. To avoid
127 evaporation of the irrigation solutions, pots and trays were covered with film. The amount of
128 absorbed solution was estimated through the difference between tray weight at the beginning of
129 the experiment and tray weight after each evaluated treatment time (7h and daily for 8d). In
130 addition, root fresh weight (FW) was determined and the results were expressed as mL of
131 absorbed solution g^{-1} root FW.

132

133 **2.2.2 Total and relative water content**

134 Total (WC) and relative water content (RWC) were determined in roots and leaves, respectively, by
135 the gravimetric method (Llanes et al., 2013).

136 For WC determination, roots were separated and FW was immediately registered. Then, they were
137 dried in an oven at 60-80°C for 18 h until constant weight and dry weight (DW) was obtained.
138 Finally, WC was calculated as $WC = (FW - DW) / DW$.

139 For RWC determination, leaf discs of 1 cm diameter were obtained and FW was measured. Leaf
140 discs were always collected from the mid-section of each seedling in order to minimize age effects.
141 Turgid weight (TW) and DW were obtained after they had been incubated for 5h in Eppendorf
142 tubes containing distilled water and subsequently dried in an oven at 60-80°C for 13 h until
143 constant weight, respectively. RWC was calculated with the following equation:

$$144 RWC = (FW - DW) / (TW - DW) \times 100.$$

145

146 **2.2.3 Stomatal conductance**

147 Stomatal conductance ($\text{mmol m}^{-2} \text{s}^{-1}$) was measured on the abaxial side of fully expanded leaves
148 using a leaf porometer (model SC-1, Decagon Devices, Inc., Pullman, WA, USA) with an accuracy of
149 $\pm 10\%$. Instrument calibration was done prior to each set of measurements according to the
150 manufacturer's guidelines.

151

152 **2.2.4 Water retention of excised leaves**

153 Water retention (WR) ability of detached leaves was evaluated in soybean plants after 8 d of As
154 treatment, following Kholodova et al. (2011). Fully expanded leaves were separated and the cut
155 was sealed with parafilm. Leaves were incubated at 28°C under light. FW was registered
156 immediately after separation and after 5, 10, 20, 30 and 40 min.

157 It is assumed that leaf weight reduction after incubation represents water loss. Thus, WR was
158 calculated as the percentage of the initial water content that was retained in the freshly detached
159 leaf after incubation.

160

161 **2.2.5 Osmotic potential**

162 For osmotic potential (Ψ_o) determination, leaves and roots were cut into small pieces,
163 immediately frozen with liquid N_2 , heated at 100°C for 5 min and centrifuged at 10,000 rpm for 30
164 min (Llanes et al., 2013). This was repeated three times. An aliquot (150 μL) of cellular fluid was

165 placed in a capsule and osmolarity was measured with a freezing point osmometer (Semi Micro K-
166 7400; Knauer, Berlin, Germany). Osmotic potential was calculated as $\Psi_o=CxRxT$, where C is
167 osmolarity, R is the universal gas constant ($0.00831 \text{ MPa osmol}^{-1} \text{ K}^{-1}$) and T is absolute
168 temperature. Seven independent biological replicates were performed, each one composed with
169 material from 3 plants.

170

171 **2.2.6 Shoot water potential**

172 Shoot water potential (Ψ_w) was determined as described by Scholander et al. (1965). Shoots were
173 placed in a pressure bomb (model 10; Bio-Control, Buenos Aires, Argentina) and gradually
174 pressurized until the meniscus of the xylem sap became visible on the cut tissue surface. At this
175 time the pressure reading was recorded. This equilibrium pressure represents the water potential
176 value.

177

178 **2.3 EFFECT OF AS ON COMPATIBLE SOLUTES**

179 Plants treated with As solutions for 8 d, as described before, were harvested. Roots and leaves
180 were separated, immediately frozen and homogenized with liquid N_2 and kept at -80°C to be used
181 for proline, glycine betaine and total soluble sugar determination as detailed below. Three
182 independent biological replicates were performed, each one composed with material from 6
183 plants.

184

185 **2.3.1 Proline**

186 Proline content was determined following Bates et al. (1973). Homogenized tissues (150 mg) from
187 leaves and roots were mixed with 3 mL of 3% sulfosalicylic acid and centrifuged at 10,000 rpm for
188 15 min. One mL of supernatant was mixed with 1 mL of glacial acetic acid and 1 mL of acid-
189 ninhydrin (1.25 g ninhydrin dissolved in 30 mL glacial acetic acid and 20 mL 6 M phosphoric acid),
190 incubated for 1h at 100°C and then cooled in an ice bath. The reaction mixture was extracted with
191 2 mL of toluene and mixed vigorously for 20 s. The chromophore containing toluene was aspirated
192 from the aqueous phase and the absorbance was measured at 520 nm. Reference standards of
193 proline from 5 to 60 μM were prepared and analyzed in the same way to obtain a calibration
194 curve.

195

196 **2.3.2 Glycine betaine**

197 Quaternary ammonium compounds (QACs) were determined as glycine betaine (GB) equivalents,
198 following Grieve and Grattan (1983). Leaves (200 mg) and roots (300 mg) were mechanically
199 shaken with 3 mL and 1.5 mL of deionized water, respectively, for 24 h at 25°C. Then, they were
200 centrifuged at 10,000 rpm for 10 min and the supernatants were diluted (1:1) with 2N H₂SO₄.
201 Aliquots of 1 mL were taken into Eppendorf tubes and cooled in ice for 1 h. Then, 0.4 mL of cold
202 KI-I₂ reagent was added and gently stirred (KI-I₂ reagent: 15.7 g of iodine and 20 g of potassium
203 iodide dissolved in 100 mL of distilled water). The tubes were stored at 4°C for 16 h and then
204 centrifuged at 10,000 rpm for 15 min at 0°C. The supernatant was discarded and periodide crystals
205 were dissolved in 9 mL of 1,2-dichloroethane. After 2 h, absorbance was measured at 365 nm. A
206 calibration curve was obtained using GB reference standards from 30 to 75 µg mL⁻¹ dissolved in 1N
207 H₂SO₄.

