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Physiological response of *Cistus salviifolius* L. to high arsenic concentrations

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Abstract

Arsenic is a trace element found in the environment which can be particularly toxic to living organisms. However, some plant species such as those of the genus *Cistus* are able to grow in soils with high As concentrations and could be used in the sustainable rehabilitation of mining areas through phytostabilization. In this work, the growth and the physiological response of *Cistus salviifolius* L. to As induced oxidative stress at several concentrations (reaching 30 mg L^{-1}) in an hydroponic system were evaluated for 30 days. Several growth parameters, chlorophyll content, chemical composition, one indicator of oxidative stress (H_2O_2) and two of the major anti-oxidative metabolites (ascorbate and glutathione) were analyzed.

The toxic effect of As was better perceived in the plants submitted to treatments with concentrations of 20 and 30 mg As L^{-1}. Plants subjected to these treatments had higher concentration of As in roots and shoots. The concentrations of Ca, Mg, K and Fe in the plants, as well as a large part of the evaluated growth parameters were also affected. Arsenic did not interfere with the ability of the plant to perform photosynthesis, as there were no significant differences in the contents of chlorophyll *a*, *b* and total between the different treatments. Plants from all treatments accumulated higher amount of As in roots than in shoots, and it was also in the roots that the concentrations of H_2O_2, AsA and GSH were higher. *Cistus salviifolius* showed high tolerance to As up to the concentration of 5 mg L^{-1}, which makes it a species with high potential to be used in the phytostabilization of soils contaminated with As and presenting high concentrations of the element in the soil solution.

Introduction

Plants are, during their life span, exposed to a countless variety of abiotic stresses, as widespread and diverse as drought, salinity, high or low temperatures, soil pH, lack of nutrients and potentially hazardous elements in excess (Mittler et al. 2004). In fact, the mere location of a plant can cause stress if the plant does not adapt to changes in the environment or if those changes occur fast or forcefully. One important abiotic stress factor is the presence of metal(loid)s in excess in the plant's environment. An element that is present in small concentrations (<100 mg/kg) in living organisms is termed trace element (Alloway 2013). Despite their occurrence at such small concentration, they may affect biological processes both positively as well as deleteriously depending on the element essentiality threshold limit and organism (Robinson et al. 2006). Some potentially hazardous elements that are not necessary for plants to complete their life cycle, such as As, are toxic even in minute quantities. In fact, As is one of the contaminants found in the environment which is particularly toxic to man and other living organisms (Chutia et al. 2009) and its toxicity depends on the species of As present. However, according to some authors As, in very low concentrations, seems to be beneficial for plants (Evans et al. 2005) and essential for animals (Uthus 1992). The soil properties as pH, redox conditions, mineral composition, and microbial activity influence the oxidation state and whether As is in the organic or inorganic form. The predominant species of As in the environment are the inorganic species arsenate(III) and arsenate(V), although the organic forms may also exist (Andrianisa et al. 2008). Usually, inorganic species of As are considered more toxic than the organic forms (Chutia et al. 2009; Vaclavikova et al. 2007) although the trivalent methylated arsenic species are in fact more toxic than inorganic As species because they are highly harmful to DNA (Vaclavikova et al. 2007). The As(V) is also less toxic than As(III), but it is able to endure longer in the environment, easily accumulating to toxic levels because it is more stable and it is also carcinogenic to humans (Yusof and Malek 2009).

Potentially hazardous elements occur naturally in soil derived from volcanoes and continental dusts to processes of weathering of parent materials, but their levels are low (<1000 mg kg^{-1}) and rarely attain toxic values (Wuana and Okieimen 2011). According to Kabata-Pendias (2011), the concentration of As considered normal for leaves of most plants is 1–1.7 mg kg^{-1}, while concentrations in the range of 5–20 mg kg^{-1} are considered as phytotoxic. When the internal concentration of an element, even if essential, exceeds a threshold limit, it becomes toxic to the plant and can stimulate the production of ROS (Reactive Oxygen Species; Rout et al. 2014). The symptoms of As toxicity observed in plants range from internerval chlorosis, followed by foliar necrosis (Melo et al. 2007) to the decrease in plant growth and fruit size (Carbonell-Barrachina et al. 1995; Kabata-Pendias 2011; Sneller et al. 1999); discoloration and root plasmolysis, wilting and necrosis of leaf tips and margins (Kabata-Pendias 2011); decrease in leaf area and photosynthetic capacity (Marin et al. 1993); leaf blight and reddening (Sneller et al. 1999). Because As is an analogue of P, it can enter the plant and be translocated using phosphate carriers, leading to phosphate deficiencies (Meharg and Hartley-Whitaker 2002; Tu and Ma 2003).

Mediterranean shrub species such as *Erica andevalensis* Cabezudo and Rivera and *Erica australis* L. have been found to be tolerant to As and low soil pH values, typical in mining areas (Abreu et al. 2008; Márquez-García et al. 2012). Species such as *Cistus ladanifer* L., *Cistus monspeliensis* L., *Daphne gnidium* L., *Rumex induratus* Boiss. & Reut. and *Genista hirsutus* Vahl are also tolerant and are indicated as potential phytostabilizing plants (Anawar et al. 2011, Abreu and Magalhães 2009, Carvalho et al. 2019). The concentration of As in the tissues of these plants is significantly lower than that present in the soil, although it exceeds the toxic limit for other species. Several studies point to the possible use of *Cistus salviifolius* L. in the phytostabilization of degraded and contaminated areas, as is the case of mining areas, due to its tolerance to high concentrations of metals and metalloids (Santos et al. 2011; Abreu et al. 2012a,b).

85 The present work aims to understand the ability of *C. salviifolius* to withstand high
86 concentrations of As and to evaluate its potential to be used in phytostabilization programs in areas
87 contaminated with As and having high concentrations of the element in the soil solution, particularly
88 those in the vicinity of mines. To assess this, the species germination capacity, growth parameters
89 and physiological response were evaluated in a hydroponic system with aqueous solutions containing
90 different As concentrations and low pH values.

91

Material and Methods

Experimental material

Cistus salviifolius L. seeds were collected in the mining area of São Domingos (SE of Portugal). The seeds undertook a treatment to break their dormancy that consisted of 10 min heating at 100 ºC. The seeds were disinfected with 5% sodium hypochlorite for 5 min under agitation at 300 rpm and then washed with distilled water.

Germination treatments

Two germination experiments were performed, the first in aqueous solutions at different pH values of the acid range and the other in aqueous solutions at different As concentrations. The pH treatments were the following: 2.5, 3.0, 4.0, 5.0, 6.0 and 7.2. Solutions were prepared from dilutions of a sulfuric acid solution with distilled water except the last one that corresponds to water alone. Arsenic treatments were the following: 0.0, 0.025, 0.1, 0.5, 1.0, 1.5, 3.0 and 5.0 mg L^{-1}. Solutions were prepared from dilutions of $Na_2HAsO_4 \cdot 7H_2O$ in distilled water and set to pH 4. The last one was the control. In both experiments 25 seeds were placed in Petri dishes of 11 cm Ø, over three leaves of filter paper Whatmann nº1, previously autoclaved and soaked with 5 mL of the aqueous solution to test. For each pH value and As concentration four Petri dishes were used. The seeds were placed to germinate in a growth chamber with 16/8 h photoperiod and 25/22 °C temperature, under a light intensity of 50 ± 5 µmol quanta m^{-2} s^{-1}. The number of germinated seeds was registered every other day for 21 days (pH treatment) or 30 days (As treatment), at which moment the roots, shoots and largest leaf were measured in all germinated seedlings.

Experimental set-up and monitoring

After performing the germination treatments the As concentrations and pH values for the hydroponic experiment were chosen. Seeds were germinated on 1.5 mL Eppendorf tubes with the lids and bottoms

115 removed. The tubes were filled with enough rock wool to hold five seeds. Germination occurred in a
116 passive hydroponic system with deionized water at pH 4 adjusted with sulphuric acid, in a growth
117 chamber (500L Aralab, Porto Salvo, PT) with 70% humidity 12/12 h photoperiod, 25/22 ºC and 130
118 ± 5 µmol quanta m^{-2} s^{-1}. After individuals had reached a height of 2 cm (*circa* two weeks) they were
119 moved to the hydroponic system with nutrient solution (Rossini et al. 2010) and forced air provided
120 by aquarium pumps. When plants reached 3 cm the hydroponic system with the As treatments began,
121 with one plant per Eppendorf tube and 20 plants per treatment. Growth conditions were the same as
122 described for germination. The treatments had a 30 day duration and sampling of four plants per
123 treatment took place every ten days (d0, d10, d20 and d30) and the As treatments were obtained by
124 applying As, in the form of $Na_2HAsO_4 \cdot 7H_2O$ in the concentrations of 0.0 (As0, control), 0.5 (As0.5),
125 1.5 (As1.5), 5.0 (As5), 20 (As20) and 30 (As30) mg L^{-1} to the nutrient solution.

