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EFFECT OF ARSENIC ON GERMINATION OF *ISATIS CAPPADOCICA* DESV., A NEWLY DISCOVERED ARSENIC HYPERACCUMULATOR

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Isatis cappadocica Desv. is a newly found As-hyperaccumulator showing very high remediation efficiency in polluted soils. We studied the effects of arsenic at 0–1400 μM concentrations on seed germination, relative root length, relative shoot height, and root and shoot biomass in young seedlings of *I. cappadocica*. The seeds were from Iranian arsenic-contaminated mine spoils and from a non-contaminated population. The control for reference was brassica (*Descurenia sofia*). Germination decreased significantly versus the control with increasing arsenic concentrations. The response to arsenic exposure differed between the *I. cappadocica* populations and *D. sofia*. *I. cappadocica* from mine spoil seeds showed strong resistance to the highest As concentration, with no adverse effects until the 1000 μM dose. Germination from non-mine seeds of *I. cappadocica* decreased to 89.6% at 1400 μM As. *D. sofia* germination was completely inhibited at 400 μM As. Relative root length (RRL) and relative shoot height (RSH) decreased with increasing As concentration. Overall, RRL correlated with RSH. Shoot height and root length were more sensitive to arsenic than other endpoints, and might be used as arsenic toxicity indicators. *I. cappadocica* showed more As tolerance than the reference brassica.

Key words: Arsenic, *Isatis cappadocica* Desv., germination, seedling, tolerance, hyperaccumulation.

INTRODUCTION

Arsenic (As) toxicity has been known for centuries and has recently received increased attention because of its chronic and epidemic effects on human health. It can enter the environment through weathering, biological pathways and volcanic activity. Anthropogenic inputs from agriculture and industry, such as pesticides and chemical fertilizers, wastewater used for irrigation, dustfall from coal combustion and smelter wastes, and residues from metal mining, increase the level of As contamination in soil, groundwater and surface water (Zhang et al., 2002; Reza et al., 2010; Zandsalimi et al., 2011). Arsenic contamination of soil, streams and underground water poses a major environmental and human health risk. Groundwater contamination with arsenic is reported from many regions of the world, and it is a serious problem in China (WHO, 2001). Arsenic-contaminated groundwater is used as a source of drinking water and also extensively for irrigation in some regions.

Elevated levels of As in irrigation water or soil can hamper the normal growth and development of plants. When exposed to excess As in soil or in culture solution, plants can develop toxicity symptoms: reduced germination, seedling growth, the tolerance index, plant height (Abedin and Meharg, 2002; Karimi et al., 2010) root and shoot growth (Carbonell-Barrachina et al., 1998), leaf area and photosynthesis, and can cause wilting and necrosis of leaf blades (Knauer et al., 1999; Liu et al., 2005).

Phytoremediation is an emerging technology which employs higher plants to clean up contaminated environments. Phytoextraction represents one of the largest economic opportunities for phytoremediation technology, with many advantages such as low cost, preservation of soil structure, landscape beautification, public acceptance and the absence of secondary pollution (Tripathi et al., 2007; Zhao et al., 2009). Phytoextraction involves the use of hyper-

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accumulators to accumulate metals from soil into plant roots and translocation/accumulation (phytoextraction) of the contaminants into plant shoots until the metal concentration in the soil decreases to acceptable levels (Williams et al., 2007). Great progress has been made in identifying hyperaccumulators, but phytoextraction technology has not yet been widely applied in remediation practice. The main limitation has been the low remediation efficiency of hyperaccumulators due to their low biomass and long growing seasons (Gonzaga et al., 2006; Karimi et al., 2009).

Isatis cappadocica is a robust perennial established in temperate regions of Asia. It is characterized by fast growth, high biomass (in its native habitat producing densely branched inflorescence-bearing stems up to 60 cm high) and easy cultivation (Karimi et al., 2010). It can thrive in highly impacted arsenic-contaminated areas and hyperaccumulate arsenic in its aerial parts (Karimi et al., 2010). In our previous research, the data on total arsenic concentration of shoots, bioaccumulation factors and translocation factors showed steady arsenic accumulation in *I. cappadocica* growing on arsenate-contaminated media (Karimi et al., 2009, 2010, 2013). *I. cappadocica* is an arsenic hyperaccumulator of potential use for phytoremediation in As-contaminated areas.

