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Effect of Phosphorus on Arsenic Accumulation and Detoxification in Arsenic Hyperaccumulator, *Isatis cappadocica*

Naser Karimi · Zahra Souri

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Abstract Arsenic (As) and phosphorus (P) interactions are important for better understanding their uptake and accumulation by plants due to similar chemical behaviors. In this study, we investigated the interactions of arsenic and phosphorus on plant biomass and uptake of arsenic and phosphorus by *Isatis cappadocica*, a newly discovered arsenic hyperaccumulator. In a 28 day hydroponic experiment with varying concentrations of arsenate (0, 50, 200, 800, and 1,200 μmol l^{-1}) and phosphate (5, 50, 200, 800, and 1,600 μmol l^{-1}), *I. cappadocica* accumulated As in the shoots up to 700 mg As kg^{-1} dry weight, and the shoot As to root As concentration ratio varied between 0.6 and 1.5. At low and medium As levels (50 and 200 μmol l^{-1}), phosphate had slight effects on As uptake and growth of *I. cappadocica*. However, increasing P supply decreased As uptake markedly, with the effect being greater on root As concentration than on shoot As concentration at high arsenate levels (800 and 1,200 μmol l^{-1}). Increasing As supply decreased the P concentration in the roots and shoots, especially at high levels, because of its phytotoxicity. The root P concentrations of *I. cappadocica* were greater than those of shoots, which is in contrast to non-accumulator plants. Our findings suggest that P application may be an important strategy for efficient use of *I. cappadocica* to phytoremediate As-contaminated areas. Further studies are needed on the mechanisms of interactive effects of As and P on *I. cappadocica* in the soil system.

Keywords Arsenic · Phosphorus · Interaction · Hyperaccumulator · *Isatis cappadocica*

N. Karimi (✉) · Z. Souri
Laboratory of plant physiology, Department of Biology, Faculty of Science, Razi University, Baghe Abrisham, Kermanshah, Iran
e-mail: nkarimie@yahoo.com; nkarimi@razi.ac.ir

Introduction

Arsenic (As) is a toxic metalloid that is harmful to plants, animals, microorganisms, and human beings (Liu and others 2009). It is listed as a priority hazardous substance and is considered one of the top 20 contaminants by the USEPA (Agency for Toxic Substances and Disease Registry (ATSDR) 2012). It is released into the water, soil, and air from natural and anthropogenic sources (Flora and others 2009) and can accumulate in the environment to toxic levels (Sridhar and others 2011). With greater public awareness of the implications of contaminated soil and water on human and animal health, there has been increasing interest among the scientific community in the development of technologies to remediate As-contaminated soil and water (Bolan and others 2011; Khang and others 2012).

In contrast, as the chemical analog of As, phosphorus (P) is one of 17 essential elements required for plant growth. However, P is frequently the most limiting element for plant growth and development. It has low bioavailability because it rapidly transforms to insoluble complexes with cations, particularly with aluminum and iron under acid conditions (Vance and others 2003). Both As and P belong to the VA family on the periodic table of chemical elements. Due to their similarities in chemical properties, they behave similarly in many ways in the soil–plant system. P and As compete with each other during uptake in the plant system (Stoeva and Bineva 2003), and plant uptake of As is usually suppressed by P uptake when P is added (Rahman and others 2008; Gunes and others 2009). Therefore, the physiological behaviors of As and P in the plant system are very different. However, the effect of P on As accumulation in an As-hyperaccumulator is poorly understood.

