1 **Nitric oxide improves tolerance to arsenic stress in *Isatis cappadocica* Desv.**
2 **shoots by enhancing antioxidant defenses**

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Abstract

Arsenic (As) is a toxic metalloid that severely hampers plant growth and also poses health risks for humans through the food chain. Although nitric oxide (NO) is known to improve plant resistance to multiple stresses including metal toxicity, little is known about its role in the As tolerance of hyperaccumulator plants. This study investigates the role of the exogenously applied NO donor, sodium nitroprusside (SNP), in improving the As tolerance of *Isatis cappadocica*, which have been reported to hyperaccumulate As. Exposure to toxic As concentrations significantly increases NO production and causes damage to the cell membrane, as indicated by increased hydrogen peroxide (H₂O₂) and malondialdehyde (MDA) concentration, thereby reducing plant growth. However, the addition of SNP improves growth and alleviates As-induced oxidative stress by enhancing the activity of superoxide dismutase (SOD), ascorbate peroxidase (APX), glutathione reductase (GR), glutathione S-transferase (GST), glutathione (GSH), as well as proline and thiol concentration, thereby confirming the beneficial role played by NO in enhancing As stress tolerance. Furthermore, the As-induced decrease in growth and the increase in oxidative stress were more marked in the presence of bovine hemoglobin (Hb; a NO scavenger) and N(G)-nitro-L-arginine methyl ester (L-NAME; a NO synthase inhibitor), thus demonstrating the protective role of NO against As toxicity. The reduction in NO concentration by L-NAME suggests that NOS-like activity is involved in the generation of NO in response to As in *I. cappadocica*.

Keywords: Antioxidant system, Arsenic hyperaccumulator, Hemoglobin, Oxidative stress, Sodium nitroprusside.

1. Introduction

Arsenic (As) is a highly toxic metalloid capable of contaminating soil and water, resulting in detrimental environmental consequences for plant growth and human health (Singh et al. 2013, Souri and Karimi, 2017). The contamination of soil and groundwater due to As is a serious problem in some contaminated areas in Iran like the Zarshuran gold mine, where the high levels of As create health problems, such as chronic As poisoning (arsenicosis), skin and bladder cancer (Karimi et al. 2010). Interestingly, a hyperaccumulator plant named *Isatis cappadocica* grows well in this contaminated area (Karimi et al. 2009, 2010).

Plant exposure to As toxicity presents a wide variety of toxicity symptoms such as leaf blade wilting, necrosis and reduced photosynthesis, which ultimately inhibit plant growth (Talukdar 2013, Talano et al. 2014, Xue and Yi, 2017, Souri et al. 2017, Souri et al. 2018). One of the first symptoms of As toxicity in plants is the production of reactive oxygen species (ROS), which causes oxidative stress in plants (Hartley-Whitaker et al. 2001, Shri et al. 2009, Tripathi et al. 2012, Da-Silva et al. 2013). As a result, an increase in lipid peroxidation and protein carbonylation takes place, which negatively affects metabolic pathways and even promotes disturbances in the cell ultrastructure (Gill and Tuteja; 2010; Farooq et al. 2019). In addition, As interacts with sulfhydryl (-SH) groups of enzymes and proteins, leading to the inhibition of enzyme activity, which may hamper several important cellular functions (Mehrag and Hartley-Whitaker, 2002, Nath et al. 2014). In humans, exposure to As mainly occurs through drinking As-contaminated water and eating plant-derived food products in contaminated areas, leading to serious health problems (Chakraborti et al. 2009, Zhao et al. 2010). Given its significant impact on human health, considerable efforts have been made to limit pollution by As and to remediate contaminated areas (Shukla et al. 2015). These efforts include phyto-extraction, which has been extensively studied as a viable strategy to remediate

75 metal pollutants in contaminated soils, whereby metals are absorbed by roots and
76 subsequently translocated to shoots (Da-Silva et al. 2017). In this context, metal
77 hyperaccumulating plants, which accumulate very high concentrations of metal in their
78 tissues without presenting toxicity symptoms, are an excellent system for the removal of As
79 from soil and water and can thus be regarded as an efficient model to decipher the
80 mechanisms involved in As detoxification (Da-Silva et al. 2017, Souri et al. 2017).

81 Nitric oxide (NO) is reported to enhance plant tolerance against multiple biotic and abiotic
82 stresses including metal toxicity through diverse mechanisms (Xu et al. 2010, Tewari et al.
83 2015). For instance, NO is known to form complexes with transition metal ions in aqueous
84 media that, in turn, produce metal-nitrosyl complexes, which can act as links between the
85 different redox states of NO under physiological steady-state conditions (Tewari et al. 2015).