To determine the toxicity of As to *Isatis cappadocica* plants, in this study we tested the effects of As on germination and early seedling growth from seeds from two *I. cappadocica* populations, one growing in an area with high soil loads of arsenic and the other growing under low arsenic exposure, and from *D. sofia* L. seeds as reference.

MATERIALS AND METHODS

SOURCE OF PLANT MATERIAL

Seeds of *Isatis cappadocica* Desv. were randomly collected from 50 plants in a population growing at the Zarshuran gold-arsenic mine (36°43'04" N, 47°08'02" E) 40 km north of the town of Takab in West Azarbaijan Province, northwest Iran. The total soil As concentration in this deposit area ranges from 145 to 6525 mg kg^{-1} (Modabberi and Moore, 2004). Seeds of the non-mine *I. cappadocica* population were sourced from Kouhin Hill (36°16'09" N, 50°00'10" E) in Qazvin Province, with soil total arsenic concentrations ranging from 2 to 10 mg kg^{-1}. Reference brassica (*Descurenia sofia*) seeds were purchased.

ARSENIC TREATMENTS

Healthy seeds were surface-sterilized in 0.5% sodium hypochlorite for 10 min and then rinsed four times with deionized distilled water and soaked in deionized distilled water for 24 h. Seeds were germinated on sterile filter paper (Whatman No. 1) and exposed to a gradient of As concentrations (0, 5, 10, 25, 100, 200, 400, 600, 800, 1000, 1200, 1400 μmol l^{-1}) supplied as $Na_2HAsO_4.7H_2O$. Then 50 seeds of each group per treatment were evenly dispersed in a sterile Petri dish, covered with a lid, and incubated in a dark chamber at 28±1°C. Seeds germinated on moistened filter paper with distilled deionized water were the control. Each treatment was done in triplicate.

DETERMINATION OF GERMINATION PARAMETERS

The geminated seeds were counted 7 days after sowing under treatment. The results are expressed as percentages of the control. Seeds were considered germinated when both the plumule and radicle extended more than 2 mm from their junction. Then the radicle length, plumule length, radicle weight and shoot weight of the 7-day-old seedlings were measured.

SEEDLING TOXICITY TEST

Randomly picked germinated seeds from the *I. cappadocica* populations and germinated *D. sofia* seeds were used for seedling toxicity tests with arsenate. Surface-sterilized seeds (see previous description) were germinated by placing 40–50 seeds on sterile filter paper in Petri dishes with 5 mL distilled water. All the Petri dishes were covered with lids and placed in an incubator at 25°C. Five days later, when the plumule and radicle of the seeds had emerged, two viable and uniform seedlings from a single sample were transferred to a plastic pot containing 100 mL test solution and 30 g black plastic beads floating on the surface of the solution. The test solutions of different arsenate concentrations (0–1400 μmol L^{-1}) also contained 0.2 mM $Ca(NO_3)_2$, 0.2 mM KNO_3 and 0.1 mM $MgSO_4.7H_2O$. Arsenate was prepared freshly as $Na_2HAsO_4.7H_2O$ and each treatment was done in triplicate. Then the pots were placed randomly in a Conviron 30 growth cabinet to ensure normal growing conditions. Artificial light was supplied at 152 μmol m^2s^{-1} intensity under a 12 h photoperiod (32°C day, 25°C night) and 80% humidity. The test solution was renewed after 4 days in order to avoid changes in As speciation (Carbonell-Barrachina et al., 1998).