Isatis cappadocica, as a robust perennial rosette plant, has been established in temperate Asian regions. It possesses characteristics such as rapid growth, high biomass (producing strongly branched inflorescence-bearing stems up to 60 cm high in their native habitat), and easy cultivation (Karimi and others 2010). It can grow in highly impacted As-contaminated areas and hyperaccumulates As in its areal parts (Karimi and others 2010). These characteristics could make it a potential candidate for phytoremediation purposes (Karimi and others 2009; Karimi and others 2010; Karimi and others 2013; Karimi and Souri 2013). Because As hyperaccumulation in *I. cappadocica* is a newly discovered phenomenon (Karimi and others 2010), the mechanisms of As and P interaction during uptake and root to shoot translocation and specifically the mechanism of how *I. cappadocica* acquires and maintains P nutrition under high As supply have not been elucidated. It is expected that a better understanding of As uptake and, As and P distribution in *I. cappadocica* would emerge from this study, which could further elucidate the mechanism of plant As detoxification and hyperaccumulation. Most importantly, such knowledge may be of great importance to commercialize the phytoremediation technology for As polluted soils and groundwater. Therefore, the overall aim of this study was to determine As uptake and As and P distribution characteristics as influenced by P in *I. cappadocica*. To address the P × As interactions in *I. cappadocica*, we used hydroponic culture rather than soil culture, because a hydroponic experiment is needed to clearly elucidate the interactive effects of phosphate and arsenate on plant uptake because it is difficult to separate the effects.

Materials and Methods

Source of Plant Material

Seeds of *I. cappadocica* were collected from a population growing at the gold- As Zarshuran deposit mine, grid reference 36°43′04″N 47°08′02″E, 40 km north of the town of Takab in the West Azarbaijan province, northwest of Iran. The total soil As concentration in this deposit area ranged from 145 to 6,525 mg kg^{-1} (Karimi and others 2010).

Plant Culture and Arsenate and Phosphate Treatments

A complete factorial experimental design was used to study the uptake of As and P at five levels of arsenate concentration (0, 50, 200, 800, and 1,200 μM) added as Na_2HAsO_4 and five levels of phosphate concentration (5, 50, 200, 800, and 1,600 μM) added as KH_2PO_4. Seeds of *I. cappadocica* were 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 d at room temperature, below 20 °C. After germination, seedlings were then transferred to 1liter polyethylene pots (four seedlings per pot) filled with a nutrient solution (Karimi and others 2009) composed of 0.5 mM KNO_3, 0.75 mM $Ca(NO_3)_2$, 0.2 mM $MgSO_4$, 15 μM H_3BO_3, 2 μM $MnCl_2$, 1 μM $ZnSO_4$, 0.5 μM $CuSO_4$, 0.5 μM Na_2MoO_4. $2H_2O$, and 50 μM Fe-EDTA (pH 6.0). The nutrient medium was continuously aerated with an aquarium air pump, renewed twice per a week, and adjusted at the daily pH levels of 5.5 ± 0.1 with HCL or NaOH. After cultivating, the plants were amended for 10 days, the solutions with different arsenate and phosphate added for another 18 days. The plants had been growing in a growth chamber (Conviron 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^{-2} s^{-1}. Each treatment was replicated three times and each time, the pots were randomly arranged during the growth period.

Measurements of Plant Biomass

The plants were harvested after they were exposed to arsenate for 18 days. Then, plant roots were washed with tap water followed by rinsing in ice-cold phosphate buffer containing 1 mM Na_2HPO_4, 10 mM MES, and 0.5 mM $Ca(NO_3)_2$ to ensure desorption of As from material surface and the root free space. Thereafter, the plants were rinsed in tap water followed by deionized water. The *I. cappadocica* were separated into roots and shoots, and dried at 65 °C for determining dry biomass, total As and P. The root and shoot biomass $plant^{-1}$ were measured.

Analysis of Total As Concentration in Plant

Total As was determined in acid digests of ground roots and shoots (100–200 mg DW) mixed with 2 ml of HNO_3 (67 % suprapur) and 2 ml of H_2O_2 (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 analyzed using a hydride generation–atomic absorption spectrometry with a flow injection hydride generator interfaced with a Shimadzu AA-6,200 atomic absorption spectrometer (HG-AAS). The reference standard for calibration of the AAS was made using 1,000 mg l^{-1} (Beach leaves material FD8, Commission of the European Communities, Joint Research Centre ISPRA).