86 Despite several reports of its function in plant stress responses, the origin of NO in plants has
87 not been fully elucidated. Different NO biosynthesis pathways, including nitrate and nitrite
88 reduction, conversion of L-arginine to L-citrulline and NO via NO synthase (NOS),
89 polyamine- and hydroxylamine-mediated NO production and NOS-like activity, have been
90 described (Gupta et al. 2011, Tamás et al. 2018). Overall, NOS and nitrate reductase (NR)
91 have been the most studied enzymatic sources of NO in plants (Graziano and Lamattina,
92 2007, Xu et al. 2010). It has been suggested that NO production by NOS and NR is
93 associated with several cell fractions, including cytoplasm, mitochondria, chloroplasts,
94 peroxisomes, endomembranes and the plasma membrane (Corpas et al. 2004, Domingos et al.
95 2015). Most studies of arginine-dependent NOS activity in plants are based on the application
96 of various L-arginine analogs, such as L-NAME, which are efficient NOS inhibitors (Víteček
97 et al. 2012, Tamás et al. 2018). Exogenous application of the NO in different plant species
98 has been reported to enhance plant tolerance to multiple stresses by enhancing antioxidant
99 enzymes activity and, subsequently, by decreasing ROS accumulation (Zhao et al. 2004,

Zhao et al. 2007, Zhao et al. 2009, Souri and Karimi, 2017). And most such studies have used sodium nitroprusside (SNP) as NO modulator, and NO scavenger 2-phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (PTIO) (Verma et al. 2013, Kováčik et al. 2014). Nevertheless, other NO modulators, such as NOS inhibitor L-NAME and Hb, which act as NO scavengers, have not been extensively studied (Xu et al. 2010, Zhang et al. 2012, Tamás et al. 2018).

In this study, we investigated the effect of NO on As uptake, growth and antioxidant defense responses in *I. cappadocica*, which was reported to be an As-hyperaccumulating plant (Karimi et al. 2009, 2013, Karimi and Souri, 2015). Although the mechanisms involved in both As tolerance and hyperaccumulation are not fully understood, the GSH complex and phytochelatin (PC) production have been reported to be potential mechanisms induced in plants to remove As and to prevent toxicity (Karimi et al. 2009, 2013). However, the role of NO in the overall response of *I. cappadocica* to the As has not been studied previously.

2. Material and Methods

2.1. Plant materials and growth conditions

Isatis cappadocica seeds, collected from a seed population at the Zarshuran mine (gold and As deposits) at 36°43'04"N 47°08'02"E, 40 km north of the town of Takab in the western province of Azerbaijan in northwestern Iran, were germinated for 7 d. The uniform seedlings were then transferred to hydroponic pots filled with a modified Hoagland nutrient solution (1/5 Hoagland solution; Karimi et al. 2009), containing :0.5 mM KNO₃, 0.75 mM Ca (NO₃)₂, 0.5 mM KH₂PO₄, 0.2 mM MgSO₄, 15 µM H₃BO₃, 2 µM MnCl₂, 1 µM ZnSO₄, 1 µM CuSO₄, 1 µM Na₂MoO₄. 2H₂O and 50 µM Fe-EDTA, and kept in a controlled growth chamber

having light intensity of 150 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$, 14/10 h light/dark regime, 25/20 °C day/night temperature and 60% relative humidity (Karimi et al. 2009). After 5 d of growth, the plants were subjected to different treatments for another 5 d (Table 1). Briefly, plants were exposed to 1000 μM (previously selected in former works, Karimi et al. 2009, 2010) of sodium arsenate heptahydrate ($\text{Na}_2\text{HAsO}_4 \cdot 7\text{H}_2\text{O}$), and the effect of the NO donor (SNP, 200 μM), NO scavenger [bovine hemoglobin (Hb), 0.1%] and the NOS inhibitor L-NAME (50 μM) was analyzed. (Table 1). After 5 d of exposure to each treatments, plants were harvested and shoot length was recorded using centimeter scale. Subsequently, fresh weight was measured using digital electronic weighing balance.

2.2. Quantification of NO and As concentration

To quantify NO concentration, 0.5 g of the shoot was ground in 3 mL of buffer containing 0.1 M sodium acetate, pH 6.0, 1 M NaCl and 1% (w/v) ascorbic acid. The homogenate was centrifuged at $10,000 \times g$ for 20 min at 4 °C. NO was quantified in the cleared supernatant analyzing the conversion of oxy-hemoglobin to methemoglobin (Murphy and Noack, 1994; Shukla and Singh, 2015).

To determine As concentration, 0.1 g dried shoot sample was digested in 2 mL of HNO_3 (67%) and 2 mL of H_2O_2 (30%). The digest was diluted with a solution containing 10% HCl, 5% ascorbic acid and 10% KI. The mixture was then analyzed using a flow injection hydride generator interfaced with a Shimadzu AA-6200 atomic absorption spectrometer (HG-AAS), using appropriate standards (Karimi et al. 2009).

2.3. Quantification of H_2O_2 and MDA concentration

H₂O₂ concentration was determined according to the method described by (Velikova et al. 2000) using trichloroacetic acid (TCA) extracts and KI. Lipid peroxidation was estimated by measuring the level of malondialdehyde (MDA) following the method described by Alia, Prasad and Saradhi (1995).