126 On d0, d10 and d20 four plants were harvested, separated in roots and shoots, frozen in liquid
127 nitrogen and kept at –80 ºC in a deep-freezer for determination of pigments, H_2O_2, ascorbate and
128 glutathione. Four plants were monitored every ten days during the whole assay for leaf area, shoot
129 height, root length and fresh biomass and returned to the assay. At the end of the assay, the remaining
130 eight plants were harvested and the shoots were separated from the roots, and height, fresh biomass
131 and leaf area of four individuals were quantified. Roots and shoots were washed with tap water
132 followed by distilled water, they were then dried at 60 °C and finely ground for determination of
133 multielemental concentration. The roots and shoots of the remaining four plants were frozen in liquid
134 nitrogen and kept at –80 ºC in a deep-freezer for determination of pigments, hydrogen peroxide
135 (H_2O_2), ascorbate and glutathione.

136 Growth analysis

137 Plant height was measured with a ruler, fresh biomass was obtained by weighting the plants in an
138 analytical scale (Mettler Toledo) after removing surface excess water with paper towels and dry
139 biomass was quantified in the same scale after drying in a chamber at 56 ºC until constant weight was

obtained. Leaf area was obtained by measuring the length and width of five leaves and applying the equations of Nakamura et al. (2005). Leaf area ratio (LAR) is defined as the leaf area (in m^2) that is used to produce one gram of dry biomass and was calculated by dividing the measured leaf area by the quantified dry biomass.

Multielemental concentration in *C. salviifolius* plants

Samples of shoots and roots were digested with ultrapure concentrated nitric acid (69%) under pressure in a microwave digester (CEM MDS 2000) at 650 W using three phases of pressure (45 Psi for 6 min, 90 Psi for 6 min and 150 Psi for 10 min), with a total duration of circa 45 min. After digestion in a fume hood, samples were diluted to 10 mL with deionized water. Extracts were then analysed for concentrations of total As and other elements (Ca, Cu, P, Fe, K, Mg, Mn, B, Al, Zn, Mo and Na) by Inductively Coupled Plasma-Mass spectrometry (ICP-MS) (Thermo X Series). Certified reference samples of bush branches and leaves (NCSDC73348) and blanks were used to test the accuracy of the method.

Quantification of pigments, antioxidants and H_2O_2

In order to quantify pigments (chlorophylls and carotenoids), the frozen leaf samples were macerated in acetone: Tris-HCl 100 mM (80:20). Chlorophyll *a* (chl*a*), chlorophyll *b* (chl*b*), total chlorophyll (chl*t*) and carotenoids (car) concentrations were analysed by spectrophotometry in a microplate reader (Sinergy HT, Biotec, Winooski, USA) at 537, 647, 663 and 470 nm, using the equations described by Porra et al. (1989) and expressed according to Richardson et al. (2002).

In roots and shoots, hydrogen peroxide production was assayed following the method described by Jiang et al. (1990) using the peroxide-mediated oxidation of Fe^{2+}, followed by the reaction of Fe^{3+} with xylenol orange. This method produces reproducible results in the 0.1–1 mM H_2O_2 concentration range. For the determination of H_2O_2, 500 µL aliquots of the extracted material were added to 500 mL of the reaction mixture, which contained 500 mM ammonium ferrous sulphate, 50 mM sulphuric

164 acid, 200 mM xylenol orange, and 200 mM sorbitol. After 45 min at room temperature, the changes
165 in A560 were evaluated.

166 Reduced (GSH) and oxidised (GSSG) glutathione were analysed colorimetrically by the 2-
167 vinylpiridine method described by Anderson et al. (1992) using frozen leaf and root material (0.1 g)
168 ground in the presence of liquid nitrogen. Absorbance was recorded at 412 nm in a microplate reader
169 The percentage of reduction corresponds to the percentage of GSH in the total glutathione pool and
170 is defined as GSH/(GSH + GSSG) ×100.

171 Ascorbic (AsA) and dehydroascorbic (DAsA) acids were assayed using a method adapted from
172 Okamura (1980) by Carvalho and Amâncio (2002) using frozen leaf and root material (0.1 g) ground
173 in the presence of liquid nitrogen. Absorbance was recorded at 525 nm in a microplate reader.
174 Standard curves of AsA in the range of 10–60 mM were prepared in 5 % metaphosphoric acid. The
175 concentration of DAsA was calculated by subtracting the AsA concentration measured from the total
176 ascorbate assayed.

177 Data analysis

178 Quality control of the analysis was made by analytical replicate samples (technical triplicates of four
179 biological replicates), use of certified standards solutions and reference plant samples. For statistical
180 purposes, the results below the detection limit were assumed as half of the detection limit.

181 Results were analyzed for a confidence level of 95% ($p < 0.05$) through *One-way* ANOVA
182 followed by the *post-hoc* test Tukey HSD when the sample distribution was found to be normal
183 (Kolmogorov-Smirnova test with Liliefors and Shapiro-Wilk correction). For non-parametric
184 samples, the Kruskal-Wallis test was performed, followed by the non-parametric Wilcoxon-Mann-
185 Whitney test for average comparisons.

186 Spearman non-parametric correlations were used to correlate the concentrations of As and other
187 elements in shoots and roots as well as the physiological characteristics (correlations were found
188 significant at the 0.05 level).

189 The translocation coefficient (TranslC = [total element in shoots] / [total element in roots]) was
190 calculated in order to characterize the translocation capacity of an element from roots to leaves
191 (Huang and Cunningham, 1996).
192

Results

Germination of *Cistus salviifolius* at different pH values and arsenic concentrations

Germination rates, presented in Table 1, were in general low. When considering germination at different pH values, rates were highest at pH 5.0 and lowest at pH 3.0 while As concentrations of 0.5 mM (at pH 4.0) were the most favorable for germination while the highest concentrations and the controls showed very low germination rates.

Arsenic and nutrients concentration in plants of *Cistus salviifolius*

The concentration of As in roots and shoots of plants subjected to the As treatments and in controls at the beginning and at the end of the treatments is shown in Fig. 1. Arsenic concentration was higher in roots than in shoots in all treatments and increased steadily with As concentration in the hydroponic solution. In control plants, the As concentration in roots and shoots decreased from the beginning (As0, d0) to the end (As0, d30) of the treatment. With the exception of the control, all As concentrations in shoots are considered toxic (5 to 20 mg kg^{-1} leaf dry weight; Kabata-Pendias 2011). In fact, in As20 and As30 values were above 30 mg kg^{-1}, the value considered as the maximum tolerable level for cattle ingestion (Mendez and Maier 2008). Table 1 shows the concentration of the studied macro- and micronutrients in roots and shoots of plants subjected to the As treatments and in controls at the end of the treatments. All nutrients are present at higher concentrations in roots than in shoots with the exception of Mg and K that have higher concentrations in shoots and Mn that has similar values in roots and shoots (Table 1).

Growth and visual symptoms of As toxicity in plants

Plants growing in the hydroponic solutions of As30 showed clear visual symptoms of toxicity, beginning as early as ten days of growth, where it is already possible to see dead small leaves and dry tips of the larger leaves (Fig. 2, white arrows). At the end of the As30 treatment, some plants were dead, as the one shown in Fig. 2. In fact, the death rate in As30 on d30 was 80%. The treatment As20

also had high death rate but the surviving plants only had visible symptoms beginning on d20 (Fig. 2, red arrows). The surviving plants in those two treatments did not grow at all (in shoot height, root length and biomass) nor did their leaf area increase (Fig. 3), while plants from the other As treatments showed similar shoot height and root biomass than the control. Arsenic at 0.5 mM actually stimulated shoot biomass while at 5 mM root growth was slightly increased by As. Leaf area also increased steadily from the control up to As5 and then abruptly declined to values lower than in the control.

Arsenic at the concentration of 0.5 mM also stimulated the production of chlorophyll, with a slight shift towards chl*b* (Fig. 4A). Also As20 showed an increase in chlorophyll quantity (Fig. 4 and image of d30 in Fig. 2), while As30 had the lowest values and the lowest chl*a*/chl*b* ratio (Fig. 4B). Carotenoids content followed the same trend as that of total chlorophyll (Fig. 4C), making the ratio chl/car relatively stable throughout the treatments (Fig. 4D).