Quantification of Total P Concentration in Plant

Because As interferes with P determination, plant P was determined by a modified method (Cavell 1995). Briefly,

the pH of the digestion solution was adjusted to around 5 with NaOH and HCl. Ten milliliters of the solution was pipetted into a 50 ml-glass test tube; to this 0.5 ml of HNO_3 was added. The solution was cooled to room temperature, and P was measured spectrophotometrically at 450 nm using the ammonium vanadate reaction at a fixed time (20 min) following addition of the color reagent.

Bioaccumulation Factor (BF) and Translocation Factor (TF)

The effect of P on the bioavailability of As in solution culture was examined by calculating the As bioaccumulation (Eq. 1) and translocation (Eq. 2) coefficients.

$$Bioaccumulation\ factor\ BF = Plant\ tissue\ concentration / Growing\ medium\ concentration \quad (1)$$

$$Translocation\ factor\ TF = Shoot\ As\ concentration / Root\ As\ concentration \quad (2)$$

where the growing medium concentration is expressed in mg l^{-1}, and plant tissue concentrations in mg kg^{-1}.

Statistical Analysis

Statistical analysis was performed using the SPSS statistical package version 16.0. Analysis of the 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 at $P < 0.001$. All the values presented in this paper were expressed as the means of three replicates ± standard error (SE).

Results and Discussion

Arsenic Accumulation in Shoots and Root Tissues

I. cappadocica is very efficient in absorbing As from soil and translocating it from roots to shoots (Karimi and others 2009, 2010). The As concentration in *I. cappadocica* is generally higher in the shoots than in the roots. These findings were also observed in the present study (Fig. 1). During the 28 day exposure to As, no visible symptoms of inhibition of plant growth or death tissue sections were observed on *I. cappadocica*. The concentration of As in the shoots was up to 700 mg kg^{-1} dry weight with no visible phytotoxic symptoms. This threshold value is similar to that reported by Karimi and others (2009), who grew *I. cappadocica* plants on a soil spiked with different sources of As. The tolerance of *I. cappadocica* to As is far greater than that observed for many non-hyperaccumulating plant species, which have a threshold concentration for phytotoxicity of between 5 and 100 mg kg^{-1} dry weight (Kabata-Pendias and Pendias 1992). Internal detoxification of As must be an important feature of this hyperaccumulator species.

Effect of Phosphorus Concentrations on Arsenic Toxicity

The ability of *I. cappadocica* to uptake and sequester As into the shoots depends highly on plant P status. The highest As concentrations in the shoots were observed in plants treated with 1200 μmol l^{-1} As and 5 μmol l^{-1} P, which it reached 700 mg kg^{-1} (Fig. 1). Such high affinity for As at high As concentration by *I. cappadocica* indicated that it was equipped with detoxification mechanisms that enabled it to accumulate additional As. Interactive effects of As and P were observed in root As concentrations (Fig .1). However, at high arsenate levels (800 μmol), phosphate treatment (1,600 μmol) decreased As concentrations by 61–82 % and 53–77 %, respectively (Fig. 1). The inhibitory effect was more apparent when the concentrations of phosphate and arsenate in nutrient solution were comparable, and affected the root As concentration more than the shoot As concentration. This was because As and P are chemical analogs, the suppression of arsenate uptake by phosphate was expected as it is a common phenomenon in many plant species. For instance, plant As reduction by phosphate has been observed in *Pteris vittata* (61–82 %) and *Nephrolepis exaltata* (53–77 %) where phosphate greatly inhibited As uptake (Tu and Ma 2004). Furthermore, Huang and others (2007) found that phosphate addition reduces arsenate translocation, as well as As accumulation in *P. vittata* fronds.