DETERMINATION OF As CONTENT

Seven days after initiation of treatments, uniform plant seedlings were immersed in K_2HPO_4 solution for 15 min, thoroughly rinsed with deionized water, blotted with tissue paper, weighed, transferred to flasks and digested in a 10 mL mixture of concentrated nitric acid and H_2O_2 (by volume). The arsenic content of the seedlings was determined by hydride gen-

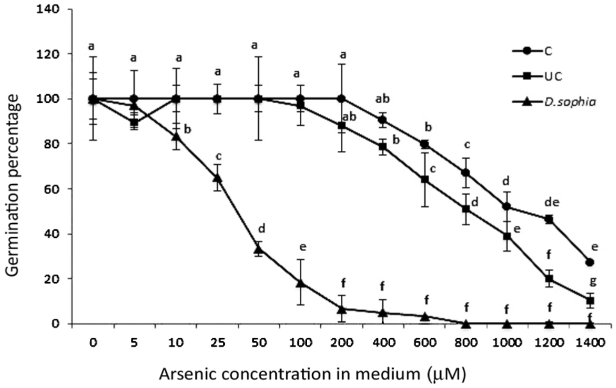

Fig. 1. Effect of arsenic dose on seed germination (% of zero-dose control) in *I. cappadocica* mine (C) and non-mine (UC) populations and in *Descurenia sofia*.

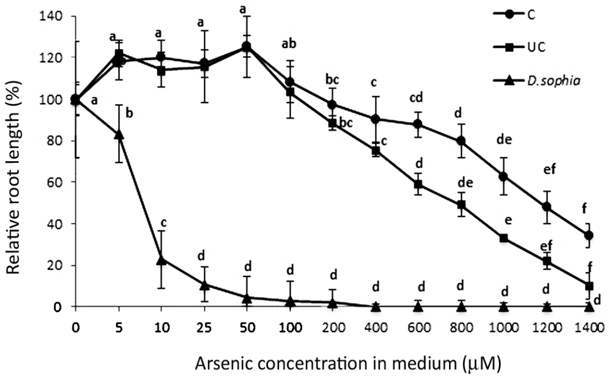

Fig. 2. Relative root length under different arsenic doses in *I. cappadocica* mine (C) and non-nmine (UC) populations and in *D. sofia*.

eration atomic absorption spectrometry with a flow injection hydride generator interfaced with a Shimadzu AA-6200 atomic absorption spectrometer (HG-AAS). All measurements were made in triplicate. Measurement quality was assured by determining total As in certified reference materials (Beech leaves material, BCR 100, commission of the European Communities, Joint Research Centre, Ispra).

DETERMINATION OF RELATIVE ROOT AND SHOOT HEIGHT

After 7 days of growth, shoot height was measured from the culm base to the tip of the longest leaf and root length was measured from the root-shoot junction to the tip of the longest root. Root length and shoot height of plants grown in As test solution are expressed as relative root length (RRL) and relative shoot height (RSH), both calculated by dividing the length of As-treated plants by the length of control plants ×100. For determination of biomass (fresh weight), tissue samples were blotted on Whatman paper and weighed.

STATISTICAL ANALYSIS

Statistical analyses used SPSS ver. 16.0. One-way ANOVA was performed to test the significance of differences in all measured variables. Duncan's multiple range test (DMRT) was performed to reveal significant differences within groups. All values are means of three replicates ±SE.

RESULTS

EFFECT OF ARSENIC ON GERMINATION

The mean germination percentage versus the control decreased significantly ($P<0.01$) with increasing As concentration in both populations of *I. cappadocica* and in *D. sofia* as the reference brassica (Fig. 1). *D. sofia* showed poor germination at high As concentrations (≥200 µM) but plants sourced from both the mine and the non-mine populations of *I. cappadocica* showed good resistance to the highest As concentration (1400 µM), with no adverse effect at 200 µM As in the mine-sourced population (Fig. 1). Below 600 µM As, arsenic had no significant effect on germination of the mine-sourced seeds. In the 600 µM As treatment, germination was 90.3% for mine-sourced seeds, 78.5% for non-mine-sourced seeds, and only 5% for *D. sofia* seeds (Fig. 1). In the 1400 µM As treatment, germination decreased significantly in *I. cappadocica* to 72.7% (mine-sourced seeds) and 89.6% (non-mine-sourced seeds), and *D. sofia* did not germinate at all.