208

209 **2.3.3 Total soluble sugars**

210 For soluble sugar determination, 50 mg of tissue (leaves and roots) were boiled in 1 mL of 80%
211 (v/v) ethanol at 70°C for 15 min and then centrifuged at 10,000 rpm for 15 min. The supernatant
212 was diluted (1:10) with distilled water. Sugar content of the resulting solution was determined
213 following Dische (1962): 250 µL of diluted extract were mixed with 1.25 mL of anthrone reagent
214 (120 mg anthrone dissolved in 60 mL of sulfuric acid solution: 250 mL of H₂SO₄ in 100 mL distilled
215 water) and heated at 95°C for 10 min. Absorbance was measured at 620 nm. Standards of glucose
216 from 20 to 100 µg mL⁻¹ were prepared and analyzed in the same way to obtain a calibration curve.

217

218 **2.4 EFFECT OF AS ON LEAF ANATOMY**

219 A histological and anatomical analysis of leaves was performed in plants treated with As solutions
220 after 8 d. For that, the youngest fully expanded leaves were processed following the method
221 described by D'ambrogio de Argüeso (1986).

222 First, the leaves were placed in FAA (95% ethanol/glacial acetic acid/37-40% formaldehyde/water;
223 50/5/10/35, v/v/v/v). Then, the samples were dehydrated according to the procedures outlined in
224 Johansen (1940), using solutions of ethanol and xylene. Finally, fully infiltrated tissues were
225 embedded in Histowax (highly purified paraffin wax blended with polymer additives). A series of
226 transverse sections with a thickness of 12 µm were obtained from sample blocks using a Minot
227 rotary microtome. The sections were placed on slides and triple-stained with hematoxylin,

228 safranin O and fast green FCF. Then, a coverslip was added to the slides with Depex. Leaf,
229 mesophyll and epidermis thickness, as well as xylematic vessel diameter were calculated.

230 For stomata analysis, an acrylic layer (synthetic colorless nail coating) was brushed onto the
231 abaxial leaf side, allowed to dry and carefully extracted, obtaining epidermal impressions.
232 Stomatal index (SI) and length and width of guard cells were determined. The SI was calculated as
233 $SI = SD / (SD + DEC) \times 100$, where SD is stomatal density and ECD epidermal cell density per unit area.
234 Leaf cross sections and epidermal impressions were assessed and photographed using a Zeiss
235 AxioPhot microscope equipped with image capture and digitization (AxioVision 4.3, with camera
236 AxioCam HRc 200x magnification) and images were analyzed with the Image-ProPlus program.

237

238 **2.5 STATISTICAL ANALYSIS**

239 Data were analyzed using the InfoStat program (v. 2012e; InfoStat, National University of Córdoba,
240 Argentina). ANOVA was performed and significant differences between treatments were
241 calculated by Tukey test with a significance level of 0.05 ($p < 0.05$). Prior to the significance test,
242 normality and homogeneity of variance were verified using the Shapiro-Wilk and Levene tests,
243 respectively. If the assumption of homogeneity of variance was not confirmed, data were
244 transformed using an appropriate function.

245

246 **3. RESULTS AND DISCUSSION**

247

248 **3.1 EFFECT OF AS ON WATER RELATIONS**

249

250 **3.1.1 Root absorption rate**

251 As can be seen in Fig. 1, plants treated with AsV and AsIII showed considerable AR reduction at all
252 times analyzed. AR for treated plants decreased between 25 and 39% in comparison with control
253 plants.

254 Minor AR is often caused by the presence of salts in soils. When salts are at a high concentration
255 osmotic water potential is reduced, affecting the ability of plants to absorb water, since water
256 flows by osmosis. However, the As treatment applied in the present work did not decrease the
257 osmotic potential, as checked by an osmometer, due to the low concentration used (25 μ M). For
258 this reason, AR reduction could be regulated instead by physiological or morphological changes in
259 these plants.

260 Regarding morphological changes which can explain minor AR, we previously demonstrated that
261 soybean plants treated with AsV and AsIII showed root cells with thicker and more lignified walls
262 and abundant dark deposits in the xylem vessels, mainly those treated with AsIII, plus a reduction
263 in root length and biomass (Armendariz et al., 2016a). A similar effect on root growth and
264 structure was observed in other plants exposed to As and other heavy metals (Rai et al., 2011;
265 Finnegan and Cheng, 2012; Yoon et al., 2015; Rui et al., 2016). Intercellular contact rupture and
266 membrane permeability decrease due to changes in plasmodesmata and aquaporins, respectively,
267 were also observed (Przedpelska-Wasowicz and Wierzbicka, 2011; Piršelová et al., 2012; Devi et
268 al., 2016). These structural changes reduce the capacity of plants to explore the soil and increase
269 the resistance to symplastic and apoplastic water transport.

270 These changes in water flow have been associated with the regulation of toxic metal uptake, and
271 might therefore work as adaptation strategies to avoid the entry of As into the root and its
272 translocation to aboveground tissues in non-hyperaccumulating or metal-sensitive plants. Sultana
273 et al. (2016) compared how several crops with different water requirements uptake As, and they
274 revealed that As accumulation was higher in those plants with higher water demand. Besides,
275 transpiration rate reduction produced low Cd accumulation in *Nicotiana tabacum* L. leaves (Liu et
276 al., 2016) and reduced As content in *Pteris vittata* L. shoots (Wan et al., 2015). Therefore, the low
277 water flow induced by As in soybean plants may be a mechanism for reducing metalloid uptake
278 and its translocation to the aerial part, as was suggested in a previous work from our laboratory
279 (Armendariz et al., 2016b).

280

281 **3.1.2 Total and relative water content**

282 It has been reported that the reduction in water absorption rate induced by metals such as Cd, Ni,
283 Pb, Cu, Co, Cr and Zn causes root and leaf dehydration in several plant species (Rucińska-
284 Sobkowiak, 2016 and references therein). In this sense, a significant decrease in leaf RWC (Fig. 2a)
285 and root WC (Fig. 2b) was observed in As-treated plants compared to control plants after 1 d of
286 treatment, mainly with AsIII. However, at 4 and 8 d there were no significant differences between
287 control and treated plants. These responses show that the reduced water absorption rate in the
288 treated plants causes a decrease in WC at the beginning of the treatment. This WC decrease could
289 act as a signal to activate mechanisms that allow a reduction in the loss of water and the
290 restoration of the water status. Spagnoletti et al. (2015) showed that a high As concentration in
291 soil considerably enhances the survival of soybean plants under extreme water stress, supporting

292 the hypothesis that the metalloid participates in the induction of responses tending to maintain
293 water equilibrium.