Oxidative stress and antioxidative defense

Control plants had higher content of H_2O_2 in roots than in shoots, with some oscillation during the treatments but no significant differences (Fig. 5). In shoots, H_2O_2 content did not increase much from the control with the exception of As30 on d20. Conversely, in roots, H_2O_2 content was significantly lower than the control in As0.5 and increased on d10 of As1.5 and As5, decreasing thereafter in those two treatments. Only in As20 did H_2O_2 content increase significantly, on d10 and d20 while on d30 it regained control values. The same occurred on As30, but with slightly lower values than the previous treatment.

Ascorbate and glutathione contents were significantly higher in roots than in shoots, especially in plants at the beginning of the treatments (Fig. 6 and 7). In control roots, ascorbate decreased significantly on d10, increased again and attained very low values on d30 while in shoots, it decreased steadily until d30. In the treatments up to As5, ascorbate increased slightly with time, in roots while in shoots it decreased. In As 20 and As30, ascorbate increased significantly and was kept high for the

241 duration of the treatments, with the exception of As20 on d30. In the shoots of As1.5 to As20
242 ascorbate reduction increased with time while in roots this was only true for As1.5, in the other
243 treatments ascorbate reduction decreased with time.

244 On average, glutathione content was three fold higher in roots than in shoots. Values increased
245 slightly with time, in control plants while in the roots of the As treatments, from As0.5 to As20, it
246 decreased steadily with time. In shoots, glutathione levels were higher on d10 in all treatments.
247 Despite the great variation in reduced and oxidized glutathione contents, the redox state of glutathione
248 was kept very constant in shoots, with treatments from As0.5 to As5 showing %reduction of *circa*
249 85% at all time points. In As20 and As30, however, %reduction decreased significantly. In roots, the
250 reduction of glutathione oscillated more with time but As20 and As30 also had significantly lower
251 values than the other treatments.

252

Discussion

Cistus salvifoliius is a species that can withstand high total metal(loid) concentrations in soil, accumulating some of these elements in their tissues but without showing significant symptoms of toxicity (Abreu et al. 2012a; 2012b). We set out to understand the physiological mechanisms underlying this ability and to assess the potential of using *C. salviifolius* in phytostabilization programs of areas contaminated with As, particularly in the vicinity of mines and containing high As concentrations in the soil available fraction. In order to try to unravel the resilience of this species and also the maximum levels of contamination it can withstand, we evaluated its germination, growth, development and physiological response in an hydroponic system with different As concentrations and the acid pH typical of As contaminated soils.

Seed of the genus *Cistus* are characterized by their small size and mass and by physical dormancy (Troumbis and Trabaud 1986), which is usually broken by high temperatures generated by fire (Ferrandis et al. 2001; Bastida and Talavera 2002). At ambient temperatures seed germination is low (Scuderi et al. 2010; Luna and Chamorro 2016). In this experiment, seed germination at various acid pH values gave rise to germination rates that were relatively average, with a maximum at pH 5.0, what can be justified by the fact that low pH may help break the testa of the seeds, replacing high temperatures. Significant increases of germination ratio were also found by Trigueros-Vera et al. (2010) for seeds of *Erica australis* at pH 4.0, however in *Erica andevalensis*, pH 3.5 did not stimulate germination (Rossini et al. 2009), indicating a strong species specific response. At low concentrations As may have a stimulation effect on plant growth and this was observed in this experiment at concentrations up to 0.5 mM. At higher As concentrations its toxicity led to decreased germination rates.

The concentration of As in the roots was significantly higher than in shoots, as also reported by Abreu et al. (2012a; 2012b) who found that *C. salviifolius* growing in soils from mine areas did not translocate As to shoots, storing it mainly in the roots, suggesting a mechanism of tolerance to As

that inhibits the translocation above a certain threshold. Arsenic accumulation in roots was higher than that reported by Carvalho et al. (2019) in *Cistus monspeliensis* L., for the same As treatments. In As5 roots accumulated 452 mg kg^{-1} As while Carvalho et al (2019) reported 254 mg kg^{-1}. This can be an effect of the hydroponic system, which makes elements more readily available for plants. However, As translocation to the shoots was much lower than observed in Carvalho et al. (2019). Thus, As can accumulate in roots, in organelles where damage can be restrained, such as vacuoles, or immobilized through complexation in forms that are less harmful such as phytochelatins (Hartley-Whitaker et al. 2002) or metallothioneins (Hall 2002). However, for higher As values these tolerance mechanisms are no longer effective and there is a significant increase of As in shoots (high values of translocation ratio), which induces leaf necroses followed by the death of the plant, as in As30. Considering the low values of the translocation ratio of As (roots to shoots) and the As concentrations in shoots, *C. salviifolius* is not an As hyperaccumulator (Rascio and Navari-Izzo 2011; Sarma 2011).

In As20 and As30 treatments there was a significant increase in the concentration of Ca in the roots, above the reference average concentration (1 to 50 g kg^{-1} dry weight; Kirkby and Pilbeam 1984). Under oxidative stress the amount of free cytosolic Ca^{2+} increases with the increase of ROS, because Ca is responsible for a stress response signaling system that triggers systemic molecular responses in the target organs and contributes to plant tolerance to stress (Choh et al. 2014; Mittler and Blumwald 2015; Steinhorst and Kudla 2013). In view of the above, the significant increase of Ca may have been an attempt to respond to the oxidative stress generated by the high concentrations of As. Also, the significant decrease of K in the roots of As20 and As30 can be a result of the increase of Ca (Marschner 2012; Silva and Trevizam 2015). In fact, in those two treatments K concentration was below the values considered adequate (20 to 50 g kg^{-1} dry weight; Varennes 2003), although still within the average range (1 to 50 g kg^{-1} dry weight; Kirkby and Pilbeam 1984).

Arsenic is an analog of P and therefore competes for the same uptake carriers in the roots (Meharg and Hartley-Whitaker 2002). In fact, As actually led to an increase in the uptake of P at concentrations lower that 5 mM because at low As concentrations, arsenate can replace P in the

304 unavailable fraction of the soil, thus increasing the available P for the plant (Gao and Mucci 2001).
305 However, at the highest As concentrations P uptake decreased, as expected, because As is unable to
306 actually substitute for P in its physiological roles in energy transfer (Tuand Ma 2003; Madeira et al.
307 2012), leading to toxicity and decreased growth, as seen on As20 and As30. Iron concentrations were
308 significantly higher in roots (especially in As1.5 and As5) but even the much lower Fe concentrations
309 in shoots were above the reference values (0.05 to 0.10 g kg^{-1} dry weight in shoots; Mengel and
310 Kirkby 2001). Abreu et al. (2012a) also obtained values of Fe between 0.160 and 0.247 g kg^{-1} dry
311 weight in *C. salviifolius* from contaminated and uncontaminated areas, without symptoms of toxicity,
312 and de la Fuente et al. (2010) reported values of 0.330 g kg^{-1} dry weight for the same species in mine
313 areas, which are in accordance with the ones obtained in this work, with the exception of As30, that
314 had an extremely high Fe concentration (1.27 g kg^{-1}).

315 In As30 the mortality rate was high and the surviving plants had severe symptoms of toxicity,
316 with internerval chlorosis and leaf necrosis (Kabata-Pendias 2011). Root length and dry weight were
317 significantly affected at As20 and above, as expected (Sneller et al. 1999). Shoot length was only
318 affected in As30, as those plants were unable to grow and had height similar to the control at the
319 beginning of the treatments, while dry weight was affected also in As20, common growth
320 impairments under As toxicity (Carbonell-Barrachina et al. 1995; Kabata-Pendias 2011). In natural
321 conditions, leaf area can vary with the season, usually lower in the summer to minimize transpiration
322 (Correia and Ascenção 2017). In *C. salviifolius* leaf area can change between 1.43 cm^2 in winter and
323 0.67 cm^2 in summer, with a year average of 1.06 cm^2 (Correia and Ascenção 2017). The present work
324 was performed under artificial conditions, with plentiful water availability and constant temperature
325 and the leaf area reached a maximum of 3.1 cm^2 in As5, a value that is close to the one observed by
326 Puglielli et al. (2017) in *C. salviifolius* growing under high solar exposure (2.77 cm^2). In As20 and
327 As30 there was a decrease in leaf area probably related to the accumulation of As in the plant that
328 reached a limit that became toxic with a consequent decrease in leaf area and photosynthetic capacity
329 (Marin et al. 1993).