2.4. Determination of antioxidant enzyme activity

Shoots (0.1 g) were homogenized in 2 mL Tris-HCl buffer (pH 7.0) containing 0.1 mM ethylenediaminetetraacetic acid (EDTA), 0.1% soluble polyvinylpyrrolidone (PVP), 1 mM DTT, 0.5 mM PMSF and 2% Triton X-100, with the addition of 1 mM ascorbate for the APX assay. The homogenate was centrifuged at 12,000 × g for 30 min, and the supernatant was used to determine antioxidant enzymes activity. SOD (EC 1.15.1.1) activity was assayed by measuring the inhibition of nitro blue tetrazolium (NBT) photochemical reduction (Beauchamp and Fridovich, 1971). APX (EC 1.11.1.11) activity was assayed in the presence of ascorbate by monitoring the decline in the absorbance of oxidized ascorbate at 290 nm, in accordance to Chen and Asada (1989). GR (EC 1.6.4.2) activity was measured according to the method used by Foyer and Halliwell (1976). The assay medium contained 50 mM Na-phosphate buffer (pH 7.8), 0.5 mM GSSG, 0.12 mM NADPH Na₄ and 0.1 mL enzyme extract in a final assay volume of 1 mL. GST (EC 2.5.1.18) activity was determined spectrophotometrically (Rohman et al. 2009). The reaction mixture contained 50 mM K-phosphate buffer (pH 7), 1.5 mM GSH, 1 mM 1-chloro-2,4-dinitrobenzene (CDNB) and enzyme solution with a final volume of 700 µL. CAT (EC 1.11.1.6) activity was estimated using the method described by Aebi (1984) in a reaction mixture containing 50 mM sodium phosphate buffer (pH 7.0), 20 mM H₂O₂ and a suitable aliquot of enzyme extract. POD (EC 1.11.1.7) activity was determined by monitoring guaiacol oxidation using the method

described by Chance and Maehly (1995). For protein extraction, 100 μ L of extracts were mixed with 5 mL of a solution containing 0.01% (w/v) Coomassie Brilliant Blue G-250 (as dye), 4.7% (w/v) ethanol and 8.5% (w/v) phosphoric acid. After mixing thoroughly for 2 min, the absorbance was measured at 595 nm. The Bovine gamma-globulin was used as standard protein (Bradford, 1976).

2.5. Determination of GSH and proline concentration

To determine GSH concentration, 0.1 g shoot sample was ground in 3 mL of 0.5 mM EDTA solution containing 3% TCA at 4 °C. The homogenate was centrifuged at $15,000 \times g$ for 10 min. 0.2 mL of the supernatant was then added to 1.5 mL of 50 mM potassium phosphate buffer (pH 7.0) containing 0.2 mM 5,5-dithio-bis (2-nitrobenzoic) (DTNB). The mixture was incubated at 30 °C for 2 min, absorbance was determined at 412 nm (Ellman, 1959).

To quantify proline, 0.2 g shoot samples were homogenized in 3% (w/v) sulphosalicylic acid, and the homogenate was centrifuged at $4,000 \times g$ for 20 min. Proline was extracted and determined according to the method used by Bates et al. (1973) based on the proline reaction with ninhydrin.

2.6. Determination of total, non-protein and protein thiol concentration

Samples of shoot were homogenized in 0.02 M EDTA in an ice bath. 0.5 mL aliquots of the homogenates were mixed with 1.5 mL of 0.2 M Tris buffer (pH 8.2) and 0.1 mL of 0.01 M DTNB. The mixture was brought to 10 mL by adding 7.9 mL of absolute methanol. The absorbance of the clear supernatant was read at 412 nm after 15 min. Total sulphydryl concentrations were calculated using an extinction coefficient of $13.1 \text{ mM}^{-1}\text{cm}^{-1}$. To measure

non-protein thiols (NPTs), 5 mL of the homogenates was mixed with 4 mL distilled water and 1 mL of 50% TCA and, after 15 min, the tubes were centrifuged for 15 min. The supernatant (2 mL) was mixed with 4 mL of 0.4 M Tris buffer (pH 8.9) and 0.1 mL of DTNB, and the absorbance was read for 5 min at 412 nm. The protein thiols (PTs) were calculated by subtracting NPTs from total thiols (TTs) (Sedlak and Lindsay, 1968).

2.7. Statistical analysis

Statistical analysis was carried out using SPSS statistical software and one-way ANOVA. Duncan's multiple range test (DMRT) was also used to compare significant inter-group differences. Comparisons with $P < 0.05$ were considered significantly different. Correlations between the different variables were analyzed. Significance levels were denoted by * at $0.01 < p < 0.05$, by ** at $p < 0.01$ and by NS (not significant). Principal component analysis (PCA) was carried out using OriginPro software.

3. Results

3.1. NO concentration and As accumulation

The effect of As on NO accumulation in *I. cappadocica* shoots is shown in Fig. 1A. In the presence of 1000 μM As, NO concentration increased by 84% as compared to untreated control plants. The addition of SNP increased NO concentration by 26% as compared to As treatment alone (Fig. 1A). Nevertheless, NO concentration fell by 18% and 29% following the application of L-NAME and Hb, respectively, as compared to As treatment alone. The

combination of SNP with Hb or L-NAME, or both, resulted in a significant reduction in SNP-induced NO (Fig. 1A).

As shown in Fig. 1B, when *Isatis* plants were exposed to 1000 μM of arsenate, an accumulation of 535.6 mg kg^{-1} DW As in shoots was observed. Under these conditions, *I. cappadocica* did not present any significant toxic symptoms. L-NAME reduced As accumulation by 31% as compared to As treatment alone. Surprisingly, compared to the application of As alone, 0.1% of Hb increased As accumulation by 31%, an increase which was slightly reduced by the addition of L-NAME. The application of SNP to As-treated plants reduced As accumulation by 29%, although the combinations of As+SNP+L-NAME; As+SNP+Hb and As+SNP+Hb+L-NAME slightly increased As concentration as compared to plants treated with As and SNP (Fig. 1B).