294 Among such response mechanisms, an increase in cell vacuole size is a recurrent response to
295 heavy metals (Nishikawa et al., 2003; Bazihizina et al., 2015). Rucińska-Sobkowiak et al. (2013)
296 observed a relation between increased cell vacuolization and high RWC values in *Lupinus luteus*
297 treated with Pb, suggesting that water can be stored in vacuoles in response to metal in order to
298 maintain the water status. Furthermore, it was observed that heavy metals can affect other
299 morphological and physiological features that regulate water loss through events in the roots and
300 leaves, such as inhibition of root sap exudation, a decrease in stomatal conductance and
301 transpiration, and an increase in water potential (Stoeva et al., 2005; Vernay et al., 2007;
302 Kholodova et al., 2011). These mechanisms might be responsible for the re-establishment of WC
303 observed in soybean plants after 4 and 8 d of As treatment.

304

305 **3.1.3 Stomatal conductance and water retention in leaves**

306 Stomatal conductance was significantly lower in As-treated soybean plants at all times evaluated
307 (Fig. 3), reaching a reduction of between 50 and 80% in respect to control plants. A significant
308 decrease in stomata ostiole size on both leaf sides was observed by Armendariz et al. (2016b) in
309 soybean plants treated with As, suggesting that the metalloid decreased stomatal conductance
310 through stomata closure. Similarly, ostioles in *Vigna radiata* were permanently closed under As
311 treatment (Gupta and Bhatnagar, 2015).

312 Stomata closure can be induced through hydroactive (abscisic acid [ABA] pathway) or
313 hydropassive (leaf cell turgor reduction or xylem sap pH change) mechanisms (Franks, 2013). In
314 previous studies we demonstrated that As triggers the phosphatidic acid (PA) signal (Armendariz
315 et al., 2016b). PA could therefore be part of the ABA signalling pathway and these events could be
316 responsible for stomata closure. However, there is a need for more studies on signal transduction
317 pathways induced by As in soybean.

318 Decrease in stomatal conductance via stomata closure could reduce water loss by transpiration in
319 the treated plants, achieving a balance between the amount of water that enters by radical
320 absorption and that which is lost through transpiration, in order to avoid dehydration. This is
321 supported by observations carried out in *Glycine max* plants exposed to Cd (Marchiol et al., 1996),
322 in which stomata closure contributed to the maintenance of high RWC under conditions of low
323 water uptake in treated plants.

324 Increased water retention ability in plants exposed to As was confirmed by WR determination in
325 excised leaves. WR was significantly higher in leaves of treated plants (Fig. 4), supporting the
326 hypothesis that As induces adaptive mechanisms to prevent water loss, such as the decrease of
327 stomatal conductance previously mentioned.

328

329 **3.1.4 Osmotic and water potential**

330 Reduction in Ψ_w is one of the adaptive strategies to maintain uptake and water flow under stress
331 conditions that generate water deficit. Ψ_w is mainly determined by osmotic (Ψ_o) and pressure
332 (Ψ_p) potentials. Thus, a decrease in Ψ_w can occur because of changes in Ψ_o (by compatible solute
333 accumulation) or Ψ_p (by dehydration) (Blum, 2011).

334 In this sense, a Ψ_w decrease in leaves of As-treated soybean plants was observed in all times
335 assayed (Fig. 5c). A significant decrease in Ψ_o was observed in the leaves of treated plants after 4
336 and 8 d of treatment (Fig. 5a), while no differences were observed in root Ψ_o between treated
337 and control plants (Fig. 5b).

338 Ψ_w decrease in the leaves of As-treated soybean plants could contribute to an increase in the
339 ability to retain water and reestablish water content in these plants, as was observed. Similarly, in
340 *B. oleracea* exposed to Co and Cd, a decrease in Ψ_w was followed by an increase in RWC (Pandey
341 and Sharma, 2002).

342 The decrease in Ψ_w after 1 d of treatment could be attributed mainly to a decrease in Ψ_p , as no
343 changes in Ψ_o were observed. At that time, there was a decrease in WC, which could lead to a
344 decrease in cell volume with a corresponding decrease in turgidity. At 4 and 8 d of treatment the
345 decrease in Ψ_w correlated with a decrease in Ψ_o . Since no differences were observed in leaf RWC
346 between treated and control plants after 4 and 8 d of treatment, it is possible that changes in leaf
347 Ψ_o could be due to osmotic adjustment caused by an increase in compatible solutes
348 accumulation.

349

350 **3.2 EFFECT OF AS ON COMPATIBLE SOLUTES**

351 Compatible solutes (or osmolytes) are organic metabolites of low molecular weight involved in Ψ_o
352 regulation and subcellular structure protection, among other functions. An increase in osmolyte
353 concentration enhances the ability of cells to retain water without disturbing cellular functions
354 and it is a frequent acclimatization strategy under abiotic stresses (Yancey, 2005). It was observed

355 that the greater tolerance to Al, Mn, Cd and As stress in different maize varieties is due in part to a
356 greater osmolyte accumulation (Khan et al., 2000; Anjum et al., 2016).

357 As shown in Fig. 6, proline (Fig. 6a) and soluble sugar (Fig. 6c) content increased in the leaves of
358 As-treated soybean plants, although no change was observed in glycine betaine content (Fig. 6e).
359 The type and concentration of accumulated osmolyte depends on the plant species and the
360 stressful situation (Meloni et al., 2004; Ashraf and Iram, 2005; Çiçek and Çakırlar, 2008; Chen et al.,
361 2013; Wu et al., 2014; Farhangi-Abriz et al., 2016). For example, Tipirdamaz et al. (2006) compared
362 the osmolytes accumulated in fifty-one plant species under saline conditions and they observed
363 that those plants which accumulated proline were poor accumulators of glycine betaine and vice
364 versa. Soybean could be classified as a low accumulator of glycine betaine, with an average
365 content of less than $5 \mu\text{mol g}^{-1} \text{DW}$ (Agboma et al., 1997). Moreover, glycine betaine increased less
366 than 1 time, while proline increased more than 34 times in young soybean plants exposed to salt
367 stress (Wu et al., 2014), which supports the idea that glycine betaine may play a minor role in the
368 osmotic adjustment with respect to other osmolytes.