330 Similarly to leaf area, also chlorophyll concentration is seasonal, with total chlorophyll in *C.*
331 *salviifolius* varying between 50.1 µg cm^{-2} in winter and 25.9 µg cm^{-2} in summer, with a yearly average
332 of 38.3 µg cm^{-2} (Correia and Ascenção 2017). Unexpectedly, the highest value observed was in As20,
333 with 90 µg cm^{-2} while the lowest was quantified in As30 (9 µg cm^{-2}), totally in accordance with the
334 visual symptoms of toxicity in this treatment. At the end of the experiment, chlorophyll concentration
335 was higher than at the beginning, in the control and in As0.5 due to the natural growth of the plants
336 and the increase in light intensity (Puglielli et al. 2017). However, As toxicity over chlorophyll began
337 to be noticed as soon as on As1.5 and affecting mostly Chl*a*. The Chl*a*/Chl*b* ratio was similar to the
338 ones found by Nuñez-Oliveira et al. (1996) in *Cistus ladanifer* L. in uncontaminated soils of the
339 Iberian Peninsula (between 1.72 and 4.91) and by Arenas-Lago et al. (2016), who measured 1.86 in
340 *Cistus monspeliensis* L. also in uncontaminated soils. Therefore, with the exception of the values
341 obtained in As30, all the others are within the range reported for some species (genus *Cistus*) of the
342 Cistaceae family.

343 Exposure to As usually leads to ROS production, with the ensuing oxidative stress production
344 **(**Hartley-Whitaker et al. 2001b). One such ROS is H_2O_2 which production leads to the onset of the
345 antioxidative response at the enzyme and metabolite levels (Finnegan and Chen 2012; Meharg and
346 Hartley-Whitaker 2002). The concentration of H_2O_2 in the shoots of the control had a significant
347 increase followed by a decrease at the end of the experiment, suggesting that a factor other than As
348 was responsible for these changes, such as an initial oxidative stress caused by the transfer to a
349 hydroponics system under higher irradiance that was later overcome with the acclimatization of
350 plants, as in *Nicotiana benthamiana* Domin. and *Solanum lycopersicon* L. after transfer from *in vitro*
351 to *ex vitro* under four fold higher irradiance where increased ROS (H_2O_2 and $O_2^{\bullet-}$) production was
352 observed, followed by a decrease that indicated recovery from oxidative stress (Carvalho et al. 2008).
353 On the other hand, the plant itself can undergo fluctuations in H_2O_2 concentration that are unrelated
354 to oxidative stress, but linked to the formation of new structures (Carvalho et al. 2006).

355 At the end of the experiment, H_2O_2 concentration in shoots did not vary significantly between
356 treatments and in some cases was even lower than on d20, which suggests that the plant was able to
357 contain the production of H_2O_2 at a nontoxic level in most treatments. However, the toxicity
358 symptoms and the mortality rate in As30 point to a different scenario in this treatment; probably in
359 this situation, oxidative stress is due to the presence of other ROS, such as $O_2^{\bullet-}$, $HO^{\bullet}$ and $^1O_2^-$. Of these
360 ROS, $O_2^{\bullet-}$ is the most stable and is usually dismutated to H_2O_2 by SODs, something that may not have
361 occurred at a sufficient rate further enhancing oxidative stress (Apel and Hirt 2004) and the observed
362 symptoms.
363 Under oxidative stress, mechanisms of ROS removal, in which ascorbate and glutathione are
364 involved, are triggered (Anjum et al. 2012, Apel and Hirt 2004). Besides their involvement in the
365 ascorbate-glutathione cycle, they are also separately responsible for detoxification, such as the role
366 of GSH in reducing arsenate(V) to arsenate(III) through a non enzymatic reaction (Meharg and
367 Hartley-Whitaker 2002). Ascorbate was high at the beginning of the experiment and decreased with
368 time and As concentration, with the exception of As20 and As30, where both AsA and DAsA levels
369 were kept high, in roots and shoots. Arenas-Lago et al. (2016) also reported high values of AsA in
370 shoots of *C. monspeliensis* subjected to Zn toxicity. The percentage reductions of ascorbate were low
371 at the beginning of the experiment and increased with time in all treatments, indicating an acclimation
372 to the stress conditions, leading to more efficient ROS removal (Mittler 2002), and that is in
373 accordance with the H_2O_2 levels measured. This may be an indication that *C. salviifolius* is more
374 resilient than *C. monspeliensis* and can withstand higher As concentrations in the soil, as Carvalho et
375 al. (2019) reported that ROS scavenging systems were only able to cope with the oxidative stress
376 caused by As toxicity at concentrations of 10000 µM or lower.
377 Glutathione increased more rapidly than ascorbate with As concentration, and its levels were
378 higher in all treatments on d10. With time, they decreased but GSH% did not, an indication that the
379 high amounts of GSH reported on d10 were probably not joining the ascorbate-glutathione cycle but
380 were the start material for the production of phytochelatins (Hartley-Whitaker et al. 2002) and for the

reduction of arsenate(V) to arsenate(III) (Meharg and Hartley-Whitaker 2002). Brossa et al. (2015) reported an increase in glutathione in leaves of *Cistus albidus* L. subjected to drought while Carvalho et al. (2019) found a decrease in GSH and in the expression levels of the *GOR* gene in *C. monspeliensis* roots subjected to 15000 µM As. This is an indication that the production of phytochelatins was not a priority in that situation and this difference may be an explanation for the apparently higher tolerance to toxic levels of As shown in the present work for C. *salviifolius.*

Conclusions

The uptake of As kept increasing with its concentrations in the solution, leading to severe toxicity above 20 mg As L^{-1} while in treatments with lower As concentration plant growth and physiological behavior were not significantly impaired. Plants subjected to 20 and 30 mg As L^{-1} had high concentration of As in roots and shoots. The concentrations of Ca, Mg, K and Fe in those plants, as well as several growth parameters were also affected. Taking the values of chlorophylls into account, it is possible to infer that As did not interfere significantly in the photosynthetic capacity of the plants. In all treatments plants accumulated higher amounts of As and H_2O_2 in roots than in shoots, and it was in roots that the concentrations of anti-oxidative metabolites (AsA and GSH) were higher. *Cistus salviifolius* showed high tolerance to As up to the concentration of 5 mg L^{-1}, which makes it a species with high potential to be used in the phytostabilization of soils that contain high concentrations of this element in the soil solution.

Author contributions

MMA and LC designed the set-up; CV and LC performed the experiments; CV performed the statistical analysis; LC wrote the paper; MMA and MCM edited the manuscript. Principal investigators: MMA and MCM.

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592 **Figure Legends**

593 **Fig. 1** Arsenic concentrations (mg kg^{-1} dry weight) in the shoots and roots of *Cistus salviifolius*
594 subjected to the control (C) and the As treatments (As0.5, As1.5, As5, As20 and As30). Inset:
595 Concentration of As in the shoots and roots of plants up to As1.5 under a different scale, for clarity
596 purposes. In roots and shoots an * indicates significant differences between C0 and any other
597 treatment ($p < 0.05$) while different letters indicate significant differences between treatments on d30
598 ($p < 0.05$).

599 **Fig. 2** A representative plant subjected to the control (C) and the As treatments (As0.5, As1.5, As5,
600 As20 and As30) monitored every ten days until d30. White arrows: dead leaves on As30, beginning
601 on d10; red arrows: dead young leaves on As20, beginning on d20.

602 **Fig. 3** Shoot height and root length (cm, A), dry biomass production (g, B), leaf area (cm^2, C) and
603 leaf area ratio (LAR, D) of *Cistus salviifolius* control plants on the beginning of the treatments (C0)
604 and of plants after 30 days of growth under control conditions (C30) and subjected to the As
605 treatments (As0.5, As1.5, As5, As20 and As30). Values for each parameter followed by a different
606 letter are significantly different ($p < 0.05$).

607 **Fig. 4** Chlorophyll *a* and *b* (A) and carotenoids (B) contents and chl*a*/chl*b* (C) and chl/car (D) ratios
608 in shoots of *Cistus salviifolius* control plants on the beginning of the treatments (C0) and in plants
609 after 30 days of growth under control conditions (C30) and subjected to the As treatments (As0.5,
610 As1.5, As5, As20 and As30). Values for each parameter followed by a different letter are significantly
611 different ($p < 0.05$).

612 **Fig. 5** Hydrogen peroxide concentration in the shoots and roots of *Cistus salviifolius* subjected to the
613 control (C) and the As treatments (As0.5, As1.5, As5, As20 and As30) measured every ten days until
614 d30. In roots and shoots lower case letters indicate significant differences between time points for

each treatment ($p < 0.05$) while different upper case letters indicate significant differences between treatments for each time point ($p < 0.05$).