A hydroponic experiment by Meharg and Macnair (1990) showed that P at 5,000 μM reduced arsenate uptake by 75 % in both tolerant and non-tolerant plant genotypes of soft grass (*Holcus lanatus* L.) grown in 50 μ*M* arsenate solution. Alfalfa (*Medicago sativa* L.) shoot arsenate concentrations were also decreased by phosphate (Khattak and others 1991). Even for Indian mustard (*Brassica juncea* L.), a hyperaccumulator, grown in 500 μ*M* arsenate hydroponic solution with phosphate addition at 1,000 μ*M*, a reduction of arsenate uptake by 55–72 % (Tu and Ma 2003) was recorded. Based on these results, therefore, to enhance plant As accumulation, the quantity of P in the growth medium should be limited. However, studies conducted under soil culture revealed an opposite phenomenon. Cao and others (2003) and Caille and others (2004) have reported that P addition increases As

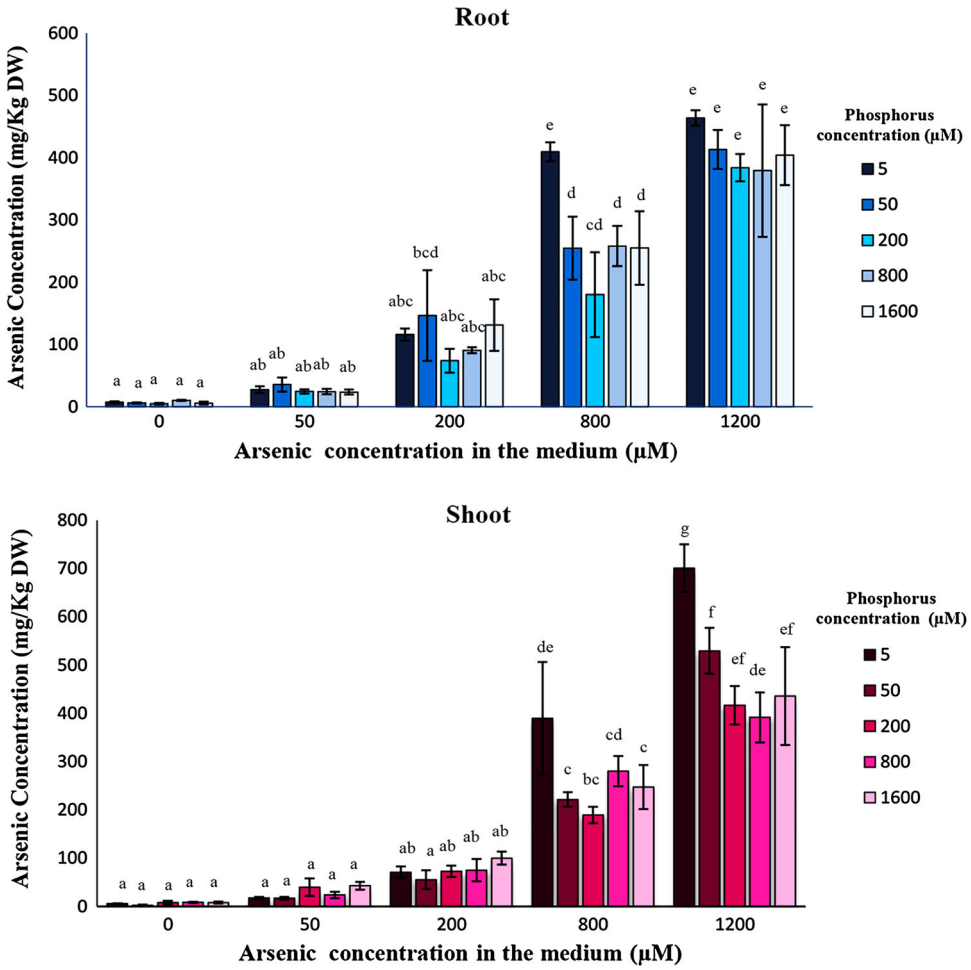

Fig. 1 Total arsenic concentrations (mg/kg, DW) in roots and shoots of *I. cappadocica* grown for 28 days in As- and P-amended media. Values represent mean ± SD. *Different letters* indicate significant differences at the 5 % level according to the Duncan test

removal from soil by *P. vittata* and presumed that, due to competition between As and P desorption in soils, P could improve As removal in soil culture, despite the observation that As and P compete with each other under hydroponic or sand experimental conditions; the relative ratio of As to P may also have played a role. Tu and Ma (2003) reported that the addition of 2.67 mmol arsenate kg^{-1} soil (soluble As at 0.589 mmol kg^{-1}) increases P uptake, but a higher As addition (5.34 mmol As kg^{-1} soil, soluble As at 1.339 mmol kg^{-1}) decreases P concentration.