369 Apart from being involved in drought and salt stress, proline has also been associated with heavy
370 metal stress (Slama et al., 2015). Proline accumulation is normally regulated by the activity of
371 enzymes Δ^1 -pyrroline-5-carboxylate synthetase (P5CS) and proline dehydrogenase (ProDH),
372 involved in its biosynthesis and catabolism, respectively, as observed in wheat under stress by Cu,
373 Cd and Zn (Li et al., 2013; Singh et al., 2016). On the other hand, several authors have reported
374 that sugars also tend to accumulate in shoots of plants exposed to different heavy metals due to
375 alterations in enzyme activity involved in carbohydrate metabolism, a decrease in the utilization of
376 carbohydrates for growth or a reduction in their translocation from the source (leaves) to sink
377 organs (root) (Jha and Dubey, 2004; Devi et al., 2007; Mishra and Dubey, 2013). Therefore,
378 considering our results, Ψ_o decrease induced by As in leaves of soybean plants could be achieved
379 through the accumulation of proline and soluble sugars.

380 In roots, an increase in proline (Fig. 6b), a decrease in soluble sugars (Fig. 6d), and no change in
381 glycine betaine content (Fig. 6f) was observed in treated plants, compared to control. The fact that
382 the increase of proline did not correlate to a decrease in root Ψ_o , suggests that it could have other
383 functions besides that of acting as a compatible solute. It has been reported that proline plays
384 significant roles in metal chelation, antioxidant defense and signaling under stress conditions
385 (Hayat et al., 2012). Exogenous application of proline increased antioxidant enzyme activities and
386 decreased the levels of oxidative stress markers such as superoxide radical, hydrogen peroxide

387 and malondialdehyde (lipid peroxidation) in *Solanum melongena* plants exposed to As (Singh et al.,
388 2015). Similar responses to proline were also found in *Phaseolus vulgaris* (Aggarwal et al., 2011)
389 and *Phoenix dactylifera* (Zouari et al., 2016) treated with Se and Cd respectively, confirming the
390 role of this amino acid in antioxidant defense responses under metal stress. Thus, soybean plants
391 treated with As (25 μ M) did not show oxidative damage (Armendariz et al., 2016a), a result which
392 may be related to an increase in proline.

393 The decrease in soluble sugar content in As-treated soybean roots could be due to changes in
394 photoassimilate production and translocation. As-induced stomata closure may negatively affect
395 CO₂ diffusion. In addition, a decrease in photosynthetic pigment content and damage to
396 chloroplast structure were observed under As treatment (Miteva and Merakchiyska, 2002;
397 Armendariz et al., 2016a). These events reduced the photosynthesis rate and, consequently,
398 photoassimilate production, as observed by other authors (Myśliwa-Kurczel et al., 2004; Stoeva et
399 al., 2005; Vernay et al., 2007). However, under adverse environmental conditions sugar transport
400 through the phloem is affected even before the inhibition of the photosynthesis process
401 (Ainsworth and Bush, 2011). In addition, it can be hampered by the disruption of the integrity
402 (e.g., callose deposition) or the underdevelopment of the phloem as suggested for metals such as
403 Cd (Mendoza-Cozatl et al., 2011; Lemoine et al., 2013 and references therein). These facts could
404 explain the lower sugar concentration in roots of As-treated plants.

405

406 3.3 EFFECT OF AS ON LEAF ANATOMY

407 It is becoming increasingly evident that heavy metals induce structural alterations with potential
408 functional consequences which can affect water relations. In a previous work, we analyzed the
409 effect of As on radical structure in soybean plants (Armendariz et al., 2016a). Here, we present As-
410 induced leaf structural changes.

411 In the analysis of leaf epidermis, there were no significant differences in stomatal density and
412 index between treated and control plants (Table 1), unlike what has been observed by other
413 authors. Stomatal index was reduced in *Beta vulgaris* (Sagardoy et al., 2010) under Zn treatment
414 and in *Sorghum bicolor* treated with Cd and Cu (Kasim, 2006), while metals like Pb, Cd, Cu or Zn
415 produced an increase in stomata number in *Helianthus annuus* (Kastori et al., 1992) and in *Vigna*
416 *radiata* treated with As (Gupta and Bhatnagar, 2015). Thus, the number of stomata is specific to
417 the type and concentration of heavy metals/metalloids, as well as to the plant species.

418 Although no differences were detected in the number of stomata, a high frequency (15%) of
419 anomalous stomata was observed in soybean plants treated with AsV (Fig. 7). Among the
420 abnormalities, round shaped stomata formed by larger guard cells and with thickened inner
421 periclinal walls were found. In addition, stomata formed by undifferentiated guard cells and with
422 smaller ostiole were observed, probably because the division between both guard cells was not
423 completed. Anomalous and non-functional stomata have been documented in response to several
424 heavy metals (Souza et al., 2005; Sagardoy et al., 2010; Gupta and Bhatnagar, 2015).

425 There is evidence to suggest that As may affect the cytoskeleton, interfering with microtubule
426 assembly (Dho et al., 2010; Gupta and Bhatnagar, 2015). Microtubules play an important function
427 in the development and differentiation of guard cells and in the opening-closing mechanism of
428 ostiole (Lucas et al., 2006; Yu et al., 2001). Thus, anomalous and non-functional stomata in As-
429 treated plants can be attributed to microtubule failure. The thickening of the periclinal wall in
430 guard cells of treated plants, which seems to be responsible for keeping the stomatal ostiole
431 partially closed (Gupta and Bhatnagar, 2015), and the smaller ostioles of undifferentiated stomata
432 could contribute to the reduction of water loss in soybean plants treated with AsV.