Fig. 6 Concentration of reduced (AsA) and oxidised (DAsA) ascorbate in the roots (A) and shoots (B) of *Cistus salviifolius* subjected to the control and the As treatments (As0.5, As1.5, As5, As20 and As30) and percentage reduction of AsA (C, roots; D, shoots) for each As treatment, measured every ten days until d30. In roots and shoots lower case letters indicate significant differences between time points for each treatment ($p < 0.05$) while different upper case letters indicate significant differences between treatments for each time point ($p < 0.05$).

Fig. 7 Concentration of reduced (GSH) and oxidised (GSSG) glutathione in the roots (A) and shoots (B) of *Cistus salviifolius* subjected to the control and the As treatments (As0.5, As1.5, As5, As20 and As30) and percentage reduction of GSH (C, roots; D, shoots) for each As treatment, measured every ten days until d30. In roots and shoots lower case letters indicate significant differences between time points for each treatment ($p < 0.05$) while different upper case letters indicate significant differences between treatments for each time point ($p < 0.05$).

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Table 1 Average germination rates (± standard error) of *C. salviifolius* from As and pH treatments (As treatments expressed in mM As).

pH treatment (21 days)	germination rate (%)
pH 2.5	39.0 ± 8.2 ac
pH 3.0	20.0 ± 3.3 b
pH 4.0	31.0 ± 2.0 ac
pH 5.0	45.0 ± 13.6 c
pH 6.0	37.3 ± 19.7 ac
pH 7.2	29.0 ± 8.2 ab
As treatment (30 days)	**germination rate(%)**
0.0	17 ± 12.8 a
0.025	10 ± 12.0 a
0.1	32 ± 21.4 ab
0.5	45 ± 18.7 b
1.0	18 ± 11.5 a
1.5	24 ± 8.0 ab
3.0	21 ± 5.0 ab
5.0	12 ± 8.0 a

Values followed by different letters in each treatment indicate significant differences between As concentrations or pH values ($p < 0.05$).

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Table 2 Concentrations of macro- and micronutrients in the roots and shoots of *C. salviifolius* from As treatments (n=4); (treatments expressed in mM As).

Treatment	Ca (g kg^{-1})	Cu (mg kg^{-1})	Fe (g kg^{-1})	K (g kg^{-1})	Mg (g kg^{-1})	Mn (g kg^{-1})	P (g kg^{-1})	Zn (mg kg^{-1})
Roots								
C0	51.89b	102.59b	12.78ab	19.20ab	3.19	0.22	10.53a	265.62c
C30	35.00b	37.22a	18.01b	30.99b	2.33	0.19	10.93a	104.24b
As0.5	41.26b	35.67a	18.71b	34.64b	3.62	0.25	16.43ab	107.13b
As1.5	18.96a	14.91a	26.21c	28.22b	2.60	0.25	13.40a	71.37a
As5	21.66a	23.39a	60.61d	55.29c	4.63	0.33	21.35b	124.52b
As20	125.20c	89.18b	17.94b	11.00a	2.80	0.16	11.11a	170.66c
As30	178.25c	150.00b	7.77a	13.24a	3.32	0.25	10.71a	210.94c
Shoots								
C0	33.45b	30.03c	5.04d	47.78b	7.82c	0.30b	4.94b	131.97c
C30	11.57a	10.14b	0.32b	38.79a	3.73b	0.22ab	3.72ab	90.60b
As0.5	11.06a	5.43a	0.28ab	28.82a	3.25ab	0.17a	3.03a	56.20a
As1.5	9.74a	6.25a	0.25a	31.07a	2.99a	0.14a	2.83a	59.82a
As5	10.75a	5.49a	0.29ab	35.91a	4.10b	0.19ab	4.73b	76.26b
As20	12.70a	3.21a	0.22a	50.58b	3.85b	0.26b	2.99a	66.65a
As30	45.82b	15.15b	1.27c	59.09b	9.13c	0.26b	10.48c	227.34d

Values of the same element and plant part followed by different letters indicate significant differences between treatments ($p < 0.05$). No significant differences were registered for Mg and Mn in roots.

Table 3 Translocation coefficient of macro- and micronutrients and of As in *C. salviifolius* control (C0 and C30) and at the end of the As treatments (treatments expressed in mM As).

Treatment	As	Ca	Cu	Fe	K	Mg	Mn	P	Zn
C0	0.23b	0.64c	0.29b	0.39b	2.49b	2.45c	1.36b	0.47a	0.50a
C30	0.09a	0.33b	0.27b	0.02a	1.25a	1.60b	1.16b	0.34a	0.87ab
As0.5	0.07a	0.27b	0.15ab	0.01a	0.83a	0.90a	0.68a	0.18a	0.52a
As1.5	0.10a	0.51c	0.42c	0.01a	1.10a	1.15a	0.56a	0.21a	0.84ab
As5	0.03a	0.50c	0.23b	0.00a	0.65a	0.89a	0.58a	0.22a	0.61a
As20	0.27b	0.10a	0.04a	0.01a	4.60c	1.38b	1.63	0.27a	0.39a
As30	1.99c	0.26b	0.10a	0.16b	4.46c	2.75c	1.04b	0.98b	1.08b

Values of the same element and plant part followed by different letters indicate significant differences between treatments ($p < 0.05$).

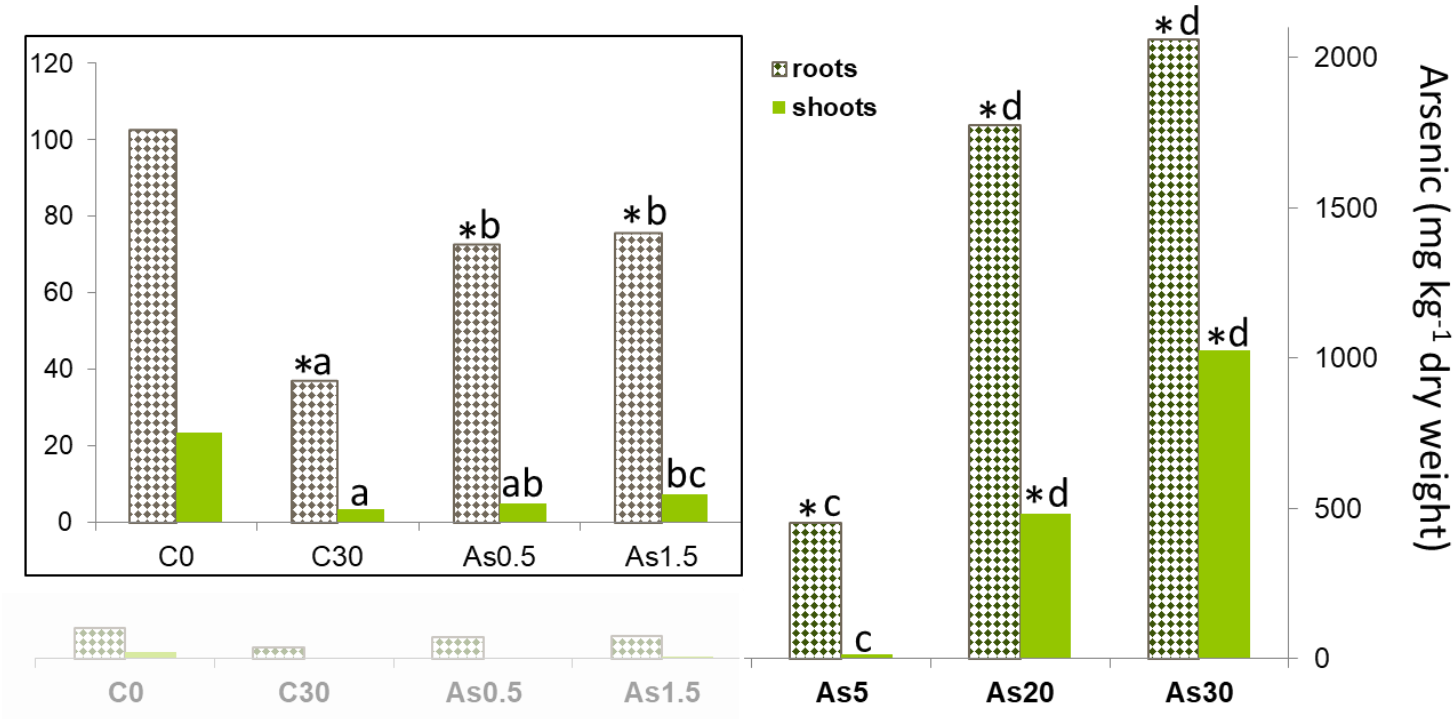

Fig. 1 Arsenic concentrations (mg kg^{-1} dry weight) in the shoots and roots of *Cistus salviifolius* subjected to the control (C) and the As treatments (As0.5, As1.5, As5, As20 and As30). Inset: Concentration of As in the shoots and roots of plants up to As1.5 under a different scale, for clarity purposes. In roots and shoots an * indicates significant differences between C0 and any other treatment ($p < 0.05$) while different letters indicate significant differences between treatments on d30 ($p < 0.05$).