Total Phosphorus Concentration in Plant Tissues

The concentrations of P in the roots and shoots of *I. cappadocica* are shown in Fig. 2. Increasing arsenate concentration in the solution decreased the concentration of P in the roots significantly ($P < 0.05$), particularly when the P supply was high (800 and 1,600 µM). In contrast, the arsenate treatments had a lower effect on the P concentration in the shoots. The highest P concentration in *I. cappadocica* was found in 0 µmol l^{-1} arsenate and 1,600 µmol l^{-1} phosphate treatment and the lowest in the 1,200 µmol l^{-1} arsenate and 5 µmol l^{-1} phosphate. The pattern of P distribution between roots and shoots was markedly different from that of As and those that occur in most other plants. Arsenate rates and the interaction of arsenate and phosphate significantly influenced P concentrations in both roots and shoots of *I. cappadocica* (Fig. 2). At high rates, As decreased P accumulation in the roots and shoots, obviously due to phytotoxicity. In most plants, P is generally concentrated in the upper parts or reproductive organs (Pederson and others 2002; Watson and Mullen 2007; Gunes and others 2009). On the contrary, the concentration of P in the roots of *I. cappadocica* was greater than that in the shoots. Such a pattern was also observed in *P. vitatta* as evidenced by the TF values of around one (Tu and Ma 2004). The fact that the root P concentrations of *I. cappadocica* were greater than those of non-accumulator plants may imply that its ability to keep high P concentrations in the roots constitutes one of it's As detoxification mechanisms. More research is under way to further explore this hypothesis.