EFFECT OF As ON EARLY DEVELOPMENT OF ROOTS AND SHOOTS

Relative root length was significantly affected by arsenic concentration and plant species (ANOVA, $P<0.01$), as shown in Figure 2. RRL for both populations of *I. cappadocica* and for *D. sofia* decreased significantly with increased As. The effect was more significant in *D. sofia* than *I. cappadocica* ($P < 0.05$). At 10 µM As, for example, RRL declined by 77% in *D. sofia* while in the mine and non-mine populations of *I. cappadocica* it increased to 120% and 114% respectively. Above 400 µM As, RRL decreased significantly ($P<0.05$) in both the mine and non-mine populations of *I. cappadocica*. Root growth in the mine population showed considerable resistance to the effects of arsenic. At the highest As concentration, RRL was significantly lower in the non-mine population (13%) than in the mine population (34%).

Figure 3 summarizes the effects of As on relative shoot height in *I. cappadocica* and *D. sofia*.

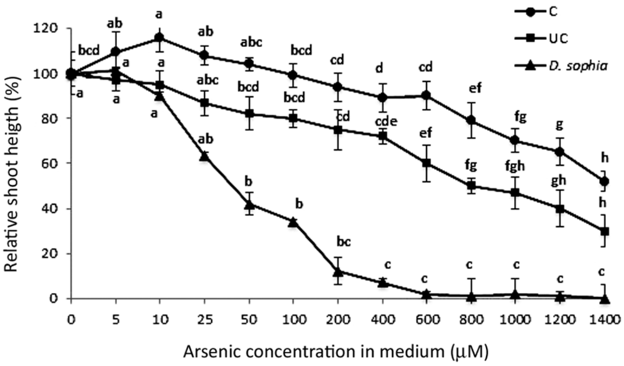

Fig. 3. Effect of arsenic dose on relative shoot height in *I. cappadocica* mine (C) and non-mine (UC) populations and in *D. sofia*.

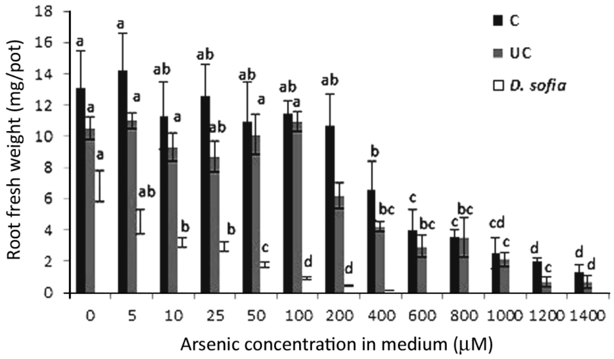

Fig. 5. Effect of arsenic dose on seedling root fresh weight in *I. cappadocica* mine (C) and non-mine (UC) populations and in *D. sofia*.

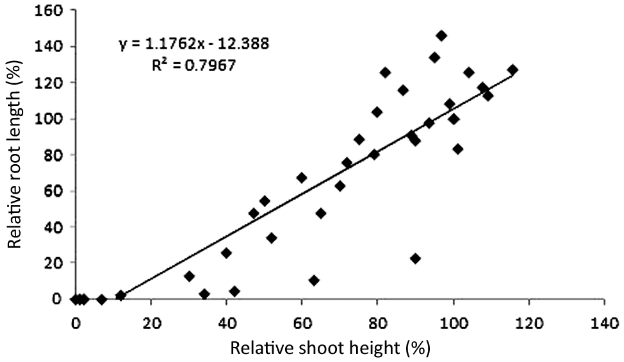

Fig. 4. Relationship between relative shoot height and relative root length under different arsenic doses.

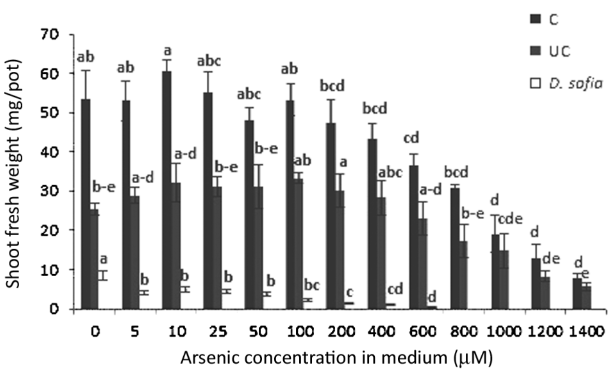

Fig. 6. Effect of arsenic dose on seedling shoot fresh weight in *I. cappadocica* mine (C) and non-mine (UC) populations and in *D. sofia*.

