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Arsenic toxicity: cell signalling and the attenuating effect of nitric oxide in *Eichhornia crassipes*

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Abstract

Nitric oxide (NO) is an important molecule involved in the perception of stress induced by toxic compounds such as arsenic (As). The present study investigated the role of NO applied as sodium nitroprusside (SNP) in cell signalling and the ability of NO to attenuate the toxic effects of As (in the form of sodium arsenate) in water hyacinth (*Eichhornia crassipes*). Water hyacinth plants were collected and assigned to one of the following treatments: control; 100 μM SNP; 20 μM As; or 20 μM As + 100 μM SNP. The plants remained under these conditions for 0, 4, 12, and 24 h. After each time interval, the plants were collected and As absorption, production of reactive oxygen species (ROS), integrity of membranes, and antioxidant enzyme activities were evaluated. The plants were able to absorb and accumulate large amounts of As, even after only four hours of exposure to the pollutant. The absorption and bioaccumulation factor of As was even greater when plants were exposed to both As and SNP. The accumulation of As triggered increases in ROS production and cell membrane damage. In the presence of SNP, the tolerance index to As increased and damage was mitigated. Therefore, from the present work, it was possible to conclude that exogenous NO influenced the ability of plants to tolerate As; this finding has implications for phytoremediation in areas contaminated by As.

Additional key words: antioxidant enzymes, oxidative stress, phytoremediation, reactive oxygen species, water hyacinth.

Introduction

The contamination of potable water with arsenic (As) is a worldwide problem with implications for human health and the conservation of biodiversity (Litter *et al.* 2011). One viable option for the decontamination of the water involves the use of plants capable of absorbing this pollutant; this procedure is known as phytoremediation (Shah and Nongkynrih 2007). In the environment, As is found in inorganic and organic forms. Overall, organic arsenicals are much less toxic than inorganic arsenicals such as arsenite (a predominant form in reducing environments) and arsenate (a predominant form in aerobic soils and superficial water) (Zhao *et al.* 2009). Many plant species including water hyacinth [*Eichhornia crassipes* (Mart.) Solms] can absorb and accumulate As in their tissues (Iqbal 2011). A number of aquaporin nodulin26-like intrinsic proteins are able to promote absorption of arsenite by roots. Arsenate is taken up by phosphate transporters. In root cells, arsenate is promptly reduced to arsenite which is effluxed to external medium, complexed by thiol peptides, or translocated to shoots (Zhao *et al.* 2009).

The accumulation of As by plants causes cellular damage, thus complicating and limiting the application of phytoremediation strategies (Farnese *et al.* 2014a). In fact, As absorption compromises the entire process of cellular metabolism, which leads to a greater production of reactive oxygen species (ROS). The ROS are highly reactive and can damage cellular macromolecules (such as membrane lipids) or cause cell death when produced in excess (Leão *et al.* 2014b). Enzymatic and non-enzymatic antioxidant systems are involved in eliminating excess ROS (Leão *et al.* 2014a) and these mechanisms are activated by secondary messengers as nitric oxide (NO) (Ismail 2012). NO is synthesized in plants by a range of enzymatic and chemical processes. Although the half-life of NO is short, it diffuses quickly in the cell and

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Abbreviations: CAT - catalase; MDA - malondialdehyde; POX - peroxidase; ROS - reactive oxygen species; SNP - sodium nitropruside; SOD - superoxide dismutase; TI - tolerance index.
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can interact with many cell components triggering signal transduction pathways which are particularly important in stressful situations (Hermes *et al.* 2013, Farnese *et al.* 2014a). However, it is still not entirely known how NO increases As tolerance in plants. Therefore, the aim of this study was to examine the role of NO in attenuation of As-induced toxicity in *E. crassipes*. Our results can have important implications both for the process of phytoremediation and for understanding the role of NO in plants subjected to stress conditions.

Materials and methods

Plants and treatments: Water hyacinth [*Eichhornia crassipes* (Mart.) Solms] plants were collected from non-polluted dams at the Federal University of Viçosa, Viçosa, Minas Gerais State, Brazil. The plants of a similar size (a fresh mass about 8.0 g) were maintained in deionized water for 24 h. Subsequently, the plants were transferred to polyethylene pots with 10 dm^3 of a nutrient solution proposed by Clark (1975), pH 6.5, with a half of the full ionic strength. The plants were then maintained in a growth room with controlled temperature, irradiance, and relative humidity (25 ± 2 ºC; 230 µmol m^{-2} s^{-1}; 16-h photoperiod; 60 %) for an adaptation period of three days. After this period, the plants were transferred to 1.0 dm^3 polyethylene pots and assigned to one of the following treatments: control (containing the Clark nutrient solution); 100 µM sodium nitroprusside (SNP); 20 µM As; 20 µM As + 100 µM SNP. Arsenic was supplied in the form of sodium arsenate ($NaHAsO_4 \cdot 7\ H_2O$). After 0, 4, 12 and 24 h of exposure to the treatments, the plants were harvested, leaves and roots were separated, washed in deionized water and immediately analyzed or stored at -80 ºC. Experiments were set up in five repetitions.

