



## Metabolic adaptations to arsenic-induced oxidative stress in *Isatis cappadocica*

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### Abstract

Arsenic is considered as one of the most important environmental contaminant elements. Some plant species can grow in arsenic contaminated soils and they are able to reduce arsenic toxicity. In this study, a hydroponic experiment was conducted on *Isatis cappadocica*, a newly-discovered As hyperaccumulator. Accordingly, we conducted this experiment to compare the interaction of effect of arsenic and phosphorus on total chlorophyll and antioxidant compounds (carotenoids, anthocyanin and proline) of *I. cappadocica* for better understanding the mechanisms applied by this species. Therefore, the plants were grown for 6 weeks in a medium, embedded with combinations of 50, 200, 800 and 1200  $\mu\text{mol l}^{-1}$  arsenic and 5, 50, 200, 800 and 1600  $\mu\text{mol l}^{-1}$  phosphorus, respectively. The highest concentration of arsenic was obtained in plants treated with 1200  $\mu\text{mol l}^{-1}$  As and 5  $\mu\text{mol l}^{-1}$  phosphorus. Increasing arsenic concentration in the medium led to increase in antioxidant compounds. The ability of *Isatis* to accumulate more than 700  $\text{mg kg}^{-1}$  arsenic in the shoots, illustrates the high resistance of this plant to arsenic and the existence of efficient mechanisms including increase of antioxidant compounds.

**Keywords:** antioxidant compounds; arsenic; chlorophyll; hyper accumulator; *I. cappadocica*; phosphorus

**Karimi, N. and Z. Souri.** 2013. Metabolic adaptations to arsenic-induced oxidative stress in *Isatis cappadocica*. *Iranian Journal of Plant Physiology* 3 (4), 785-792.

### Introduction

In recent years, arsenic (As) has received great attention as a global problem mostly stemming from the crisis in South-East Asia. Remediation of As-contaminated soils and water is necessary to protect both human life and agricultural production. Nowadays, use of phytoremediation techniques to clean up As-polluted sites including groundwater is gaining attention due to the environmental concerns and remediation costs associated with conventional remediation processes. Numerous studies have been carried out in relation to uptake and

translocation of arsenic from soil to plants (Tripathi et al., 2007). Recent discovery of several As hyperaccumulators makes it possible to phytoremediate arsenic-contaminated soils (Ma et al., 2001; Zhao et al., 2002; Meharg, 2003). Among the known arsenic hyperaccumulators, *Isatis cappadocica* L. is one of the most efficient in accumulating high As concentration in its aboveground parts (karimi et al., 2009, 2010), indicating its high tolerance to As and efficient mechanisms of As detoxification.

Plants vary greatly in their sensitivity and resistance to arsenic (Meharg and Hartley-Whitaker, 2002) and some As resistant plants have been identified in a range of plant species (Sharples et al., 2000; Meharg and Hartley-

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Received: March, 2013  
Accepted: June, 2013

Whitaker, 2002). The primary response of plants to metal stress is the generation of reactive oxygen species (ROS) such as superoxide ( $O_2^-$ ), hydrogen peroxide ( $H_2O_2$ ) and the hydroxyl radical ( $OH^\cdot$ ), the major contributors to oxidative damage (Gill and Tuteja, 2010; Yadav, 2010). These ROS are highly reactive and damage membrane lipids, proteins, pigments and nucleic acids, resulting in dramatic reductions of growth and productivity, and eventually causing the death of plants (Foyer et al., 1994).

To minimize the harmful effects of ROS, plants have evolved an effective scavenging system composed of antioxidant molecules and antioxidant enzymes (Meharg, 1994). Accordingly, hyperaccumulators or accumulators should take an effective arsenic tolerance strategy using different tolerance strategies under arsenic stress (Karimi et al., 2009). To better understand the mechanisms of plant arsenic detoxification, we chose *Isatis cappadocica*.

In the present work, we tested the hypothesis that arsenic hyperaccumulator *I. cappadocica* has evolved metabolic adaptations to cope with arsenic-induced oxidative stress. Information obtained from this study should enhance our understanding of the arsenic detoxification mechanisms by this hyperaccumulator species.