ANOVA indicated significant effects of As concentration and species on RSH ($P<0.05$). RSH declined with the increase of arsenic concentration. In the 100 μM As treatment, RSH was highest in the mine population (99.6%), followed by the non-mine population (80.1%) and *D. sofia* (34.3%). At 600 μM As, RSH fell sharply in both *I. cappadocica* populations; as expected, root growth was completely inhibited by arsenic. At the highest As concentration (1400 μM), relative shoot height was 52% for mine and 30% for non-mine *I. cappadocica*. At the different concentrations of As there is a strong relationship ($r^2 = 0.796$) between RRL and RSH (Fig. 4): RSH increases with increasing RRL. Reduction of shoot growth is highly correlated with reduction of root growth.

EFFECT OF As ON ROOT AND SHOOT BIOMASS

Figure 5 shows the root biomass of *I. cappadocica* mine and non-mine populations and *D. sofia* under treatment with As in nutrient solution. Both *I. cappadocica* populations were considerably more tolerant than the reference brassica species. At 10 μM As the root growth of the *D. sofia* was not more than 48% of the value from the 0 μM As treatment, while in the *I. cappadocica* populations it was at least 86% of the control value. At 400 μM As, root growth was totally inhibited in the reference brassica but more than 40% in all *I. cappadocica* treatments. At the highest arsenate dose, 1400 μM, there was a distinct difference between mine and non-mine *I. cappadocica*: the mine population still had more than 25% root growth, while root growth in the non-mine population ceased.

Shoot fresh weight showed similar patterns of response to As concentration in *I. cappadocica* and *D. sofia* (Fig. 6). In the reference brassica, gain of shoot fresh weight was at maximum in the 5 μM As treatment and then decreased dramatically at As levels higher than 400 μM.

At 5 μM As there were no significant differences in shoot biomass versus the control in the *I. cappadocica* populations. Shoot fresh weight in the *I. cappadocica* populations significantly decreased versus the control at 600–1200 μM ($P<0.05$). At 1400 μM As, maximum shoot fresh weight was only 14.4% in the mine population and 22.3% in the non-