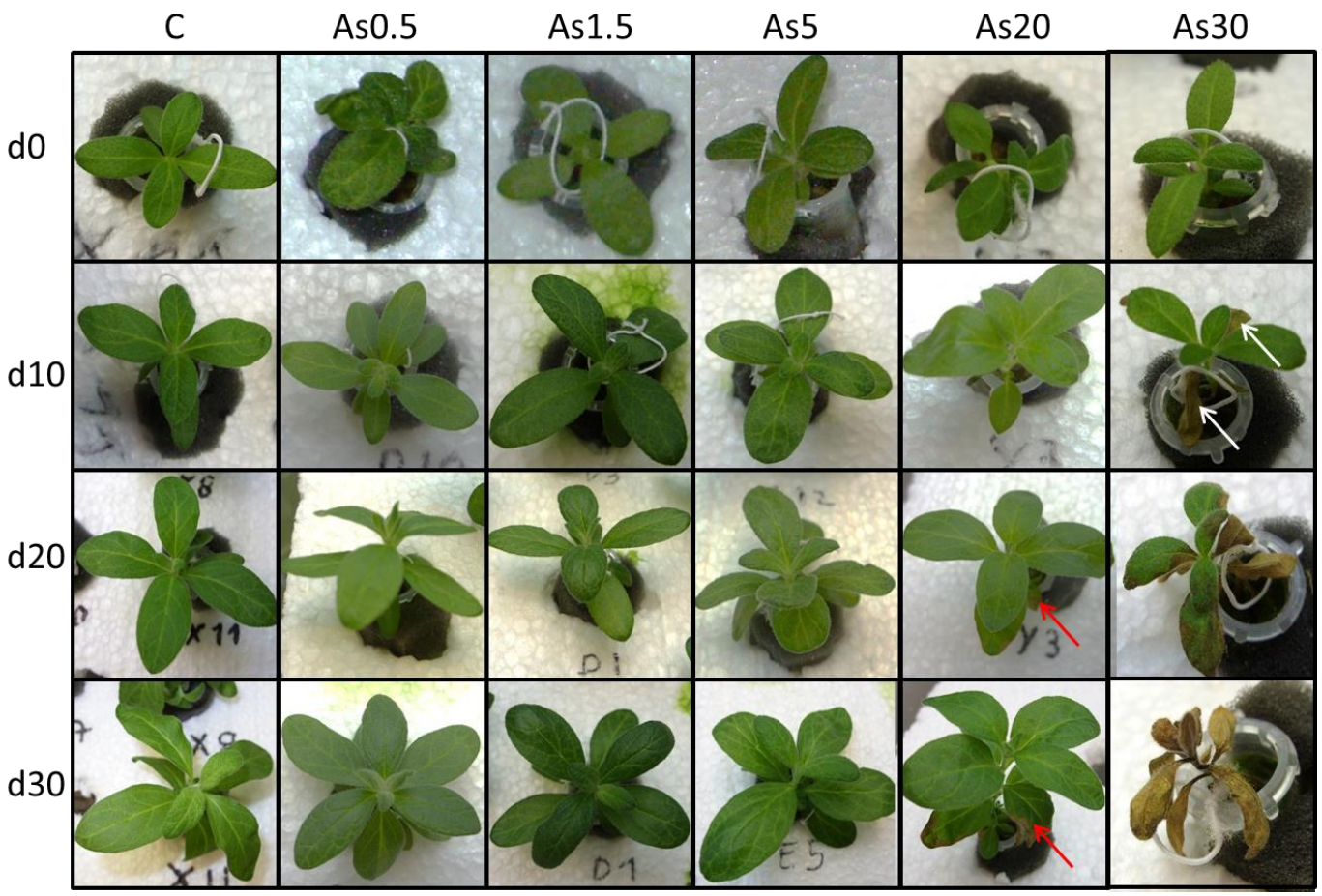

Fig. 2 A representative plant subjected to the control (C) and the As treatments (As0.5, As1.5, As5, As20 and As30) monitored every ten days until d30. White arrows: dead leaves on As30, beginning on d10; red arrows: dead young leaves on As20, beginning on d20.

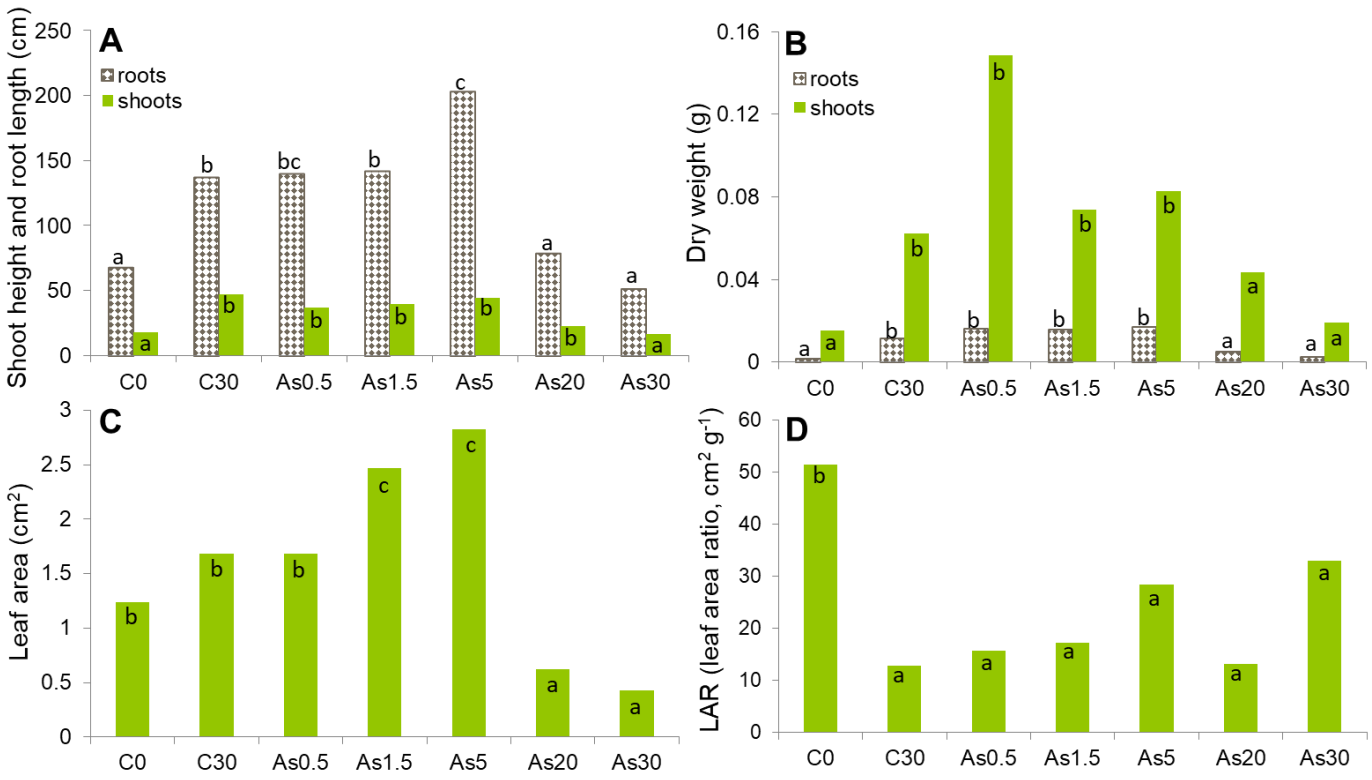

Fig. 3 Shoot height and root length (cm, A), dry biomass production (g, B), leaf area (cm^2, C) and leaf area ratio (LAR, D) of *Cistus salviifolius* control plants on the beginning of the treatments (C0) and of plants after 30 days of growth under control conditions (C30) and subjected to the As treatments (As0.5, As1.5, As5, As20 and As30). Values for each parameter followed by a different letter are significantly different ($p < 0.05$).