## Materials and Methods

### Source of plant material

Seeds of *I. cappadocica* Desv. were collected from a population growing at the gold-arsenic Zarshuran deposit mine, grid reference 36° 43' 04" N, 47° 08' 02" E, 40 km north of Takab in the West Azerbaijan province, northwest of Iran. The total soil As concentration in this deposit area was ranged from 145 to 6525 mg kg<sup>-1</sup> (Modabberi and Moore, 2004).

### Plant culture

Seeds of *I. cappadocica* surface sterilized in 50% (v/v) commercial bleach (LODA; 4% NaOCl) for 2 min, followed by rinsing three times for 5 min in sterilized distilled water. Seeds were germinated on distilled water moistened filter

paper in Petri dishes for 4 days at room temperature, <20 °C. After germination, seedlings were then transferred to 1 litre polyethylene pots (four seedlings per pot) filled with a nutrient solution (Karimi et al., 2009) composed of 0.5 mM KNO<sub>3</sub>, 0.75 mM Ca(NO<sub>3</sub>)<sub>2</sub>, 0.2 mM MgSO<sub>4</sub>, 15 μM H<sub>3</sub>BO<sub>3</sub>, 2 μM MnCl<sub>2</sub>, 1 μM ZnSO<sub>4</sub>, 0.5 μM Na<sub>2</sub>MoO<sub>4</sub>, 2H<sub>2</sub>O and 50 μM Fe-EDTA (pH 6.0). The nutrient medium was continuously aerated with an aquarium air pump, renewed twice a week, and adjusted at the daily pH levels of 5.5±0.1 with HCL or NaOH. After cultivating the plants were cultured for 10 days, the solutions with different arsenic concentrations (0, 50, 200, 800 and 1200 μM) and phosphorus (5, 50, 200, 800 and 1600 μM) were added for another 4 weeks. The plants were grown in a growth chamber (Convicon model CG72) with 14/10 h light/dark cycles; temperature was kept at 26 °C during the day and 20 °C during the night. Light intensity was around 280 μmol m<sup>-2</sup> s<sup>-1</sup>. Each treatment was replicated three times and each time, the pots were randomly arranged during the growth period.

### Total chlorophyll and carotenoids determination

Fresh fronds (0.2 g) were homogenized in 80% ice-cold acetone in dark and centrifuged at 10,000 × g for 10 min. For carotenoids the acetone extract was treated with ether and the acetone was completely removed by washing with water. The absorbance of the supernatant was measured. The chlorophyll and carotenoids were estimated by the following formula:

$$\text{chl a (mg/g leaf)} = (12.7 \times \text{Abs 663}) - (2.6 \times \text{Abs 645}) \times \text{ml acetone/mg leaf}$$

$$\text{chl b (mg/g leaf)} = (22.9 \times \text{Abs 645}) - (4.68 \times \text{Abs 663}) \times \text{ml acetone/mg leaf}$$

$$\text{Total chl} = \text{chl a} + \text{chl b}$$

$$\text{Total carotenoids content (mg/g leaf)} = (1000 \times \text{Abs 470} - 1.8 \times \text{chl a} - 85.02 \times \text{chl b}) / 198 \times \text{ml acetone /mg leaf}$$

### Total anthocyanin determination

Water-soluble pigment (anthocyanin) was determined using the method of Nogués and Baker (2000). Leaves were ground to a powder in liquid nitrogen before extraction in 10 cm<sup>3</sup> of acidified methanol (HCL: methanol, 1:99, v/v). The anthocyanin contents were estimated from absorbance at 520 nm.

### Extraction and determination of proline

Free proline content was determined according to Bates et al., (1973). 500 mg of plant sample was homogenized in 3% (w/v) sulphosalicylic acid and the homogenate was filtered through filter paper. After addition of acid ninhydrin and glacial acetic acid, the resulting mixture was heated at 100 °C for 1 h in water bath. Reaction was then stopped by using ice bath. The mixture was extracted with toluene, and the absorbance of fraction with toluene aspired from liquid phase was read at 520 nm (Tomas 302, USA). The concentration of proline was estimated by referring to a standard curve prepared using L-proline.