3.2. NO supplementation improved growth under As stress conditions

As shown in Fig. 2A, exposure to 1000 μM As significantly inhibited shoot growth by approximately 27%, as compared to control plants (Fig.2). However, SNP On the other hand, the presence of 50 μM L-NAME in the growth medium of As-stressed plants further reduced the shoot fresh weight and length (by 44% and 32%, respectively) compared to non-stressed control plants (Fig. 2A, B). Moreover, exposure of As-stressed plants to Hb (0.1%) and Hb+L-NAME had maximally reduced the *I. cappadocica* growth, particularly the shoot fresh weight (by ~80%) compared to non-stressed control plants (Fig. 2 A, B). In contrast, SNP supplementation to As-stressed plants prevented further damage, as indicated by improved shoot growth and length, which reached levels close to those of non-stressed control plants (Fig. 2A, B).

3.3. Oxidative stress and antioxidant responses

The relationship between As toxicity and oxidative damage was studied by analyzing the concentration of H₂O₂ and MDA acting as oxidative stress markers. As shown in Fig. 3, the variation in H₂O₂ concentration was similar to that in MDA concentration. When compared to control plants, exposure to As toxicity markedly increased H₂O₂ and MDA concentration approximately 9- and 9.5-fold, respectively (Fig. 3 A, B). This pattern was reversed by the presence of 200 µM SNP, which produced reductions of 51% and 57% in H₂O₂ and MDA concentration, respectively, as compared to As-stressed plants (Fig. 3). However, L-NAME and Hb did not produce any significant changes in these two parameters in As-treated plants (Fig. 3B).

Given that antioxidant enzymes are an essential component of a hyperaccumulator plant's antioxidant defense responses (Hartley-Whitaker et al. 2001), the effect of NO on antioxidant enzymes (SOD, APX, GR, GST, CAT and POD) activity under As stress conditions was investigated. Our results show that SOD, APX, GR and GST activity in the presence of 1000 µM of As increased 4-, 8-, 4- and 5-fold, respectively, as compared to untreated control plants (Fig. 4 A-F). The application of SNP led to additional increases in SOD, APX, GR and GST of 49%, 90%, 75% and 52%, respectively, as compared to As treatment alone (Fig. 4 A-D).

By contrast, Hb on its own only affected the activity of antioxidants CAT and POD when combined with SNP (Fig. 4 A-F). The application of L-NAME by itself only produced an increase in POD. However, no significant differences between SOD, APX, GR and GST activity were observed in response to L-NAME and Hb when combined with SNP as compared to treatment with L-NAME and Hb alone (Fig. 4A-E).

As shown in Fig. 4E, As treatment increased CAT activity 4.4-fold as compared to control plants; CAT activity also increased by a further 88% following supplementary treatment with Hb as compared to As on its own (Fig. 4E). The trend observed in CAT activity reflected that in As accumulation (Fig. 1B and Fig. 4E).

As GSH and proline have been reported to be a good indicator of heavy metal stress and antioxidant capacity in stressful situations, we examined the concentration of both these compounds under our experimental conditions. As shown in Fig. 5A, although As reduced GSH concentration slightly, SNP increased it significantly, indicating that exogenous NO can induce GSH production. Our results indicate that NO supplementation increased the accumulation of GSH and proline by 46% and 65%, respectively, as compared to treatment with As alone, while L-NAME reduced the levels of GSH and proline (Fig. 5).

To further assess non-enzymatic strategies for mitigating As toxicity, TT, NPT and PT concentrations were investigated. As shown in Table 2, exposure to As enhanced TT, NPT and PT concentration by 128%, 81%, and 198%, respectively, as compared to control plants, with a further increase of 18%, 27% and 10%, respectively, being recorded following the application of SNP as compared to treatment with As alone (Table 2). We found that Hb and L-NAME treatments reduced TT and PT concentration but did not cause any significant changes in NPT concentration as compared to the application of both As alone and As+SNP (Table 2). Supplementation with L-NAME and Hb reduced TT and PT concentration, while supplementation with SNP was found to reverse the inhibitory effects of L-NAME and Hb.

3.4. Correlation between antioxidants, thiols and oxidative stress markers

In order to determine differential patterns in *I. cappadocica* plants, PCA analysis was used to identify the high-variability components of antioxidants, thiols and oxidative stress markers

(Fig. 6). The biplot tool enabled us to compare the quantified variables, with lines found to be more closely correlated. While omitting control plants, PCA analysis showed two clear components (Fig. 6A), with PC1 and PC2 accounting for 62.15% and 16.7% of total variance, respectively. In the plot, the different variables, represented by a black line, were observed to vary in relation to distance from the origin. Proline, TT, PT, NPT and GR, which were found to be more closely correlated than the other variables, accounted for much of the data variance (Table 3). The principal component analysis shows changes caused by As+SNP+L-NAME in NO, GST, TT, PT, NPT, and SOD, while As+SNP and As+SNP+Hb affected mainly APX, GSH GR, and proline concentration. However, the values for CAT and POD were influenced by the non-SNP treatments As+Hb and As+L-NAME. Variables such as APX, GST and NO, which were closely correlated, were similar (Fig. 6A).