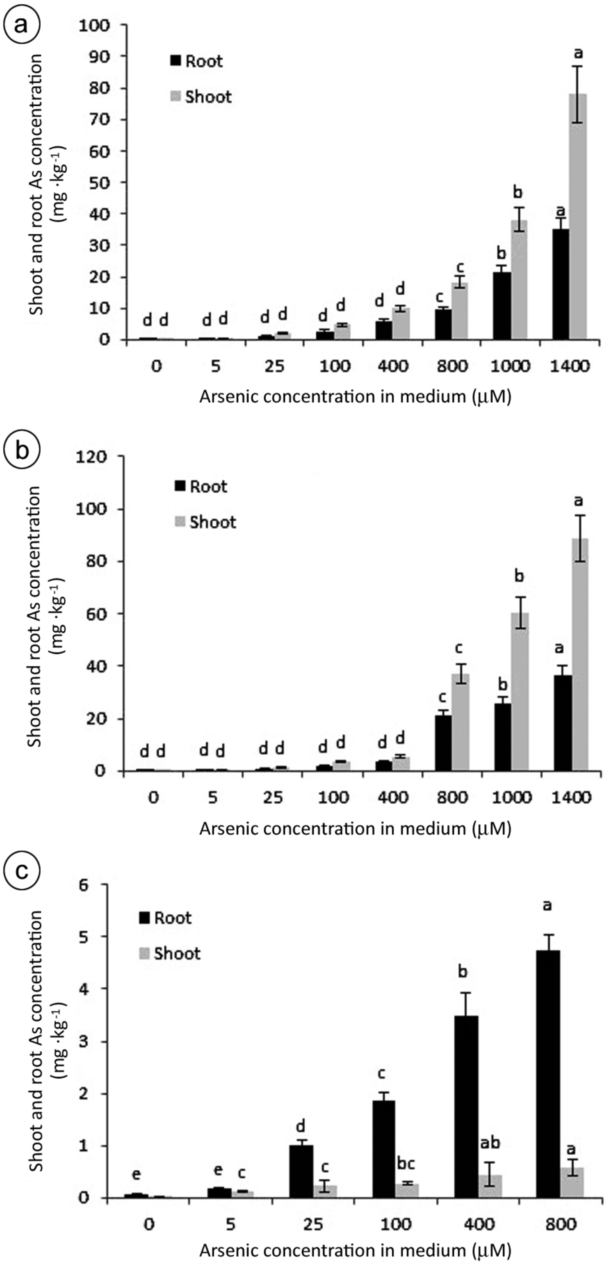

Fig. 7. Effect of arsenic dose on arsenic accumulation characteristics of *I. cappadocica* mine (**a**), non-mine (**b**) populations and of *D. sophia* (**c**).

mine population. It appears that both populations showed maximum production of shoot and root biomass at 600 μM As.

ARSENIC CONTENT IN PLANTS

We measured the arsenic content of the plants in order to determine any relationship between the level and the effect of As on seedling growth. Arsenic content increased with increasing As dose in both *I. cappadocica* and *D. sofia* (Fig. 7). Accumulation of As was significantly higher in *I. cappadocica* than in *D. sofia*, suggesting resistance or detoxification as possible mechanisms of high As tolerance in *I. cappadocica*.

At 1400 μM As the arsenic concentration reached maximum, 78 mg kg^{-1} FW, in shoots of the mine population, and it also reached maximum in the non-mine population. At higher As doses there was a significant gradient of As levels from roots to shoots. In both *I. cappadocica* populations, arsenic accumulation was higher in shoots than in roots (Fig. 7a, b), a trend observed in some hyperaccumulators. *I. cappadocica* accumulated more As than *D. sofia*, and much more rapidly. These above results indicate that *I. cappadocica* has the basic characteristics of a strong As-tolerant accumulator.

DISCUSSION

Arsenic, a ubiquitous toxic element, exerts adverse effects on the morphology, growth and photosynthetic processes of plants, reduces the biomass of root and shoots, inhibits enzyme activities and disturbs the uptake of mineral nutrients (Tripathi et al., 2007).

In this study we showed that germination of *I. cappadocica* and *D. sofia* was affected by As. Paliouris and Hutchinson (1991) observed no effect of arsenate on germination of *Silene vulgaris* (Moench) Garcke seeds even at concentrations up to 20 mg As L^{-1}. The As content of our seedlings increased markedly with the increasing As in the medium; it was significantly lower in *D. sofia* (lower As tolerance) than in *I. cappadocica* (higher As tolerance) (Fig. 7). The literature suggests that high resistance to As can be achieved by complexation of arsenic by peptides with SH-groups (Karimi et al., 2009), reduction of As influx by suppression of phosphate/arsenate uptake systems (Meharg and Macnair, 1992; Meharg, 1994), and/or enhanced production of antioxidants that detoxify reactive oxygen species (ROS) produced in response to As (Hartley-Whitaker et al., 2001; Requejo and Tena, 2005). The sensitivity of *D. sofia* to As might be due to disturbances of cell metabolism. In maize, for example, arsenic significantly inhibited amylase activity, especially α-amylase activity, retarding starch hydrolysis and reducing the supply of energy and substrates during seed germination and seedling growth (Requejo and Tena, 2005).

Under normal field conditions other factors may have adverse effects on seed germination. Soil characteristics such as texture, redox potential and total arsenic concentration can affect the solubility, mobility, bioavailability and toxicity of arsenic in soils (Anawar et al., 2006; Karimi et al., 2010). Also, continued addition of As to soils in irrigation water will lead to a steady buildup of As.