433 In addition, a decrease in the diameter of xylem vessels in vascular bundle was observed in the
434 leaves of plants treated with AsV and AsIII (Table 2). Similar changes were observed in roots in a
435 previous work (Armendariz et al., 2016a).

436 Taken together, these changes in stomata and conducting tissues can alter water relations by
437 affecting the amount of water loss through transpiration and the movement of water from root to
438 shoot.

439

440 **4. CONCLUSION**

441 Although water is essential for plant development, soybean plants showed a reduction in water
442 absorption rate when they were irrigated with solutions containing AsV and AsIII. Consequently, a
443 decrease in water content in the leaves and roots of As-treated plants was identified as an early
444 stress response. However, soybean triggers a set of adaptive events that could be responsible for
445 restoring the water status, including changes in water potential and stomatal conductance. The
446 reduction in water potential observed in leaves of As-treated plants could increase the ability to
447 retain water within the plant and could also be a consequence of osmotic potential reduction
448 through the accumulation of compatible solutes such as proline and soluble sugars. On the other
449 hand, the rapid and significant reduction in stomatal conductance observed in As-treated plants

450 could contribute to avoiding water loss through the leaves. In turn, lower stomatal conductance
451 could be due, at least in part, to abnormal stomata development, mainly under treatment with
452 AsV. This suggests that both physiological and morphological changes could be involved in the
453 complex network of responses that allow a restoration of the water status, in order to avoid
454 dehydration in soybean plants exposed to As.

455 Our results provide relevant knowledge on water relations in soybean plants exposed to AsV and
456 AsIII, contributing to the understanding of the response mechanisms developed by this crop in an
457 As-contaminated environment.

458

459 **Figure Legends**

460

461 Figure 1: Effect of As on root absorption rate of soybean plants treated for 8 d with 25 μM AsV or
462 AsIII and water (control). Results represent the mean \pm SE (n= 3). (*) indicates significant
463 differences compared to the control (Tukey test, $p \leq 0.05$).

464 Figure 2: Effect of As on relative (RWC) and total water content (WC) of leaves (a) and roots (b),
465 respectively, from soybean plants treated for 1, 4 and 8 d with 25 μM AsV or AsIII and water
466 (control). Results represent the mean \pm SE (n= 20 for RWC; n= 10 for WC). Different letters indicate
467 significant differences (Tukey test, $p \leq 0.05$).

468 Figure 3: Effect of As on stomatal conductance of abaxial leaf side in soybean plants treated for 1,
469 4 and 8 d with 25 μM AsV or AsIII and water (control). Results represent the mean \pm SE (n= 6-8).
470 Different letters indicate significant differences (Tukey test, $p \leq 0.05$).

471 Figure 4: Effect of As on water retention (WR) ability of detached leaves from soybean plants
472 treated for 8 d with 25 μM AsV or AsIII and water (control), after different incubation times.
473 Results represent the mean \pm SE (n= 6-7). (*) indicate significant differences compared to the
474 control (Tukey test, $p \leq 0.05$).

475 Figura 5: Effect of As on osmotic potential of leaves (a) and roots (b), and water potential of
476 leaves (c) from soybean plants treated for 1, 4 and 8 d with 25 μM AsV or AsIII and water
477 (control). Results represent the mean \pm SE (n= 7). Different letters indicate significant differences
478 (Tukey test, $p \leq 0.05$).

479 Figure 6: Effect of As on proline (a-b), total soluble sugars (c-d) and glycine betaine (e-f) content of
480 leaves (a-c-e) and roots (b-d-f) from soybean plants treated for 8 d with 25 μM AsV or AsIII and

481 water (control). Results represent the mean \pm SE (n= 3). Different letters indicate significant
482 differences (Tukey test, $p \leq 0.05$).

483 Figure 7: Microphotographs of abnormal stomata in abaxial leaf epidermis of soybean plants
484 treated with 25 μ M AsV: large stomata (a), round stomata and guard cells with thickened inner
485 periclinal walls (b-c), undifferentiated stomata with small ostiole (d-g). s, stomata; gc, guard cell; o:
486 ostiole. Scale 25 μ m.

487

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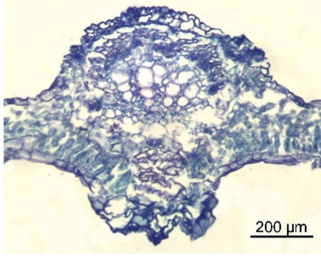
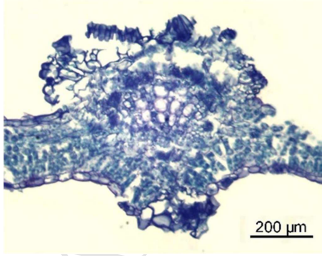
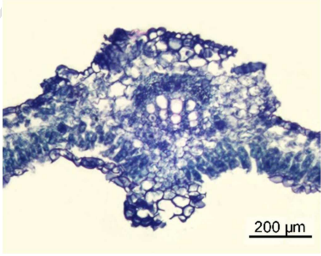
691 **Acknowledgements**