### Analysis of total arsenic concentration in plant tissues

Total arsenic was determined in acid digests of ground shoot (100–200 mg DW) mixed with 2 ml of HNO<sub>3</sub> (67% suprapur) and 2 ml of H<sub>2</sub>O<sub>2</sub> (30% by volume), and then microwave-digested at 95 °C. The digest was diluted with a solution containing 10% HCl, 5% ascorbic acid and 10% KI, and then analysed using hydride generation–atomic absorption spectrometry with a flow injection hydride generator interfaced with a Shimadzu AA-6200 atomic absorption spectrometer (HG-AAS). Reference standard for calibration of the AAS was made using 1000 mg l<sup>-1</sup> (Beach leaves material FD8, Commission of the European Communities, Joint Research Centre ISPRA).

### Statistical analysis

Statistical analysis was performed using the SPSS statistical package version 16.0. The analysis of variance (ANOVA) appropriate for the design was carried out to detect the significance of the differences ( $P < 0.05$ ) between the treatment and control means. Duncan's multiple range test (DMRT) was also performed to compare the significant difference between groups. Difference from the control was considered significant at  $P < 0.05$  or very significant as  $P \leq 0.001$ . All the values presented in this paper were expressed as the means of three replicates  $\pm$  standard error (S.E).

## Results

### Arsenic accumulation

To evaluate the extent of As-induced oxidative stress and the associated As detoxification systems in the *I. cappadocica*, it is important to know plant As concentration (Fig. 1). The As concentrations in the shoots of *I. cappadocica* grown in medium supplemented with Na<sub>2</sub>HAsO<sub>4</sub> are shown in Fig. 1. The As concentrations in the shoots of studied plant increased steadily and significantly ( $p < 0.01$ ) with As treatment. When *Isatis* was exposed to 1200  $\mu\text{M}$  of arsenate, the concentration of accumulated As reached the maximum of 700 mg kg<sup>-1</sup> in shoots. Low P levels (5 and 50  $\mu\text{M}$ ) lead to highest As accumulation in each As treatment, which could be due to their interaction.

### Effects of arsenic stress on total chlorophyll

Exposure to 50-200  $\mu\text{M}$  As did not significantly affect the chlorophyll content in *I. cappadocica* (Fig. II). However, *I. cappadocica* was affected by highest As treatment at 800 and 1200  $\mu\text{M}$ , where Chl content was significantly reduced (Fig. II). The decline in the chl content implies As-induced stress in this species. The increase in the Chl content by exposure to 50  $\mu\text{M}$  arsenate may indicate an improved growth.

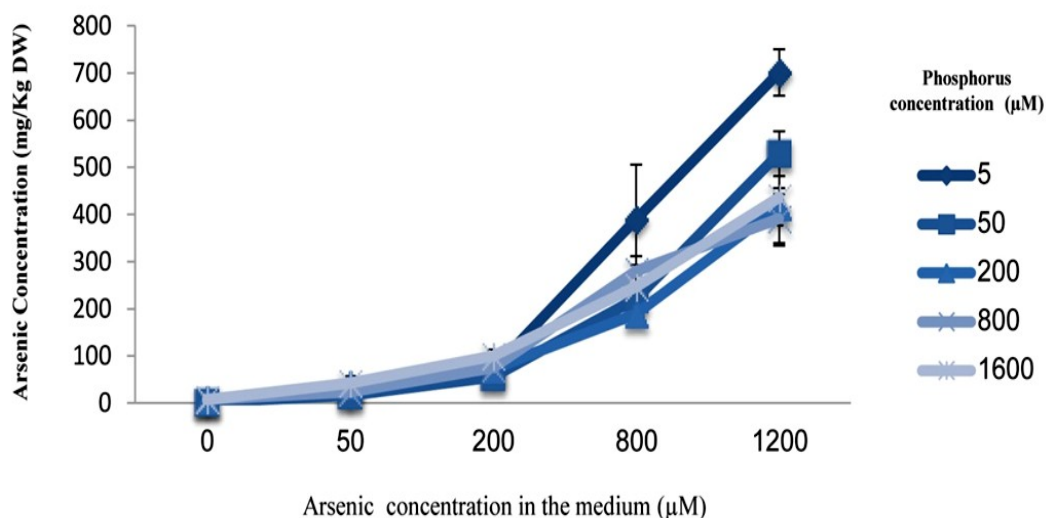


Fig. I. Total arsenic concentrations (mg/kg, DW) in *I. cappadocica* grown for 6 weeks in As & P amended medium. Values represent mean  $\pm$  S.D. Different letters indicate significant differences at the 5% level according to the Duncan test.