Determination of arsenic absorbtion and tolerance index: The leaves and roots were placed in a conventional oven at 80 °C until a constant dry mass was obtained. The dry plant material was crushed and digested in a mixture of nitric acid and perchloric acid (Marin *et al.* 1993). The concentration of As was determined through inductively coupled plasma emission spectroscopy (*Optima 3300 DV*, *Perkin-Elmer*, Norwalk, CT, USA). The accuracy of the method was verified with an analysis of certified reference materials.

The As bioaccumulation factor was calculated using the concentration of As in plant organs (leaf and root) and in the nutrient solution (bioaccumulation factor = arsenic concentration in plant tissue/arsenic concentration in solution). The calculations were performed as proposed by Huang *et al.* (2006).

The As tolerance index (TI) was estimated as proposed by Wilkins (1978): TI [%] = (Rw*/Rw) × 100, where Rw* and Rw are the relative growth rates of plants in the solution with As and without As, respectively.

Content of ROS: To determine the content of the superoxide anion ($O_2^{\cdot-}$), 50 mg of a root sample was incubated in an extraction medium proposed by Mohammadi and Karr (2001). The reaction was initiated by adding 100 mm^3 of 25.2 mM epinephrine in 0.1 M HCl, and absorbance was read at 480 nm for 5 min. Superoxide anion production was assessed by determining the amount of accumulated adrenochrome using a molar absorption coefficient of 4.0×10^3 M^{-1} cm^{-1} (Boveris *et al.* 2002).

Hydrogen peroxide (H_2O_2) content was determined by homogenizing 200 mg of a root sample in an extraction medium proposed by Kuo and Kao (2003). After centrifugation, the supernatant was added to a reaction medium containing 100 µM $FeNH_4SO_4$, 25 mM sulphuric acid, 250 µM xylenol orange, and 100 mM sorbitol (Gay and Gebicki 2000). The sample was kept in the dark for 30 min, and absorbance was determined at 560 nm. The H_2O_2 content was estimated from a calibration curve prepared with H_2O_2 standards.

Lipid peroxidation: To assess the effect of As on lipid peroxidation, a 200 mg sample of root tips was homogenized in 2 cm^3 of 0.1 % (m/v) trichloroacetic acid (TCA) and centrifuged at 10 000 *g* for 20 min. Then, 1 cm^3 of 0.5 % (m/v) thiobarbituric acid in 20 % (m/v) TCA was added to an aliquot of 0.5 cm^3 of the supernatant. This mixture was incubated in boiling water for 30 min and then transferred to an ice bath for 10 min. The sample was centrifuged at 10 000 *g* for 5 min. The absorbance of the supernatant was read at 532 nm and corrected for a nonspecific absorbance at 600 nm. The content of malondialdehyde (MDA) was calculated using a molar absorption coefficient of 155 mM^{-1} cm^{-1} (Heath and Packer 1968).

Histochemical analysis of the cell membrane integrity: The integrity of the plasma membrane of cells of the water hyacinth roots was tested histochemically using the Evans blue stain. A root sample (1 cm) was collected from root tips after 24 h of exposure to the treatments. They were washed in deionized water and then incubated in 10 cm^3 of an Evans blue solution (0.025 % m/v, in 100 µM $CaCl_2$, pH 5.6) for 30 min. After that, the roots were removed from the staining solution, washed for approximately 15 min in deionized water, and imaged under a stereoscope. The images were obtained using a digital camera *Panasonic DMC-LX3* (Japan) according to Yamamoto *et al.* (2001).

Assessment of activity of antioxidant enzymes: To assess activity of the antioxidants enzymes, 0.3 g of a root fresh matter was homogenized in an extraction medium proposed by Peixoto *et al.* (1999). The homogenate was centrifuged at 12 000 *g* and 4 °C for

15 min. The resulting supernatant was used as crude extract to assess superoxide dismutase (SOD), catalase (CAT), and peroxidase (POX) activities. Protein content in the supernatant was measured by the Bradford (1976) assay.