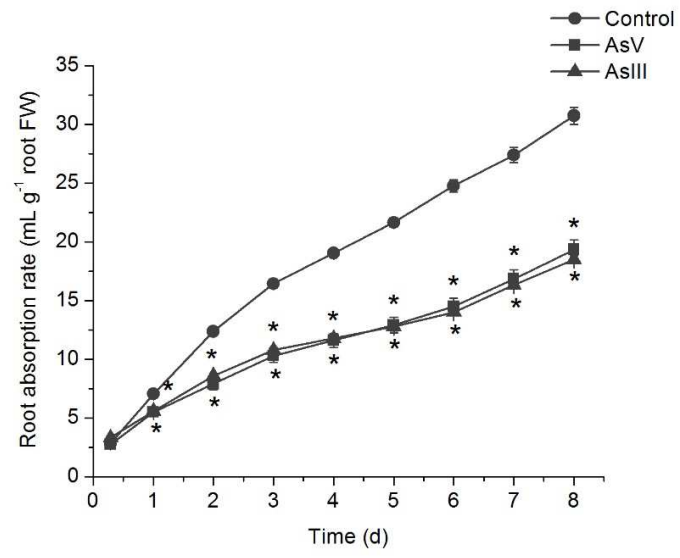
692 MV is a CONICET scholarship. AL, CT, EA and MT are members of the research career from Consejo
693 Nacional de Investigaciones Científicas y Técnicas (CONICET) (Argentina). We wish to thank
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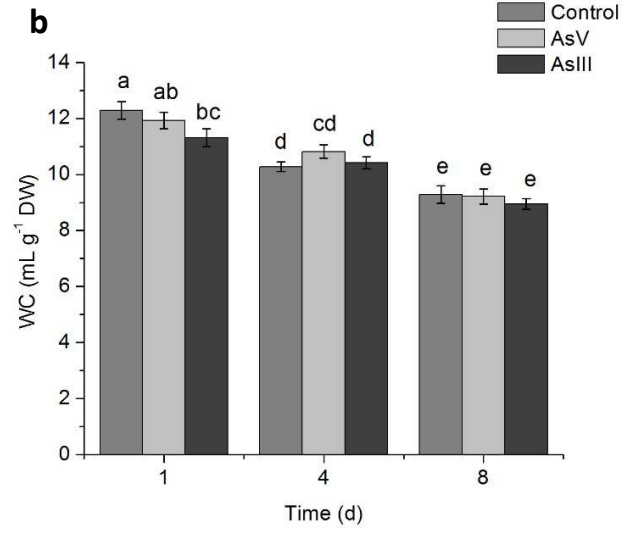
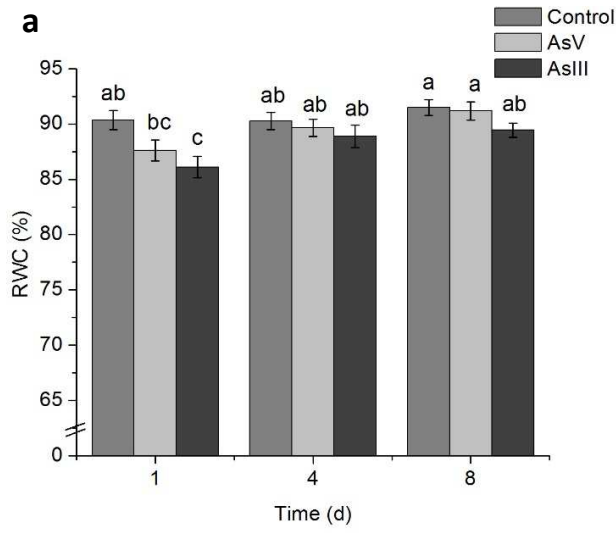
Table 1: Effect of As on stomatal density (SD), epidermal cell density (ECD), stomatal index (SI), length and width of guard cells in abaxial leaf epidermis of soybean plants treated with 25 μ M AsV or AsIII. Results represent the mean \pm SE (n= 28). Different letters indicate significant differences (Tukey test, $p \leq 0.05$).

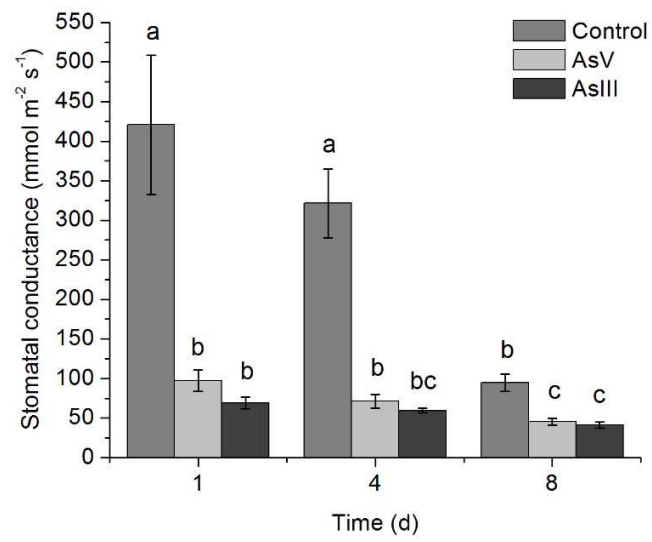
	SD	ECD	SI	Length of guard cells (μ m)	Width of guard cells (μ m)
Control	28.90 \pm 1.57 ^a	106.94 \pm 4.00 ^a	21.17 \pm 0.44 ^a	22.58 \pm 0.28 ^a	4.19 \pm 0.09 ^a
AsV	28.53 \pm 2.01 ^a	122.33 \pm 3.26 ^a	18.88 \pm 0.96 ^a	24.26 \pm 0.59 ^b	5.24 \pm 0.11 ^b
AsIII	28.10 \pm 3.30 ^a	110.52 \pm 9.93 ^a	20.19 \pm 1.23 ^a	23.48 \pm 0.45 ^{ab}	4.08 \pm 0.09 ^a

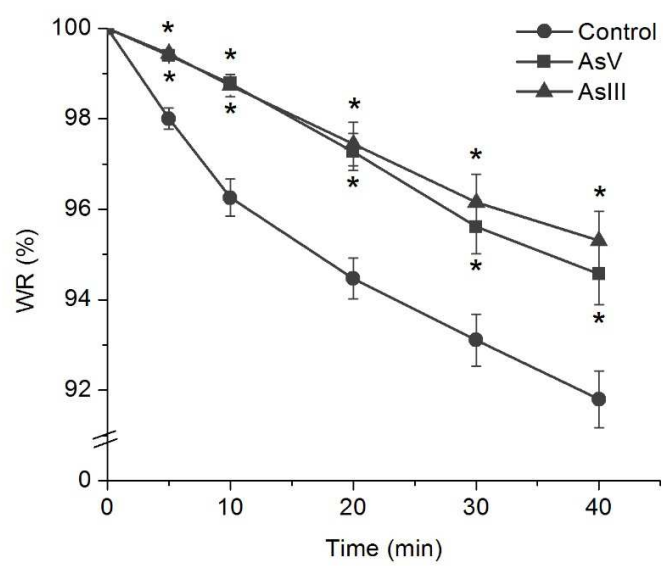
Table 2: Effect of As on leaf, mesophyll and epidermis thickness and xylematic vessel diameter in leaf from soybean plants treated with 25 μM AsV or AsIII. Results represent the mean \pm SE (n= 28). Different letters indicate significant differences (Tukey test, $p \leq 0.05$).