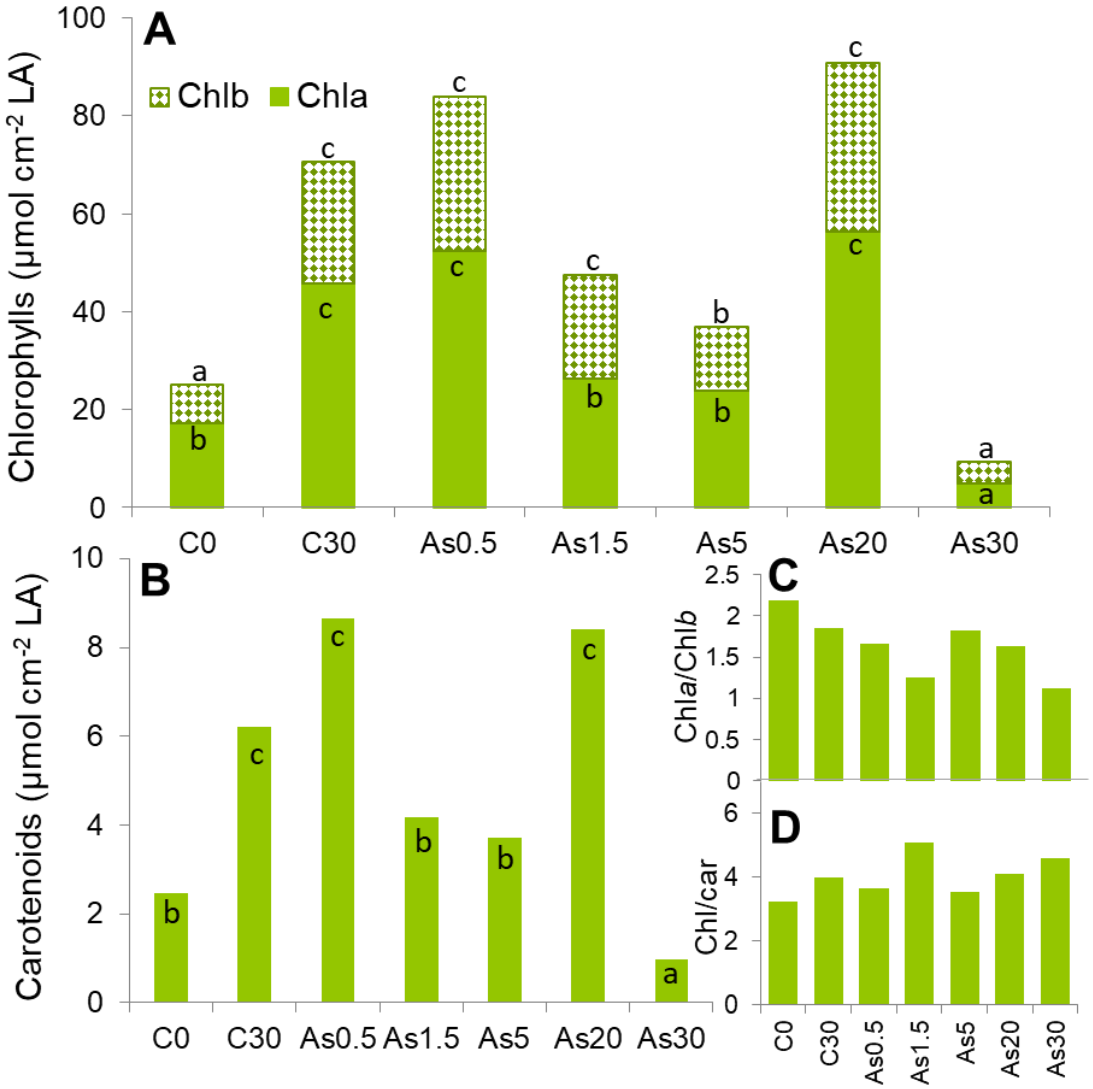

Fig. 4 Chlorophyll *a* and *b* (A) and carotenoids (B) contents and chl*a*/chl*b* (C) and chl/car (D) ratios in shoots of *Cistus salviifolius* control plants on the beginning of the treatments (C0) and in plants after 30 days of growth under control conditions (C30) and subjected to the As treatments (As0.5, As1.5, As5, As20 and As30). Values for each parameter followed by a different letter are significantly different ($p < 0.05$).

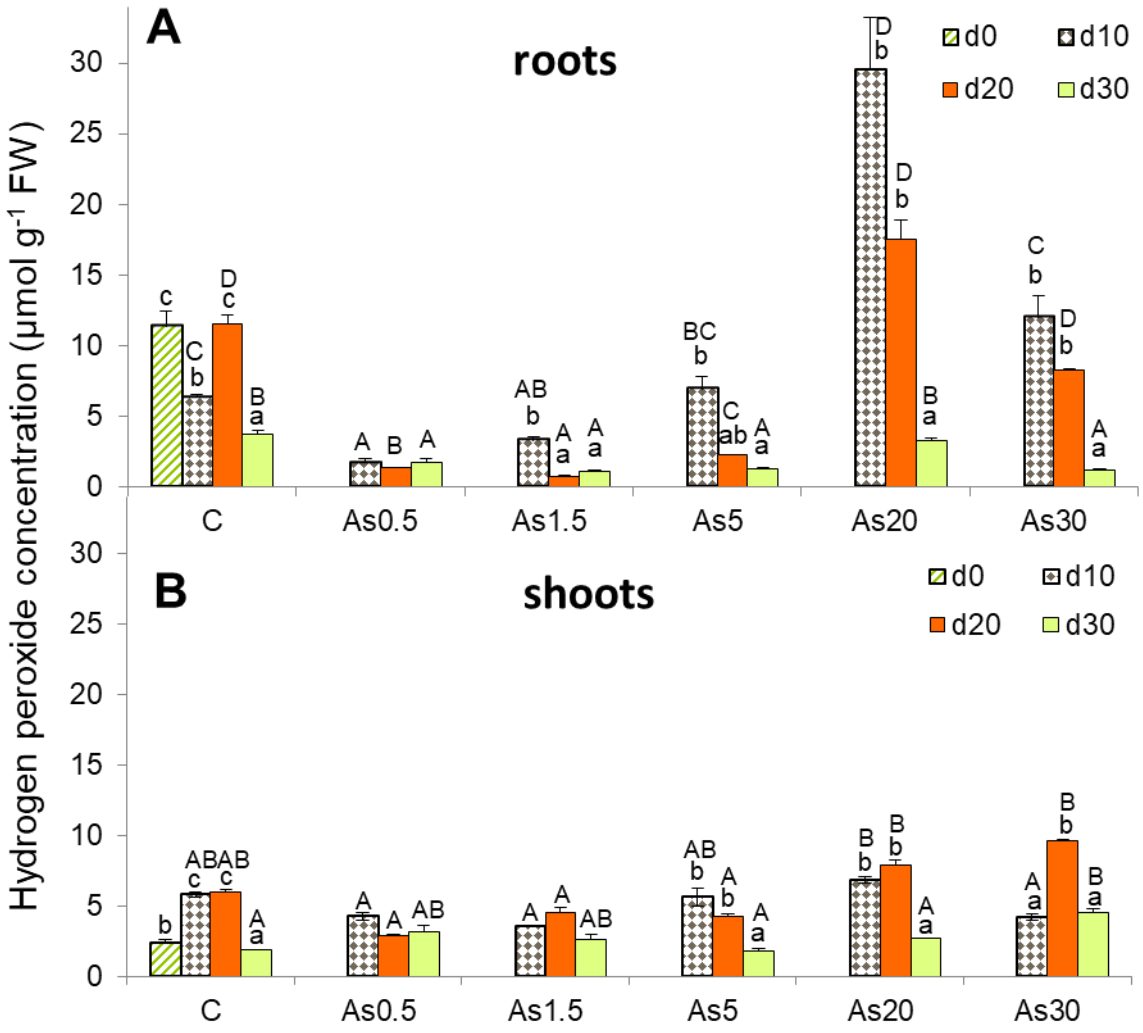

Fig. 5 Hydrogen peroxide concentration in the shoots and roots of *Cistus salviifolius* subjected to the control (C) and the As treatments (As0.5, As1.5, As5, As20 and As30) measured every ten days until d30. In roots and shoots lower case letters indicate significant differences between time points for each treatment ($p < 0.05$) while different upper case letters indicate significant differences between treatments for each time point ($p < 0.05$).