Superoxide dismutase (EC 1.15.1.1) activity was measured according to the inhibition of *p*-nitroblue tetrazolium (NBT) photoreduction (Giannopolitis and Ries 1977). A SOD activity unit corresponded to the amount of the enzyme required to inhibit 50 % of NBT photoreduction per min (Beauchamp and Fridovich 1971). Catalase (EC 1.11.1.6) activity was estimated as the rate of decomposition of H_2O_2 during the first minute of a reaction measured spectrophotometrically at 240 nm (Havir and McHale 1987). A molar coefficient of absorption was 36 M^{-1} cm^{-1} (Anderson *et al.* 1995). Peroxidase (EC 1.11.1.7) activity was estimated as the rate of production of purpurogallin at 420 nm according to the method proposed by Nakano and Asada (1981) with a molar absorption coefficient of 2.47 mM^{-1} cm^{-1} (Chance and Maehley 1955).

Statistical analyses: The experiment was performed using a randomized block design with four treatments and five replicates; each experimental unit consisted of two plants per pot. The data were collected at four time points (0, 4, 12, and 24 h) and subjected to linear regression analysis. The bioaccumulation factor and the tolerance index were analyzed using analysis of variance (*ANOVA*). The comparison of means was conducted using Tukey's test at 5 % probability. The statistical analyses were performed using the statistical program *SAS* (Cary, NC, USA).

Results

The *E. crassipes* plants were able to accumulate significant amounts of As over a short period of time; indeed, the pollutant was detected in both the roots and the leaves after only 4 h of exposure to As (Fig. 1). The pollutant accumulated even at higher rates when SNP was added. The As accumulation occurred mainly in the roots where the bioaccumulation factor, which is used to evaluate the capacity of a plant to accumulate a given pollutant, was greater than in the leaves. The addition of SNP increased the bioaccumulation factor of As in the leaves but the tolerance index to the pollutant was significantly higher when the pollutant was supplied in combination with SNP (Table 1).

Arsenic accumulation triggered many changes in the plants within 12 h of As exposure. It caused a visible damage such as chlorosis and necrosis. After 24 h, the plants exhibited dramatic symptoms of toxicity

Table 1. The bioaccumulation factor (BF) and the tolerance index (TI) in *Eichhornia crassipes* plants exposed to arsenic alone or in combination with SNP. Means followed by the same upper-case letter, between treatments for the same organ, and by the same lower-case letter, between organs for the same treatment, were not significantly different according to Tukey's test at 5 % probability.

Treatments	Exposure [h]	BF leaves	roots	TI [%]
As	4	0.07 Bb	0.14 Ba	22.50 B
	12	0.10 Bb	0.22 Aa	18.34 B
	24	0.11 Bb	0.24 Aa	17.85 B
As + SNP	4	0.11 Bb	0.22 Aa	39.61 A
	12	0.17 Aa	0.23 Aa	39.75 A
	24	0.19 Aa	0.24 Aa	38.94 A

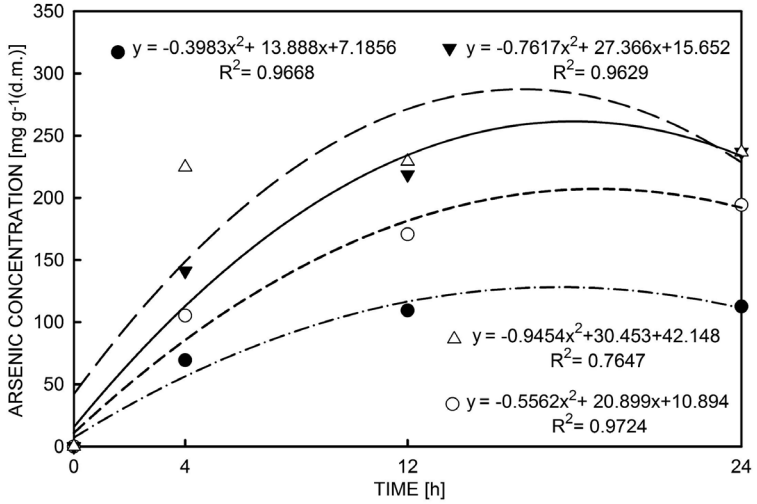

Fig. 1. The content of As in leaves and roots of *Eichhornia crassipes* exposed to As in the presence and the absence of SNP [● - As (leaves), ○ - As + SNP (leaves), ▼ - As (roots), Δ - As + SNP (roots)].

including extensive regions of necrosis on the leaves. The addition of SNP did not completely reverse the damage caused by As but it did reduce the number of leaves affected and the area covered by necrosis.