With regard to control plants, our analysis showed two principal components, with PC1 and PC2 accounting for 63.5% and 22.5% of total variance, respectively. The components of the antioxidant defense system were greatly affected by the different treatments (Fig. 6B), while the changes observed in antioxidants, thiols and oxidative stress markers are more closely related in all treatments. However, the values for GSH were closer to those for the control plants (Fig. 6B). The values for APX, SOD, GST, GR, TT, NPT, PT and NO, which were closely correlated, were similar.

4. Discussion

Although As is a highly toxic metalloid, As hyperaccumulator plants are capable of growing under high As concentration conditions in the medium (Souri and Karimi, 2017). *I. capadoccica*, which can grow in the presence of 1000 μ M of As without shoot length or fresh weight being severely affected, is one of the few As hyperaccumulator plants to have been

characterized (Fig. 2). The tolerance of *I. capadoccica* to As could be related to NO, whose application through the use SNP significantly enhances the growth of plants treated with As, while the reduction in NO production by the NOS inhibitor L-NAME and the NO scavenger Hb reduces plant growth considerably (Fig. 2). These results point to a close relationship between NO and tolerance to As, with As inducing almost twice the level of NO concentration as compared to untreated plants (Fig. 1). The slight reduction in NO accumulation caused by L-NAME suggests that, apart from NOS, sources of NO, such as nitrate reductase, could contribute to NO production induced by As. Table 4 shows a comparison of the effect of As on NO production in different plant species, which suggests that NO production in As-treated roots and leaves varies considerably in a time, dose- and species-dependent manner (Table 4). For example, in *Arabidopsis thaliana* roots, although acute As toxicity significantly enhanced NO accumulation (Leterrier et al. 2012). NO levels were reduced in leaves subjected to comparatively low doses of As for a virtually equal period of time (Gupta et al. 2011). On the other hand, in *Oryza sativa* roots, the application of SNP provides resistance to As toxicity and counteracts As-induced stress (Karimi et al. 2009, Farnese et al. 2017). Interestingly, in both these studies, NO accumulation in rice roots was quite low in the presence of As stress imposed for variable periods of time. Similar results have been reported in relation to tall fescue and water lettuce plants, in which the application of SNP reduced As-induced oxidative damage in leaves, in which, however, the increase in NO accumulation was quite significant (Jin et al. 2010, Farnese et al. 2017). Furthermore, longer exposure to arsenite [As(III)], as compared to arsenate [As(V)], has been shown to increase NO accumulation in rice roots (Singh et al. 2017a). In the case of *Pisum sativum*, As toxicity [50 μ M As (V)] suppressed As accumulation in roots, but had a stimulatory effect in leaves (Rodríguez-Ruiz et al. 2018). On the other hand, at similar levels of As, Singh et al. (2015a) observed an inhibition of NO biosynthesis in secondary leaves. Taken together, these

348 discrepancies could be explained by differences in experimental design and the species
349 studied.

350 NO, an important endogenous signaling molecule, can diffuse through biological membranes
351 and regulate various processes by altering gene transcription and through covalent post-
352 translational protein modifications (Víteček et al. 2012), although the mechanisms involved
353 in NO functioning are not fully understood. Experimental evidence suggests that plant
354 tolerance to heavy metals caused by a decrease in metal accumulation and a reduction in
355 metal-induced oxidative stress is an adaptive response, which may involve NO (Chen and
356 Asada, 1989, Singh et al. 2009, Arasimowicz-Jelonek et al. 2012). In different plant species,
357 exogenous applications of NO promote a reduction in As uptake and enhance gamma-
358 glutamylcysteine synthase expression to increase GSH concentration and to maintain the
359 GSH/GSSG ratio in plants (Song et al. 2010, Namdjoyana and Kermanian, 2013, Singh et al.
360 2017b). In this study, excess NO supplied as SNP to As-treated plants reduced As
361 accumulation compared to treatment with As alone, which is in line with previous studies of
362 rice (Singh et al. 2009), Luffa seedlings (Singh et al. 2013), watercress (Yadav, 2010) and
363 barley (Shukla et al. 2015). The lower levels of As observed in *I. cappadocica* shoots treated
364 with SNP may be caused by a reduction in As uptake and/or translocation to the shoot,
365 although information regarding NO-dependent regulation of As transporters is very scarce. A
366 proteomic study of S-nitrosylated proteins in Arabidopsis plants has identified the phosphate
367 transporter PHT3;1 (also a transporter of As) as a target for S-nitrosylation, whose effect on
368 PHT activity, however, has yet to be analyzed (Fares et al. 2011). It has also been suggested
369 that NO can modulate the expression of other metal transporters, particularly NIP, NRAMP,
370 ABC and iron transporters, in plants treated with As (Singh et al. 2017b). Interestingly, As
371 and As+SNP down-regulated NIP, which is involved in As uptake in the root, while
372 upregulating ABC transporters are involved in As vacuolar sequestration (Souri and Karimi,

2017). However, an opposite result was obtained in our study following the application of Hb, with an increase in As being observed despite the reduction observed in NO. These results indicate that the regulation of As uptake and translocation by NO is a highly complex process, while the, as yet, unknown role played by Hb in As transport in *Isatis* plants requires more in-depth study. This could partly explain the reduction in *Isatis* growth induced by Hb-dependent As. NO needs to be highly regulated in the cells and phytoglobins1 (non-symbiotic haemoglobins1, nsHbs1) have been shown to metabolize NO (Gupta et al. 2011). However, the role played by phytoglobins in plant responses to As remains unclear.