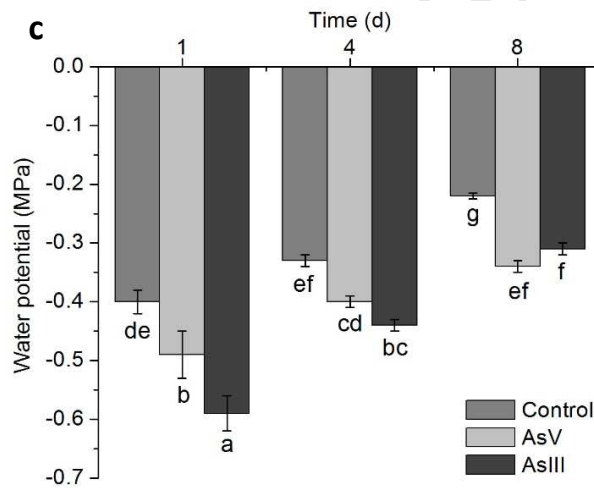
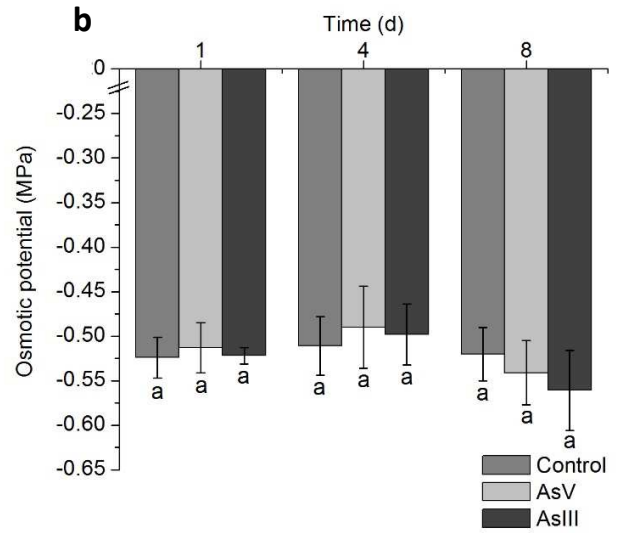
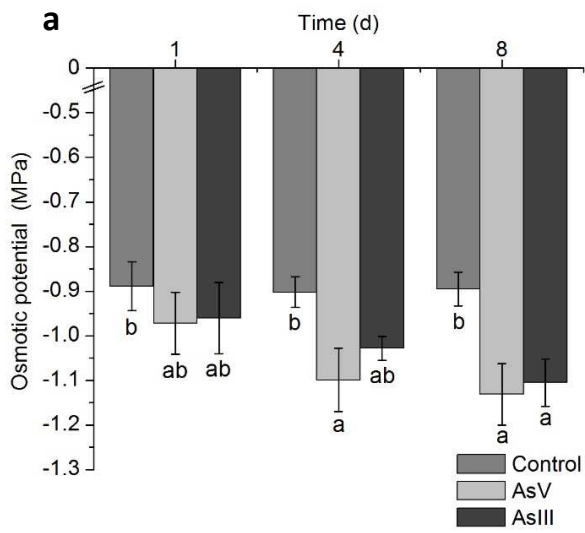
	Control	AsV	AsIII
Leaf thickness (μm)	126.53 \pm 0.88 ^a	112.25 \pm 1.15 ^c	117.10 \pm 1.46 ^b
Mesophyll thickness (μm)	91.01 \pm 0.80 ^a	82.12 \pm 1.28 ^b	84.29 \pm 1.35 ^b
Epidermis thickness (μm)	12.29 \pm 0.16 ^a	12.36 \pm 0.15 ^a	12.70 \pm 0.15 ^a
Xylematic vessel diameter (μm)	801.03 \pm 32.58 ^a	457.49 \pm 38.27 ^c	567.23 \pm 21.43 ^b
Microphotographs of leaf cross-sections.			