The content of $O_2^{\cdot-}$ in the roots of *E. crassipes* increased with exposure to As and with exposure time indicating that oxidative stress occurred. The content of $O_2^{\cdot-}$ in the plants exposed to As and SNP was similar to that in the control (Fig. 2*A*). In fact, the plants exposed to As and SNP had the content of $O_2^{\cdot-}$ in the roots 50 % lower than the plants exposed to As alone. Similarly, the H_2O_2 content increased in the plants exposed to As. The addition of SNP minimized the effect of As, especially after 12 h of exposure. After 24 h of exposure to the As + SNP treatment, the H_2O_2 content returned to that of the control (Fig. 2*B*).

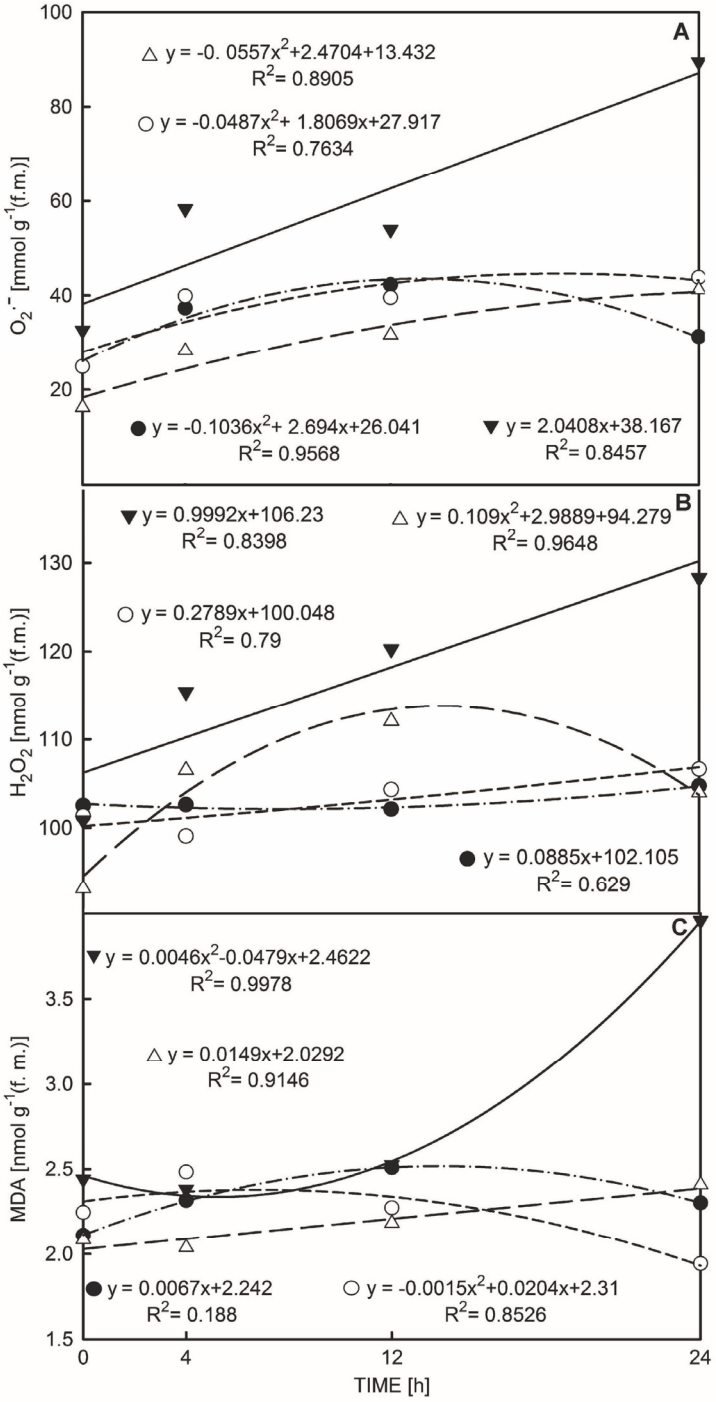

Fig. 2. The content of the superoxide anion (*A*), hydrogen peroxide (*B*), and malondialdehyde (*C*) in the roots of *Eichhornia crassipes* exposed to As in the presence and the absence of SNP (● - control, ○ - SNP, ▼ - As, Δ - As + SNP).