One of the primary responses of plants to metal stress is the generation of ROS such as H₂O₂, which is a major contributor to oxidative damage (Yadav, 2010). Some evidence shows that exposure to As leads to the generation of ROS, which can induce oxidative stress and cellular damage (Talukdar, 2013) and thus reduce biomass production (Hartley-Whitaker et al. 2001, Gupta et al. 2011, Talukdar, 2011). Heavy metals and As also stimulate reactive nitrogen species (RNS), lipid nitration and protein tyrosine nitration (Hasanuzzaman and Fujita, 2013, Corpas and Barroso, 2014). In this study, high levels of As (1000 µM) considerably enhanced H₂O₂ and MDA accumulation in *Isatis* plants, a process which was attenuated by SNP. This protective effect of SNP could be due not only to the reduction in As accumulation by NO but also to the role played by NO as a ROS scavenger (Hill et al. 2010), which reduces the oxidative damage observed in this study. Similar results have been obtained in previous studies of different plant species (Singh et al. 2009, Hasanuzzaman and Fujita, 2013, Singh et al. 2013, Da-Silva et al. 2017, Farnese et al. 2017). The supply of NO could also protect proteins from carbonylation induced by excess ROS, such it has been reported in citrus and sorghum under salinity (Tanou et al. 2012, Baena et al. 2017). On the other hand, the reduction in NO concentration by Hb or the NOS inhibitor L-NAME intensifies the

accumulation of H₂O₂ and MDA, indicating that NO plays an important role in regulating ROS accumulation induced by As.

The balance of ROS levels in cells, which is determined by the interplay between different ROS-producing and ROS-scavenging mechanisms, is maintained by enzymatic antioxidants such as SOD, CAT, POD, APX, GR and peroxiredoxin, as well as non-enzymatic antioxidants including GSH, other thiols (TT, PT and NPT) and proline (Nadgórska-Socha et al. 2013, Sandalio et al. 2013). The regulation of antioxidant capacity and redox status is an important factor in hyperaccumulator plant resistance to heavy metals (Hernández-Allica et al. 2006, Zeng et al. 2011). In this study, the protective effect of SNP correlated with an increase not only in GSH, thiol and proline concentration, but also in the enzymatic antioxidants SOD, APX, GR, GST and CAT, giving rise to a reduction in H₂O₂ and lipid oxidation. The positive effect of NO on antioxidant activities is a common response to As and other metals in different species, although the mechanisms involved remain unclear (Hasanuzzaman and Fujita, 2013, Namdjoyana and Kermanian, 2013, Singh et al. 2013, Talukdar 2013, Shukla et al. 2015, Singh et al. 2015a, b). The reduction observed in TT and PT with applications of Hb and L-NAME in As-treated plants demonstrates that endogenous NO, though not essential in NPT regulation, plays an important role in regulating both total and protein thiols.

GSH, an abundant low-molecular-weight thiol compound, is ubiquitously distributed among all organelles and plays a central role in plant responses to stress and as a buffering system against redox imbalance (Hasan et al. 2016). GSH is also involved in As tolerance PC, which binds As to impede its toxicity (Ahsan et al. 2008, Anjum et al. 2012, 2014, Souri and Karimi, 2017). It has been suggested that the hyper-tolerance and accumulation of larger amounts of above-ground As in *I. cappadocica* plants are achieved through PC complexation (Karimi et al. 2009, Souri and Karimi, 2017), which in turn, is regulated by NO. GSH and

proline share L-glutamate as a biosynthesis precursor (Anjum et al. 2012, 2014), while a causal relationship between proline accumulation and GSH metabolism in order to improve metal stress tolerance has been reported in previous studies (Anjum et al. 2012, 2014, Noctor et al. 2012, Kishor and Sreenivasulu, 2014). Proline has been reported to act as an osmoprotectant and antioxidant by scavenging ROS to protect the structure of biological macromolecules during dehydration and to store and transfer reductants, thus facilitating plant tolerance of environmental stresses (Ashraf and Foolad, 2007). Proline has also been reported to improve antioxidant capacity (Chandrakar et al. 2017) and to reduce As uptake (Singh et al. 2015c). In our study, the NO donor, SNP, increased proline concentration probably by up-regulating P5CS1 encoding 1-pyrroline 5-carboxylate synthetase, which is a key enzyme in proline synthesis in different plant species (Zhang et al. 2008, Rejeb et al. 2014, Singh et al. 2015c). However, as observed in cucumber seedlings, SNP can also downregulate proline dehydrogenase activity involved in proline degradation (Fan and Chang-Xia, 2012).

5. Conclusion

Despite the considerable increase in our knowledge of As toxicity in plants in recent years, many questions remain with regard to understanding the mechanisms involved in As detoxification and its overall impact on hyperaccumulator plants. In this respect, we studied in considerable depth the role of NO as a signaling molecule in plant responses to As stress by investigating its impact on antioxidant responses, growth and As accumulation in *I. cappadocica*.