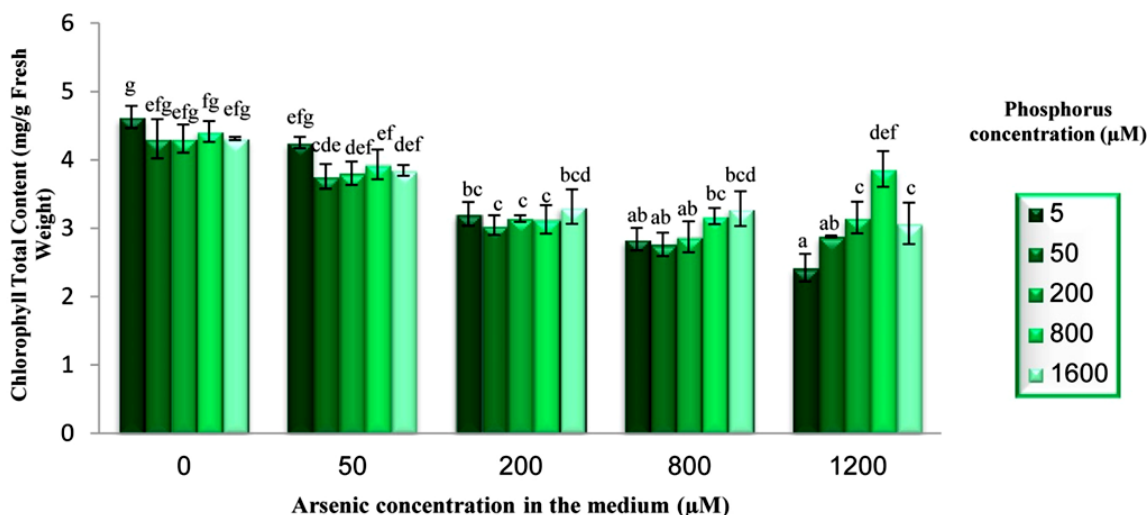


Fig. II. Chlorophyll content (mg/g, FW) in *I. cappadocica* grown for 6 weeks in As & P amended medium. Values represent mean  $\pm$  S.D. Different letters indicate significant differences at the 5% level according to the Duncan test.

### Effects of As stress on antioxidant compounds

From the comparison of the activities of the three non-enzyme antioxidants, i.e., carotenoids, anthocyanin and proline it is evident that uptake of As induced a strong antioxidative response in *I. cappadocica* (Fig. III a). A positive correlation was found between carotenoids and As accumulation in *I. cappadocica* shoots. The maximum induction was observed in treatments contain 1200  $\mu$ M As and 5  $\mu$ M P, which was 6.18 mg/g (FW). Carotenoid content in each fixed As and different P treatments showed a differential

pattern. At 800 and 1200  $\mu$ M of As treatment, the carotenoid content decreased with increasing P concentration whereas in 50  $\mu$ M As treatments, content increased with increasing P from 50 to 800  $\mu$ M (Fig. IIIa). The fact that high plant P treatment was associated with low activities of enzymatic antioxidants, suggested the role of phosphorus in maintaining plant growth.