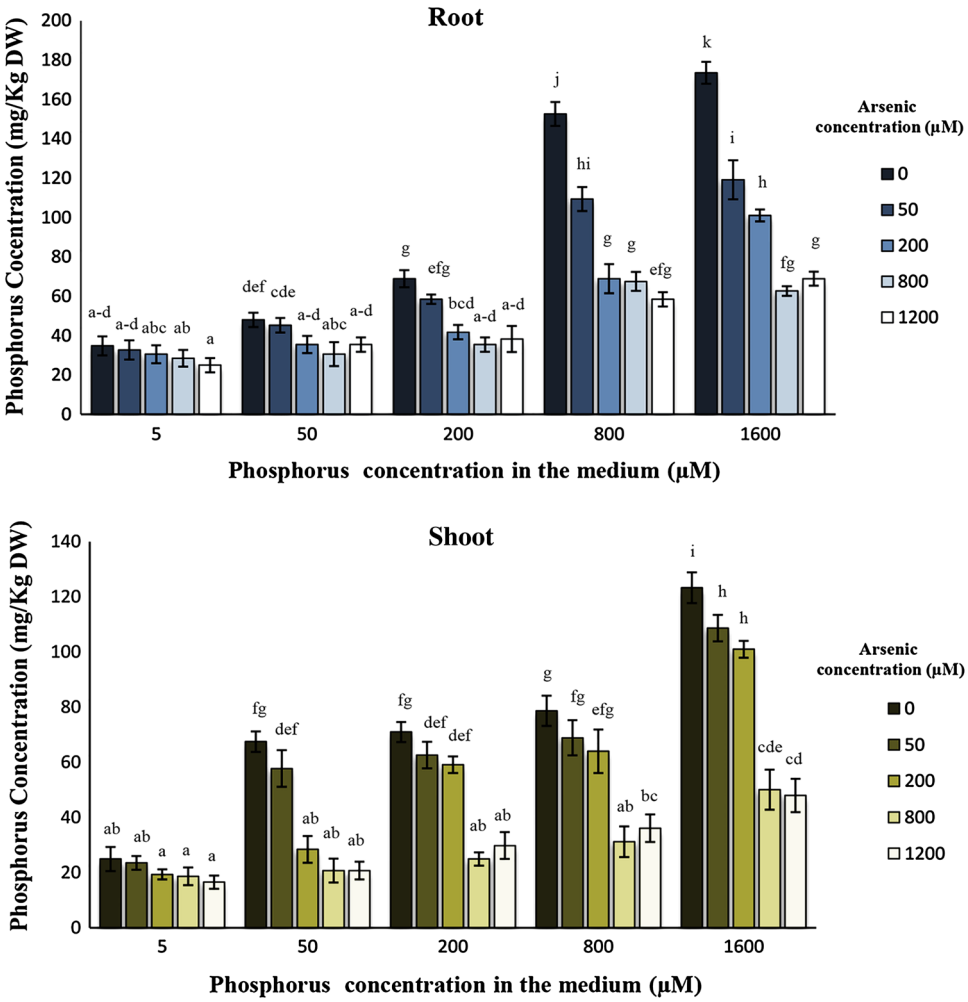

Fig. 2 Total phosphorus concentrations (mg/kg, DW) in roots and shoots of *I.cappadocica* grown for 28 days in As and P-amended media. Values represent mean ± SE. *Different letters* indicate significant differences at the 5 % level according to the Duncan test

Plant Growth

Generally, the biomass of plants may give an important index for identifying them as tolerant and an accumulator. So, this parameter should not decrease significantly at the threshold concentration of inhibiting plant growth (Karimi and others 2013). Compared to the control, a 50 µM arsenate supply in the solution enhanced the growth of *I. cappadocica* (Fig. 3). This appears to be due to the dilution effects from greater biomass production, which resulted from alleviation of arsenate phytotoxicity by phosphate (Fig. 3).The growth was lower at the highest arsenate concentration (1,200 µM), but without significant difference from the control. A possible explanation for the reduction of plant biomass at high arsenate levels (1,200 µmol l^{-1}) may be related to its high As accumulation, because the plant may have to use energy to cope with the high As concentration in the tissues. Nevertheless, no toxicity symptoms were observed for *I. cappadocica* at this level, implying that it tolerated As at 1,200 µmol l^{-1} in this medium.

Bioconcentration Factor (BF) and Translocation Factor (TF)

While evaluating hyperaccumulation, in addition to total As content, both the bioconcentration factor (BF) and the translocation factor (TF) need to be considered. The bioconcentration factor is defined as the ratio of metal concentration in the plant to metal concentration in the medium. As BFs for *I. cappadocica* were 3.38–8.54, 2.76–4.99, 2.76–3.50, and 3.26–5.84 at 50, 200, 800, and 1,200 µM arsenate, respectively (Table 1). These results show highly efficient accumulation of As from the medium by *I. cappadocica*. The bioconcentration factor of *I. cappadocica* was increased by As treatment, but the two highest levels of phosphate supply (800 and 1,600 µM) gave lower BF than the two lowest rates of phosphate supply. With the 800 µM arsenate treatment, the 1,600 µM phosphate treatment gave a lower BF than the lowest rates of phosphate supply.

Translocation factor (TF), used as an index to measure the effectiveness of plant metal translocation, is defined as the