We identified several mechanisms, such as ROS scavenging through enhanced antioxidant systems, by which NO may alleviate metal toxicity. We also showed that the addition of SNP increases *I. cappadocica* growth, which is accompanied by lower H₂O₂ and MDA

accumulation and enhanced antioxidant defense systems. However, Hb and L-NAME reversed the beneficial effects of SNP on plant growth promotion, antioxidant defenses, as well as proline, GSH and thiol compound accumulation. Our findings suggest that NO-induced antioxidant defenses are essential for As detoxification and constitutive resistance to this metalloid in *Isatis* plants. Further research is clearly required in order to gain a deeper insight into the role of NO in improving the As tolerance of hyperaccumulator *Isatis* plants, particularly in relation to the regulation of As uptake and translocation, as well as the role of endogenous nsHbs in these processes.

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Figure legends

Fig. 1: Effects of As alone or with L-NAME (50 μ M), Hb (0.1%) and SNP (200 μ M) on NO concentration (A) and As accumulation (B) in *I. cappadocica* shoots following exposure to 1000 μ M As for 5d.

Data represent means $\pm$ S.E. of n=6 from three independent experiments. Bars with different letters indicate statistically significant differences at $P<0.05$.

Fig. 2: Effects of As alone or with L-NAME (50 μ M), Hb (0.1%), and SNP (200 μ M) on *I. cappadocica* shoot fresh weight (A) and shoot length (B) following exposure to 1000 μ M As

for 5 d. Data represent means $\pm$ S.E. of n=6 from three independent experiments. Bars with different letters indicate statistically significant differences at $P<0.05$.

Fig. 3: Effects of As alone or with L-NAME (50 μ M), Hb (0.1%) and SNP (200 μ M) on hydrogen peroxide (H_2O_2) (A) and malondialdehyde (MDA) (B) contents in *I. cappadocica* following 5 d of exposure to 1000 μ M As. Data represent means $\pm$ S.E. of n=6 from three independent experiments. Bars with different letters indicate statistically significant differences at $P<0.05$.

Fig. 4: Effects of As alone or with L-NAME (50 μ M), Hb (0.1%) and SNP (200 μ M) on antioxidant enzymes activities. superoxide dismutase (SOD) (A), ascorbate peroxidase (APX) (B), glutathione reductase (GR) (C), glutathione S-transferase (GST) (D), catalase (CAT) (E) and peroxidase (POD) (F) activities in *I. cappadocica* after 5 d of 1000 μ M As treatment. Data represent means $\pm$ S.E. of n=6 from three independent experiments. Bars with different letters indicate statistically significant differences at $P<0.05$.

Fig. 5: Effects of As alone or with L-NAME (50 μ M), Hb (0.1%) and SNP (200 μ M) on glutathione (GSH) (A) and proline (B) concentration in *I. cappadocica* treated with 1000 μ M As for 5 days. Data represent means $\pm$ S.E. of n=6 from three independent experiments. Bars with different letters indicate statistically significant differences at $P<0.05$.

Fig. 6. Graphical representations of principal component analyses (PCA1 and PCA2) obtained from evaluation of all parameters studied in *I. cappadocica* without considering control plants (A) and control and As-treated plants (B).

Table 1: The Experimental design.

Table 2: Effects of As alone or with L-NAME (50 μ M), Hb (0.1 and SNP (200 μ M) on total thiol (TT), non-protein thiol (NPT) and protein thiol (PT) concentration in *I. cappadocica* shoots after 5 d of 1000 μ M As treatment. Different letters indicate statistically significant differences at $P < 0.05$.

Table 3: Correlation matrix and statistical significance among antioxidants, oxidative stress markers, thiols, NO concentration and As accumulation in *I. cappadocica* shoots grown under different treatments with As, L-NAME, Hb and SNP.

Table 4: Effect of various As concentrations and exposure times on NO generation in root and leaf tissues of different non-hyperaccumulator plant species, where changes in NO level are indicated by upward arrow (increase) or downward arrow (decrease).

Fig. 1

Fig. 2

Fig. 3

Fig. 4

Fig. 5

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Fig. 6

Table 1: The experimental design.

Treatment	Description
Control	No added SNP and As
As	1000 μ M As
As+L-NAME	1000 μ M As+50 μ M L-NAME
As+Hb	1000 μ M As+0.1% Hb
As+L-NAME+Hb	1000 μ M As+ 50 μ M L-NAME+0.1% Hb
As+SNP	1000 μ M As+200 μ M SNP
As+SNP+L-NAME	1000 μ M As+200 μ M SNP+50 μ M L-NAME
As+SNP+Hb	1000 μ M As+200 μ M SNP+0.1% Hb
As+SNP+L-NAME+Hb	1000 μ M As+200 μ M SNP+50 μ M L-NAME+0.1% Hb

L-NAME, N(G)-nitro-L-arginine methyl ester (NO synthase inhibitor); Hb, bovine hemoglobin (NO scavenger); SNP: NO donor

Table 2: Effects of L-NAME, Hb and SNP on total thiol (TT), non-protein thiol (NPT) and protein thiol (PT) content in *I. cappadocica* after 5 d of 1000 μ M As treatment. Different letters indicate statistically significant differences at $P<0.05$.