Compared to the control, the anthocyanin concentration in *I. cappadocica* was significantly ( $p < 0.05$ ) increased along with As exposure in the medium (Fig. III b). This demonstrated the involvement of anthocyanin in As detoxification by *I. cappadocica*. The highest

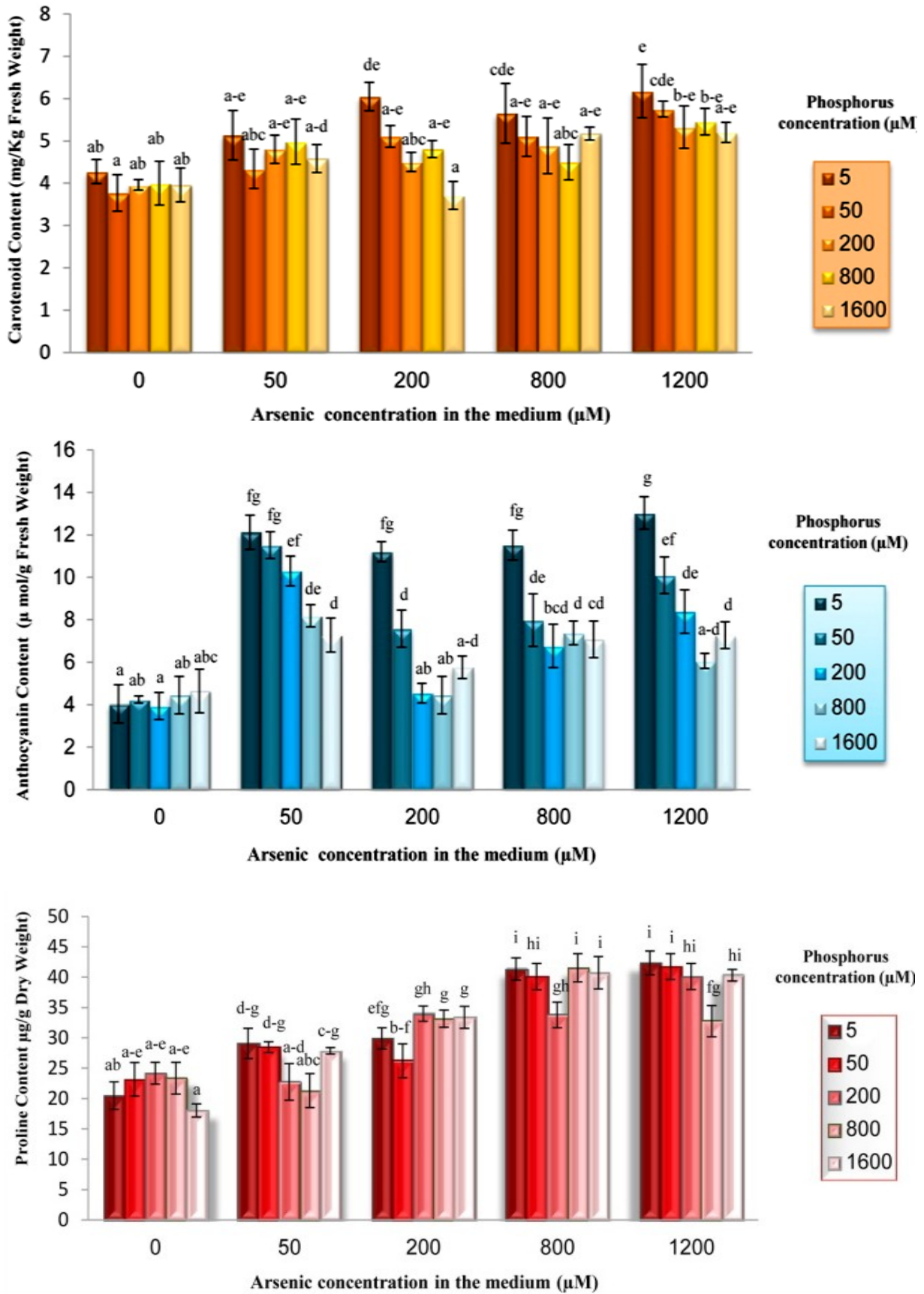


Fig. III. Antioxidant compounds including carotenoid (a), anthocyanin (b) and proline (c) content of *I. cappadocica* grown for 6 weeks in As & P amended medium. Values represent mean ± S.D. Different letters indicate significant differences at the 5% level according to the Duncan test.

achieved by plants affected with 1200  $\mu\text{M}$  As. These results clearly suggest that accumulation of anthocyanin is a sensitive indicator to changes in As exposure in *I. cappadocica*. Furthermore, the anthocyanin accumulation of *I. sasis* was influenced significantly by phosphorus addition. It was decreased sharply under high levels of phosphorous (Fig. III b).