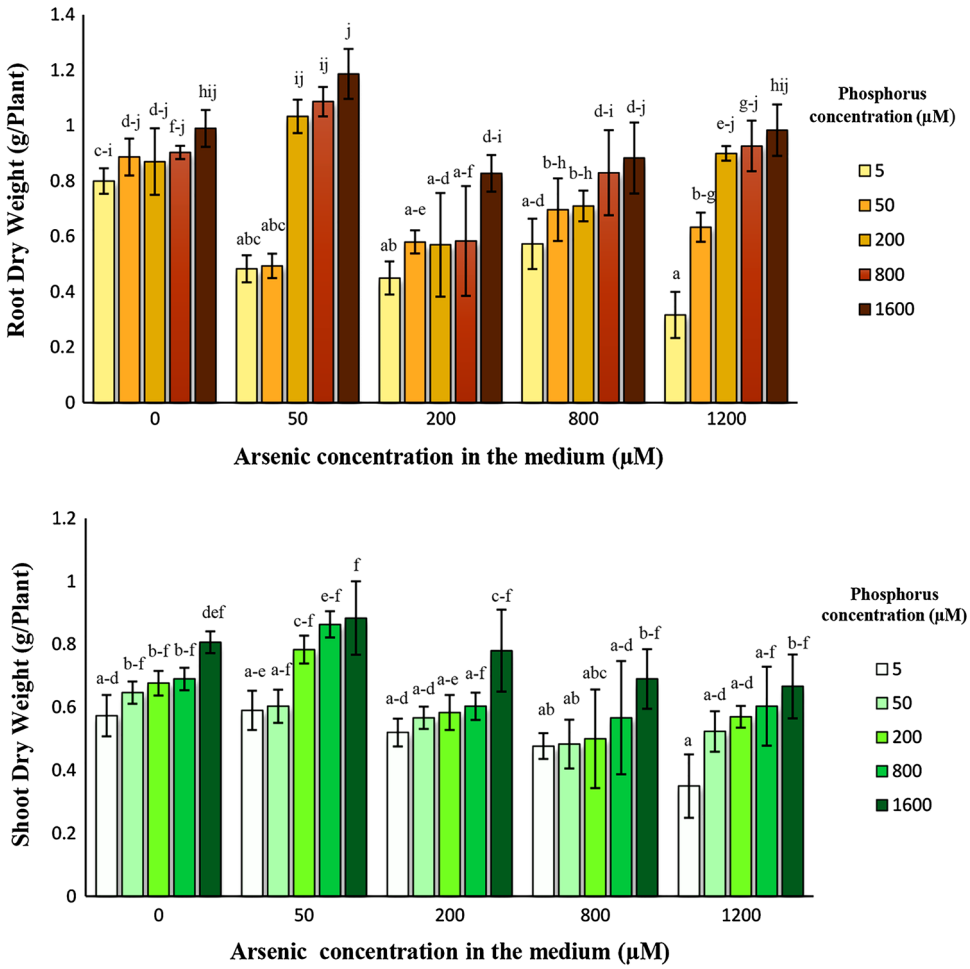

Fig. 3 Dry weight (g/plant) in roots and shoots of *I.cappadocica* grown for 28 days in As and P-amended media. Values represent mean ± SD. *Different letters* indicate significant differences at the 5 % level according to the Duncan test

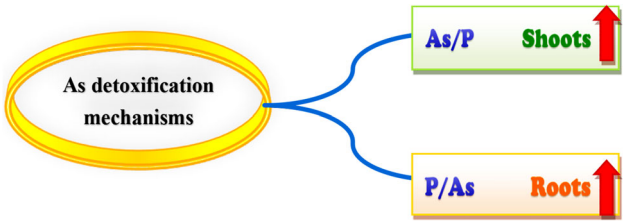

Fig. 4 The proposed pattern for As detoxification mechanisms in *I. cappadocica* against As stress

ratio of As concentrations in shoots to those in the roots. This research revealed that As TF for *I. cappadocica* were 0.538–1.87, 0.44–1.22, 0.95–1.39, 1.08–1.50 at 50, 200, 800, and 1,200 µM As (Table 1) respectively, indicating that *I. cappadocica* transferred much of the high levels of As from its roots to shoots in comparison with non-accumulator plants (Wang and others 2002; Gunes and others 2009; Karimi and others 2013). As expected, increased As levels enhanced the As TF index of *I. cappadocica*. Similar trends were observed for the increase in As TF when P was added to solutions with low As (50–200 µM).