Treatment	TT content ($\mu\text{mol}^{-1} \text{g FW}$)	NPT content ($\mu\text{mol g}^{-1} \text{FW}$)	PT content ($\mu\text{mol g}^{-1} \text{FW}$)
Control	12.51 ^a	7.43 ^a	5.08 ^a
As	28.60 ^{cde}	13.43 ^{bc}	15.16 ^{cde}
As+L-NAME	21.88 ^b	11.19 ^{ab}	10.68 ^b
As+Hb	22.18 ^b	12.64 ^{bc}	9.54 ^b
As+L-NAME+Hb	24.32 ^{bc}	12.41 ^{bc}	11.90 ^{bc}
As+SNP	33.89 ^c	17.09 ^c	16.79 ^{de}
As+SNP+L-NAME	30.94 ^{de}	13.94 ^{bc}	16.99 ^e
As+SNP+Hb	26.97 ^{bcd}	14.35 ^{bc}	12.69 ^{bcd}
As+SNP+L-NAME+Hb	29.07 ^{cde}	13.84 ^{bc}	15.16 ^{cde}

Table 3: Correlation matrix and statistical significance among antioxidants, oxidative stress markers, thiols, NO content and As accumulation in *I. cappadocica* shoots grown under different treatments with As, L-NAME, Hb and SNP.

	APX	CAT	GR	GST	SOD	POD	Proline	GSH	TT	PT	NPT	H ₂ O ₂	MDA	NO	As
APX	1.000														
CAT	0.610**	1.000													
GR	0.902**	0.635**	1.000												
GST	0.944**	0.623**	0.919**	1.000											
SOD	0.914**	0.512**	0.847**	0.920**	1.000										
POD	0.221 ^{ns}	0.515**	0.466*	0.316 ^{ns}	0.186 ^{ns}	1.000									
Proline	0.804**	0.685**	0.870**	0.848**	0.724**	0.578**	1.000								
GSH	0.311 ^{ns}	0.005 ^{ns}	0.237 ^{ns}	0.218 ^{ns}	0.299 ^{ns}	-0.28 ^{ns}	-0.025 ^{ns}	1.000							
TT	0.887**	0.493**	0.848**	0.897**	0.893**	0.261 ^{ns}	0.724**	0.121 ^{ns}	1.000						
PT	0.696**	0.345 ^{ns}	0.652**	0.697**	0.749**	0.193 ^{ns}	0.474*	0.029 ^{ns}	0.906**	1.000					
NPT	0.885**	0.545**	0.864**	0.903**	0.830**	0.276 ^{ns}	0.843**	0.205 ^{ns}	0.843**	0.537**	1.000				
H ₂ O ₂	0.063 ^{ns}	0.406*	0.133 ^{ns}	0.194 ^{ns}	0.130 ^{ns}	0.385 ^{ns}	0.243 ^{ns}	-0.511**	0.295 ^{ns}	0.388*	0.095 ^{ns}	1.000			
MDA	0.319 ^{ns}	0.644**	0.395*	0.401*	0.279 ^{ns}	0.582**	0.601**	-0.510**	0.399*	0.256 ^{ns}	0.471*	0.597**	1.000		
NO	0.600**	0.096 ^{ns}	0.622**	0.633**	0.635**	0.027 ^{ns}	0.420*	0.413*	0.679**	0.564**	0.638**	0.122 ^{ns}	-0.025 ^{ns}	1.000	
As	0.533**	0.838**	0.530**	0.599**	0.429*	0.394*	0.589**	-0.193 ^{ns}	0.511**	0.431*	0.472*	0.643**	0.648**	0.214 ^{ns}	1.000

*, **: Statistically significant at P < 0.05; and P < 0.01, respectively. ns: not significant

Table 4: Effect of various As concentration(s) and exposure time(s) on NO generation in root and leaf tissues of different non-hyperaccumulator plant species, where changes in NO level are indicated by upward arrow (increase) or downward arrow (decrease) .

Plant species	Organ/tissue	As concentration used	Exposure time	Changes in NO level	References
<i>Arabidopsis thaliana</i>	Primary roots	500 & 1000 µM As (V)	7 d	↑	Leterrier et al., 2012
<i>Arabidopsis thaliana</i>	Leaves	25 & 50 µM As (V)	1 & 5 d	↓	Gupta et al., 2013
<i>Oryza sativa</i>	Roots	25 & 50 µM As (V)	4, 8 & 24 h	↓	Singh et al., 2009
<i>Oryza sativa</i>	Roots	25 µM As (III)	7 d	↓	Singh et al., 2017a
<i>Oryza sativa</i>	Roots	25 µM As (III)	4 & 12 d	↑	Singh et al., 2017b
<i>Oryza sativa</i>	Primary roots	100 µM As (V)	10 d	↓	Kushwaha et al., 2018
<i>Pisum sativum</i>	Secondary leaves	50 µM As (V)	15 d	↓	Singh et al., 2015a
<i>Pisum sativum</i>	Roots	50 µM As (V)	10 d	↓	Rodríguez-Ruiz et al., 2018
<i>Pisum sativum</i>	Leaves	50 µM As (V)	10 d	↑	Rodríguez-Ruiz et al., 2018
Tall fescue (<i>Festuca arundinacea</i>)	Leaves	25 µM As (V)	4 & 8 d	↑	Jin et al., 2010
<i>Pistia stratiotes</i>	Leaves	1.5 mg L ⁻¹ As (V)	24 h	↑	Farnese et al., 2017