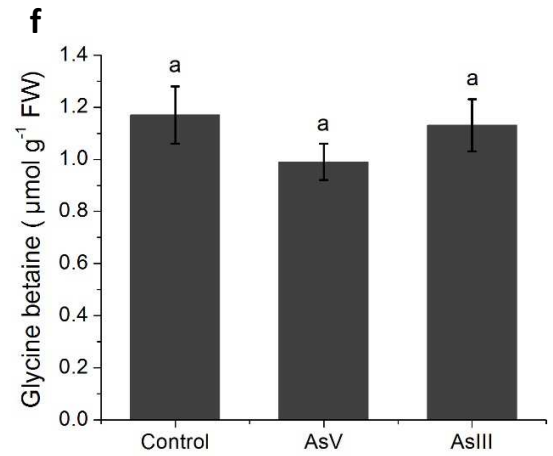
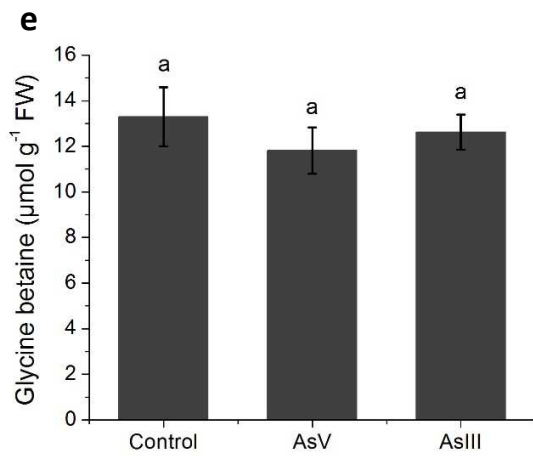
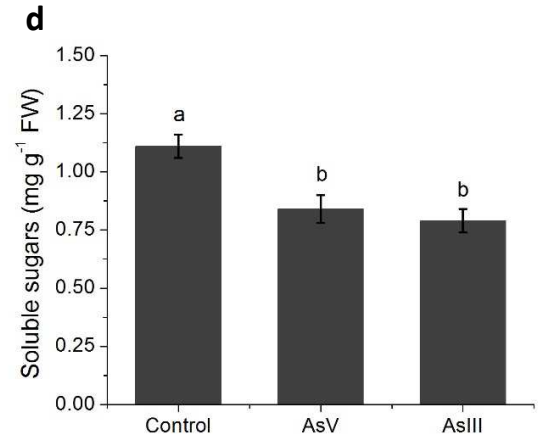
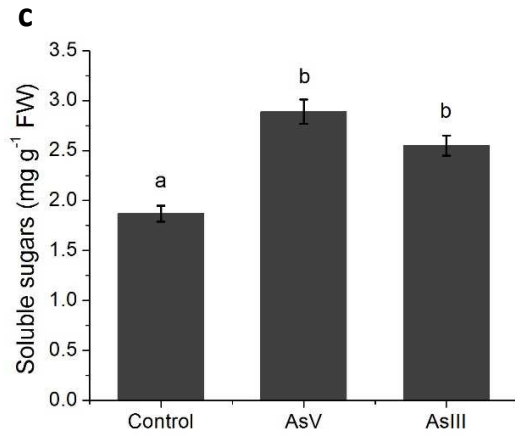
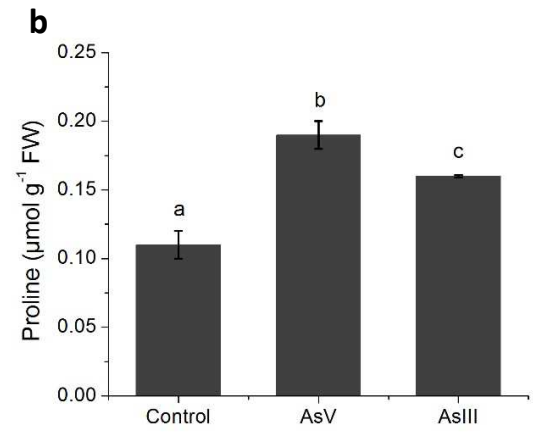
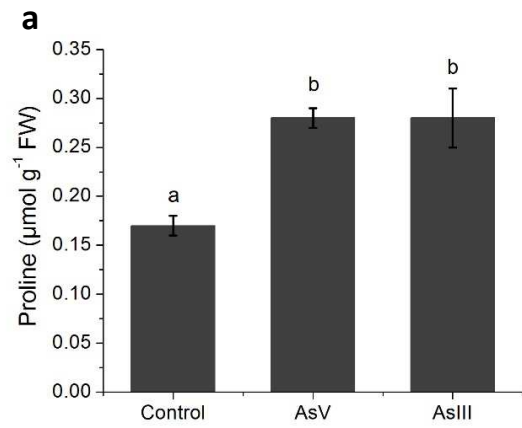


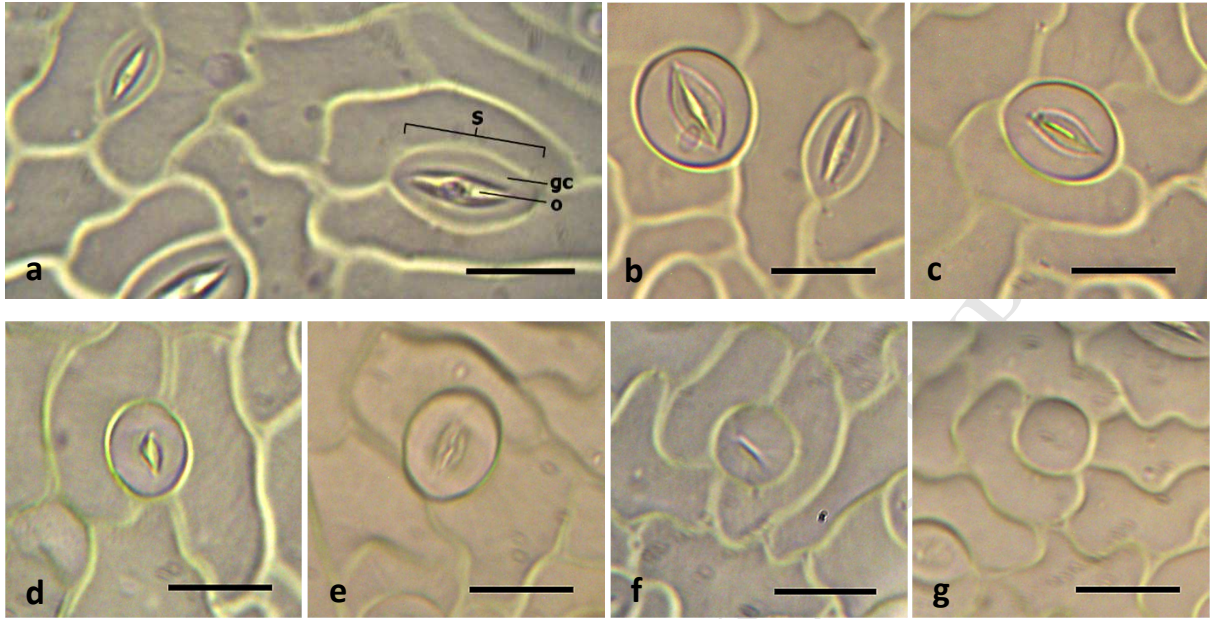












Highlights

Soybean plants reduced absorption of solutions containing As.

Arsenic caused disturbance of water balance in soybean plants as a stress response.

Adaptive mechanisms restored water status and avoided cellular dehydration.

Arsenic-treated plants showed reduction on stomatal conductance.

Increase of compatible solute production reduced osmotic potential in leaves.

Authors contribution

The research work was carried out with equal contribution from all the authors. MV did all the experiments. AL contributed with the execution and analysis of assays on water relations parameters. CT contributed with the execution and analysis of microscopical assays. MV, EA and MAT designed the experiments, analysed the results and wrote the paper.