In the current study, As accumulation in *I. cappadocica,* in low arsenate treatments, was improved obviously by adding a large amount of phosphate. However, addition of phosphate to the solution suppressed As TF significantly (Table 1) in plants affected by high As treatments. When phosphate concentrations were increased from 5 to 1,600 µM in solutions with 1,200 µM arsenate, the decline in As TF was 38 %. Therefore, the present study shows that an increase in phosphate suppressed transport of As from roots to shoots when the plants were treated with arsenate. Thus, regarding understanding the effects of P on As transport, it is more important to consider the valence of As during transport. In the plants treated with As (V), part of As presented as As (V) might compete with phosphate during transport, and suppress As transport at higher phosphate concentrations (Table 1). Thus, in addition to that phosphate could suppress As (V) uptake in *I. cappadocica* during root uptake, which has been demonstrated by a short-term uptake kinetics experiment (Karimi and others 2009), we infer that competition between phosphate and As (V) may also occur during root to shoot transport. This phenomenon was reported previously in *P. vittata* (Huang and others 2007).

Table 1 Bioaccumulation and transfer factor of *I. cappadocica* exposed during 28 days to different concentrations of As and P

As (μM)	P (μM)	(TF)	(BF)
50	5	0.647[a]	3.487[a]
50	50	0.538[a]	3.384[a]
50	200	1.495[ab]	7.922[bc]
50	800	1.073[ab]	4.70[ab]
50	1,600	1.887[b]	8.544[c]
200	5	0.626[a]	3.525[a]
200	50	0.445[a]	2.764[a]
200	200	1.229[ab]	3.619[a]
200	800	0.856[ab]	3.748[a]
200	1,600	0.973[ab]	4.994[ab]
800	5	0.953[ab]	4.871[ab]
800	50	0.951[ab]	2.768[a]
800	200	1.394[ab]	2.367[a]
800	800	1.109[ab]	3.505[a]
800	1,600	1.165[ab]	3.088[a]
1,200	5	1.509[ab]	5.841[abc]
1,200	50	1.289[ab]	4.410[bc]
1,200	200	1.080[ab]	3.471[a]
1,200	800	1.166[ab]	3.262[a]
1,200	1,600	1.135[ab]	3.632[a]

Different letters indicate significant differences at the 5 % level according to the Duncan test

In addition, P deficiency was found to significantly increase arsenate concentration in the shoots of *I. cappadocica*. Published reports indicate that, in response to P deficiency, plants may increase synthesis of additional transporter molecules (Drew and others 1984) or upregulate expression of genes encoding P transporters (Smith and others 1998), leading to a subsequent increase in transporter proteins (Muchhal and Raghothama 1999). The increase of As (V) uptake with P deficiency has been found by Wang and others (2002), Singh and Ma (2006) and Lei and others (2012), who showed that P starvation increases the maximal influx of As (V) in the rhizoid cell plasma membranes of *P. vittata*, suggesting an increased density of P/As (V) transporters in the rhizoid cell plasma membranes.

The most striking feature associated with the As hyperaccumulation by *I. cappadocica* lies in the exceedingly efficient transport from roots to shoots. This trait is observed in other As-hyperaccumulators (Ma and others 2001). The shoot to root ratio of As concentration was around 1 in the long-term As × P experiment and also As-contaminated land (Karimi and others 2010). In contrast, this was not observed in *B. Juncea* (Grifoni and others 2014) or in the five other Brassica species tested by Raab and others (2007). Thus, it seems that these species have the characteristic of an As hyperaccumulator.

Conclusion

Data of total As concentrations in shoots, total P concentrations in roots, bioaccumulation and translocation factors showed that *I. cappadocica* had steady As accumulation characteristics in the As-contaminated media. *I. cappadocica* is, therefore, a potentate As-hyperaccumulator and could be advantageous in phytoremediation. The results of this study demonstrated that *I. cappadocica* has a high tolerant ability to As based on its good growth and an effective strategies for As detoxification. Also these data suggest that P application may be an important strategy for the efficient use of *I. cappadocica* to phytoremediate As-contaminated soils. Moreover, these results imply that *I. cappadocica* is a good material for plant physiologist to conduct comparative and mechanism studies on the uptake behaviors of P and As. Obviously, further studies are required to explain the mechanisms for As detoxification in *I. cappadocica*.

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