The results pertaining to the effect of As on proline content is presented in Fig. III c. There was an almost linear increase in proline accumulation with increasing concentrations of As from 0 to 800  $\mu\text{M}$ , with the greatest increase in proline content in medium containing 1200  $\mu\text{M}$  As (Fig. IIIc ). 800 and 1200  $\mu\text{M}$  of As did not significantly affect the proline content of *I. cappadocica*. It was discovered that phosphorus addition at high level did not exhibited a significant difference in the proline accumulation of *I. cappadocica* caused by the arsenical toxicity (Fig. III c). A decrease in the proline accumulation, however, was observed under some As and P treatments (As 800 – P 200 and As 1200 – P 800), which was most likely due to a sampling error.

## Discussion

Arsenic is known to induce oxidative stress in plants by generating various ROS (Hartley-Whitaker et al., 2001) which lead to various responses in plants. It is conceivable that greater arsenic concentrations in plants cause greater oxidative stress (Srivastava et al., 2005). Therefore, high As accumulation in *I. cappadocica* should lead to greater oxidative stress and it must also have developed an efficient defense system to mitigate arsenic included oxidative stress as an efficient As plant hyperaccumulator.

Adaptation to As may depend on different mechanisms, including the capacity to maintain high levels of antioxidants. To avoid the damages effectively, the anti-oxidative system would be activated. ROS scavenging can be achieved by antioxidant compounds such as carotenoids, anthocyanin and proline. A constitutively high antioxidant capacity or increase in antioxidant level could prevent oxidative damage and improve tolerance to the established oxidative stress. These results showed that the high levels of As lead to

substantially increase in carotenoids, anthocyanin and proline concentrations (Fig. III a, b and c).

Carotenoids act as light-harvesting pigments as well, and can protect chlorophyll and membrane destruction by quenching triplet chlorophyll and removing oxygen from the excited chlorophyll – oxygen complex (Krinsky, 1994). In this study, carotenoid content of cultivars increased with increasing As concentrations which led to greater protection of *I. cappadocica* against ROS. The increase in anthocyanin concentrations is another remarkable response of *I. cappadocica* against As toxicity. Arsenic may enhance anthocyanin biosynthesis by inducing the activity of PAL (l-phenylalanine ammonia-lyase, (see Holton and Cornish, 1995), a key enzyme in flavonoid biosynthesis (Tahkokorpi et al., 2010). Flavonoids participate in plant responses under As accumulation, and their protective function is partly due to antioxidant properties against free radicals produced by metals (Sakihama and Yamasaki, 2002).

Accumulation of some kind of compatible solutes is another strategy that plant adopt to withstand stress conditions (Parida and Das, 2005). Compatible solutes fall into three major groups: amino acids (e.g., proline), quaternary amines (e.g., glycine betaine, dimethylsulfoniopropionate), and polyol/sugars (e.g., mannitol, trehalose) (Lutts et al., 2004). The result of this study showed that there was an almost linear positive relationship between proline accumulations with increasing concentrations of As in the medium of *I. cappadocica* which approved its efficiencies as a stress resistance marker. Proline acts as osmolyte, radical scavenger, electron sink, macromolecules stabilizer, and cell wall component (Matysik et al., 2002). Furthermore, proline can serve as an organic nitrogen reserve ready to be used after stress relief to sustain both amino acid and protein synthesis (Sairam and Tygai, 2004).

Considering the data obtained on studied parameters, it is clear that *I. cappadocica* was inherently tolerant to ROS in the presence of As. It is possible that As resistance of *I. cappadocica* was associated with its ability to maintain a coordinated increase in the concentration of

carotenoid, anthocyanin and proline resulting in lower ROS production and higher plant stability. Therefore, antioxidant compounds in shoots of the studied plant may play important roles to accumulate and detoxify As, although details on the detoxification mechanism remain unknown.

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