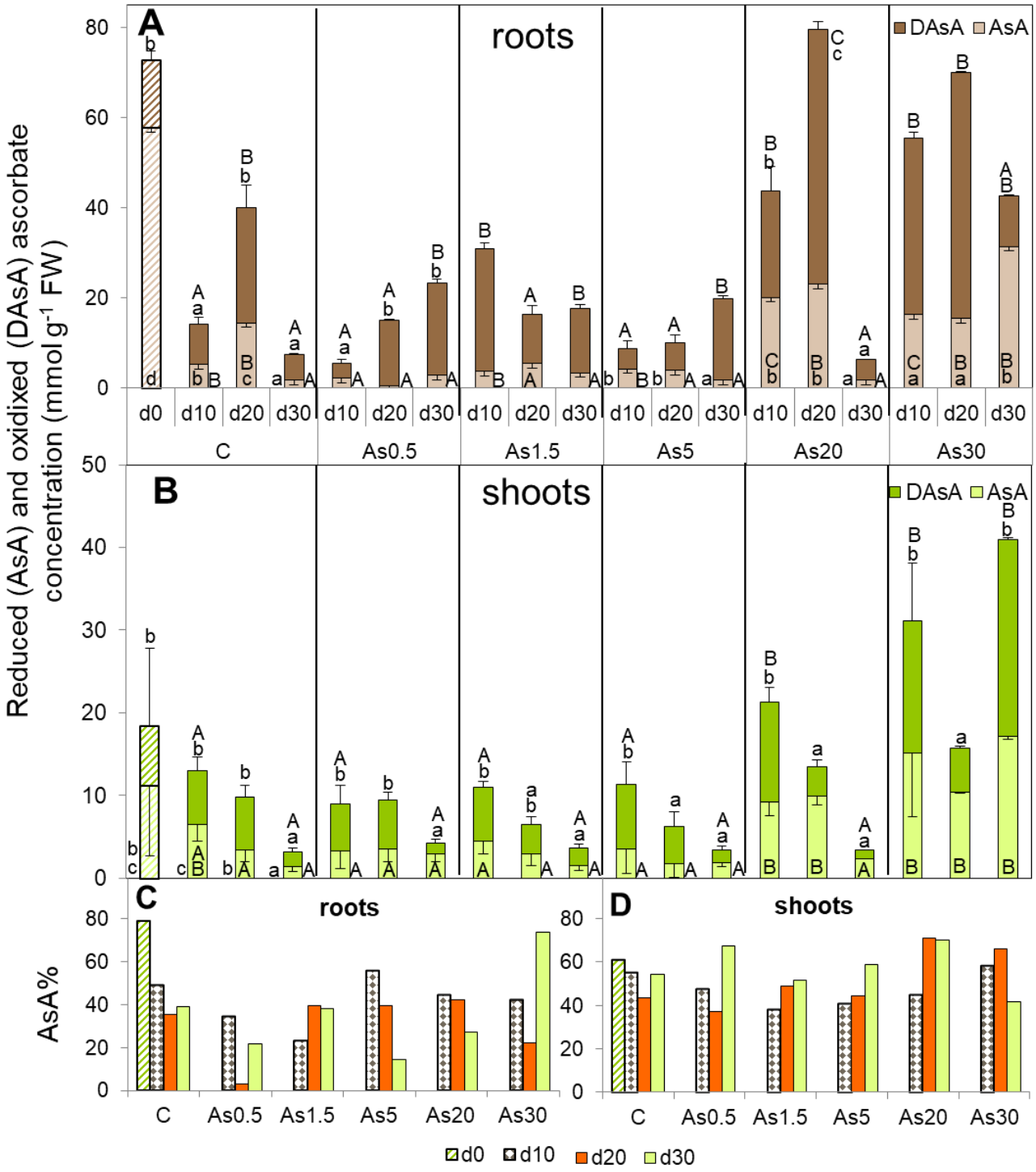

Fig. 6 Concentration of reduced (AsA) and oxidised (DAsA) ascorbate in the roots (A) and shoots (B) of *Cistus salviifolius* subjected to the control and the As treatments (As0.5, As1.5, As5, As20 and As30) and percentage reduction of AsA (C, roots; D, shoots) for each As treatment, measured every

ten days until d30. In roots and shoots lower case letters indicate significant differences between time points for each treatment ($p < 0.05$) while different upper case letters indicate significant differences between treatments for each time point ($p < 0.05$).

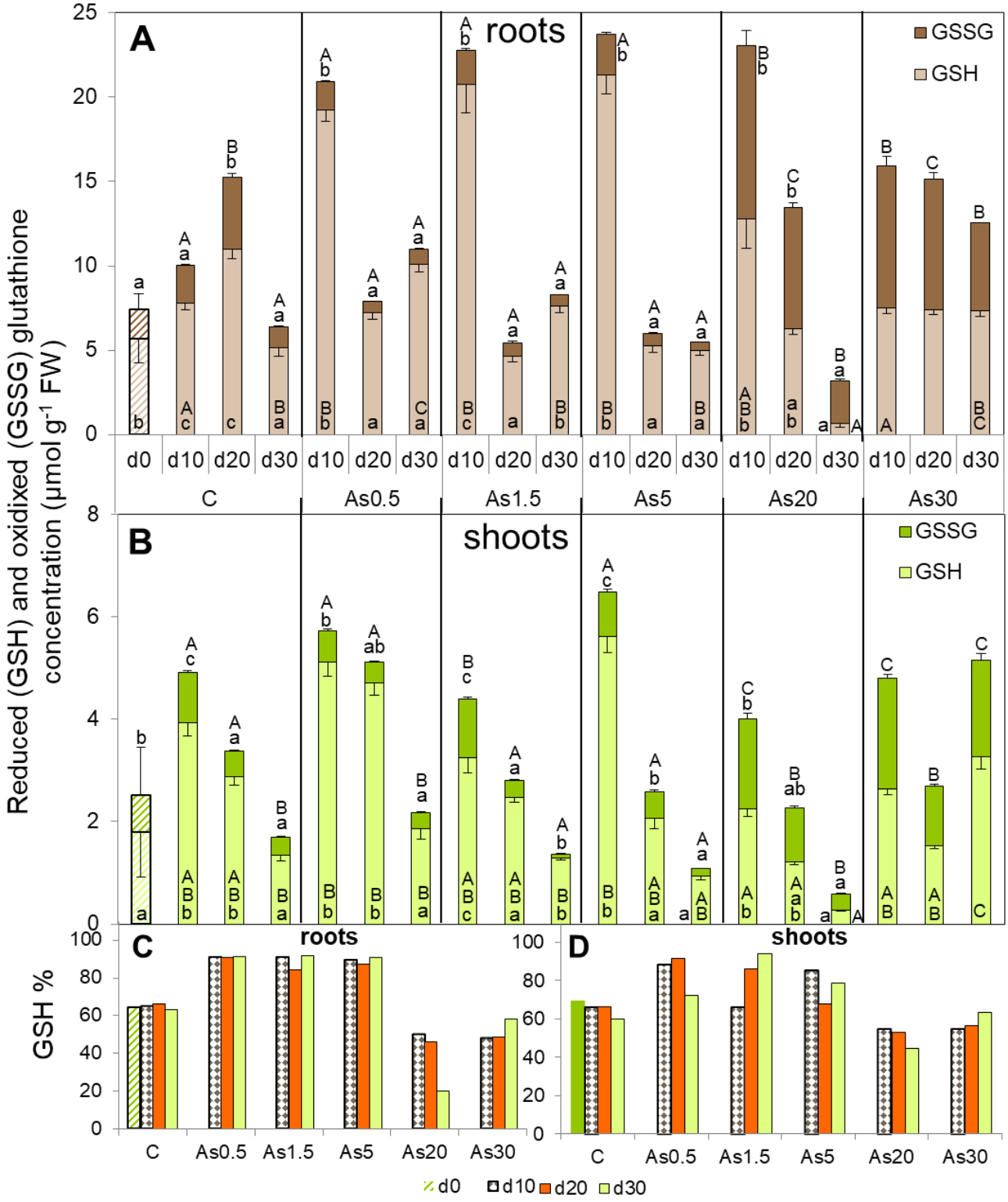

Fig. 7 Concentration of reduced (GSH) and oxidized (GSSG) glutathione in the roots (A) and shoots (B) of *Cistus salviifolius* subjected to the control and the As treatments (As0.5, As1.5, As5, As20 and

As30) and percentage reduction of GSH (C, roots; D, shoots) for each As treatment, measured every ten days until d30. In roots and shoots lower case letters indicate significant differences between time points for each treatment ($p < 0.05$) while different upper case letters indicate significant differences between treatments for each time point ($p < 0.05$).