Our results suggest that a low concentration of As (0–50 μM) can stimulate root and shoot growth in *I. cappadocica*. In rice seedlings treated with low As concentrations, root length, shoot height and biomass was slightly higher than in the control (Abedin and Meharg, 2002). In our work, root length and shoot height decreased significantly at higher As doses even in the mine population of *I. cappadocica* in treatments that did not drastically reduce the germination percentage. Reduced root elongation in response to arsenic exposure has been reported by a number of investigators (Hutchinson, 1991; Paliouris and Kapustka et al., 1995; Hartley-Whitaker et al., 2001; Abedin and Meharg, 2002; Liu et al., 2006).

The significant reduction of shoot length with increasing arsenic concentrations recommends *I. cappadocica* shoot length as a good indicator of arsenic toxicity. Marin et al. (1992) found significant reduction of rice shoot height when arsenite or monomethyl arsenic acid was applied at the relatively low dose of 0.8 mg As L^{-1}.

In this study, root growth inhibition was stronger than shoot growth inhibition, especially at higher arsenic concentrations (800–1400 μmol l^{-1}) (Figs. 3–5). When nutrient uptake is inhibited in roots the growth of the whole plant is affected, reducing biomass. Plant roots are the first point of contact for toxic As species in nutrient media (Abedin and Meharg, 2002).

At high As doses, root and shoot biomass decreased in both *I. cappadocica* and *D. sofia*. Decreased biomass in response to As stress has been reported in wheat (Wang et al., 2003; Dasgupta et al., 2006). Our plants showed inhibition of radicle elongation due to As toxicity. Others have reported inhibition of root growth and cell divisions in root tips, with mitotic abnormalities, damage to microtubules and destabilization of cell membranes (Seregin and Kozhevnikova, 2008).

In the seedlings we studied, arsenic accumulation increased with the increase in As dose except at higher concentrations. Further accumulation of As in *D. sofia* was lower than in *I. cappadocica*. In *I. cappadocica*, accumulation of As was higher in shoots than in roots under As exposure; in *D. sofia* it was higher in roots. The higher retention of As in *D. sofia* roots might be due to membrane disruption and disturbed transporter function, leading to less transport of As – and less transport of important nutrients. This might be why *D. sofia* showed reduced growth at 400 μM As, despite having lower As accumulation than in *I. cappadocica*. Inside *I. cappadocica* cells, arsenate is detoxified through reduction to arsenite, which is subsequently complexed with thiols, particularly phytochelatins. Accumulation of As by *I. cappadocica* is dominated by PC complexes, with PCs up to PC4 identified in this species (Karimi et al., 2009).

Reviews indicate considerable variation of As sensitivity among plant species (Jiang and Singh, 1994; Barrachina et al., 1995). As reflected in germination parameters, the mine population of *I. cappadocica* showed significantly higher tolerance to As than the non-mine population and *D. sofia*, though the actual differences in tolerance were not large. Tolerance to arsenic may be related to some physiological and biochemical adaptation strategies (Meharg, 1994). In general, plants' tolerance of heavy metals involves exclusion and accumulation mechanisms (Meharg and Macnair, 1992). In our previous work, *I. cappadocica* exhibited arsenate hypertolerance in both mine and non-mine populations actively growing in hydroponic solution with >1 mM arsenate (Karimi et al., 2009). In this species, tolerance was not achieved through suppression of high-affinity phosphate/arsenate root transport, unlike in other monocotyledons and dicotyledons (Karimi et al., 2009). A high percentage (>50%) of arsenic was phytochelatin-complexed in the tissues; however, Karimi et al. (2009) suggest that this is a constitutive rather than an adaptive mechanism of tolerance.

CONCLUSION

Arsenic (arsenate compound) was found to be more toxic to seedling growth than to germination. Root length and shoot height were significantly reduced with increasing arsenic concentrations in the growth media. These plant growth parameters might be used as indicators of As toxicity. There were considerable differences in As tolerance between the studied species: *I. cappadocica* populations showed higher tolerance to arsenic than the reference brassica *D. sofia*. The data for root length, biomass and total As concentrations show steady As accumulation and hypertolerance in *I. cappadocica* growing in arsenate-contaminated media. These characteristics make it a potential candidate for use in phytoremediation. Tolerance characteristics are an important criterion in selecting a plant species or variety to grow in soils with elevated As levels or in soils irrigated with contaminated water.

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