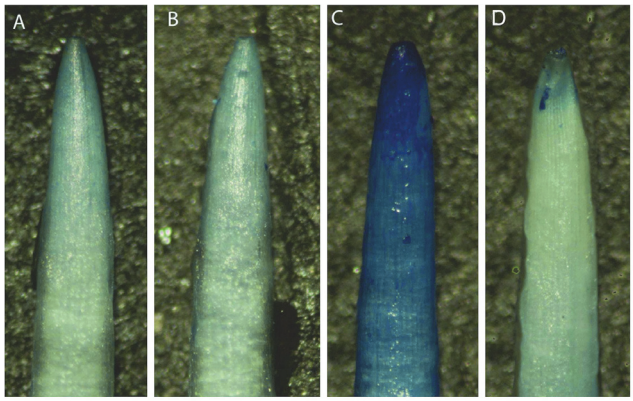

Fig. 3. The histochemical analysis of damage to root membranes triggered by As in *Eichhornia crassipes* in the presence and the absence of SNP (*A* - control, *B* - SNP, *C* - As, *D* - As + SNP).

Arsenic damaged the membranes of root cells as reflected by an increase in MDA production (Fig. 2*C*) which increased with the time of As exposure. After 24 h, the MDA content in the treated plants was nearly 50 % greater than in the control. The toxic effect of the pollutant on the cell membranes was attenuated by the addition of SNP; indeed, the MDA content in the plants exposed to As and SNP was not statistically different from the control. This finding demonstrates that NO had a protective effect on the cell membranes, most likely by decreasing ROS production.

The damaging effect of As on the membranes was evident in the histochemical analysis of the roots. The plants exposed to As had more intense blue staining in the root tips than the control plants indicating compromised membrane integrity. The plants exposed to the As + SNP treatment had more faintly stained root tips, further confirming the protective effect of NO (Fig. 3).

The activity of SOD increased in the plants exposed to As for 24 h indicating that the defence mechanisms of *E. crassipes* had been activated. However, this increase was not observed when the pollutant was supplied in combination with SNP (Fig. 4*A*). The exposure to As also significantly increased the CAT activity, especially after 12 h of exposure. Although the activity of this enzyme increased in the plants under the As + SNP treatment, the increase was not as significant as that observed in the plants treated with As alone after 24 h. The presence of SNP appeared to accelerate the response of the plants to the pollutant as changes in enzyme activity were evident after 4 h of exposure to As (Fig. 4*B*). Of the enzymes analyzed in the roots, POX showed the highest absolute and most increased activity after exposure to As. The activity of this enzyme increased linearly with increasing time of exposure. However, when As was supplied in combination with SNP, a smaller increase in enzyme activity was observed (Fig. 4*C*).

Discussion

The water hyacinth plants exposed to As were able to accumulate large amounts of this pollutant, but it triggered a series of morphological changes and cellular damage in the plants. The most obvious signs of toxicity in the leaves of *E. crassipes* were chlorosis and necrosis. It has been previously demonstrated that chlorosis and necrosis might be the result of As stress, mainly due to the formation of ROS in the region of the cell wall and within the cell (Mascher *et al.* 2002, Farnese *et al.* 2014b). Arsenic can also modify absorption and translocation of micronutrients (especially iron) resulting in nutritional deficiencies and chlorosis of leaves as observed in barley (Shaibur *et al.* 2009). The addition of SNP attenuated the damaging effect of As and the damages observed in the plants exposed to As + SNP were less severe than those observed under As alone.

An increased ROS production triggered by As affected the integrity of cell membranes and these changes may have contributed to the emergence of necrotic areas. The production of ROS may have increased because of the conversion of arsenate to arsenite, once that this conversion represents a mechanism by which the plant adapts to the pollutant (Meharg and Hartley-Whitaker 2002) and may result from the direct reduction of molecular oxygen (Zhang and Qiu 2007). However, such an increase does not occur in the presence of NO. Consequently, no cell membrane damage was observed in the plants exposed to As + SNP. The same results were observed in other species exposed to As and NO, as *Pistia stratiotes* and mung bean (Ismail 2012, Farnese *et al.* 2014a), emphasizing the protective effect of NO on cell membranes.

The maintenance of membrane integrity in the presence of NO was likely the main factor responsible for the ability of the plants to absorb greater amounts of As. Arsenic in the form of arsenate is absorbed through phosphate transporters in the plasma membrane of root cells (Gusman *et al.* 2013). Because the application of

SNP mitigated damage to cell membranes, the phosphate absorption system remained active and the plant was able to absorb greater amounts of As. The reduction in ROS content and the consequent maintenance of membrane integrity were apparently triggered by NO through two main mechanisms: the direct elimination of $O_2^{\cdot-}$ and the activation of signal transduction pathways that resulted in the response of antioxidant enzymes involved in eliminating H_2O_2. In fact, NO is able to trigger both mechanisms as demonstrated in other plants exposed to As such as *Lupinus luteus* and aquatic macrophytes (Arasimowicz and Floryszak-Wieczorek 2007, Farnese

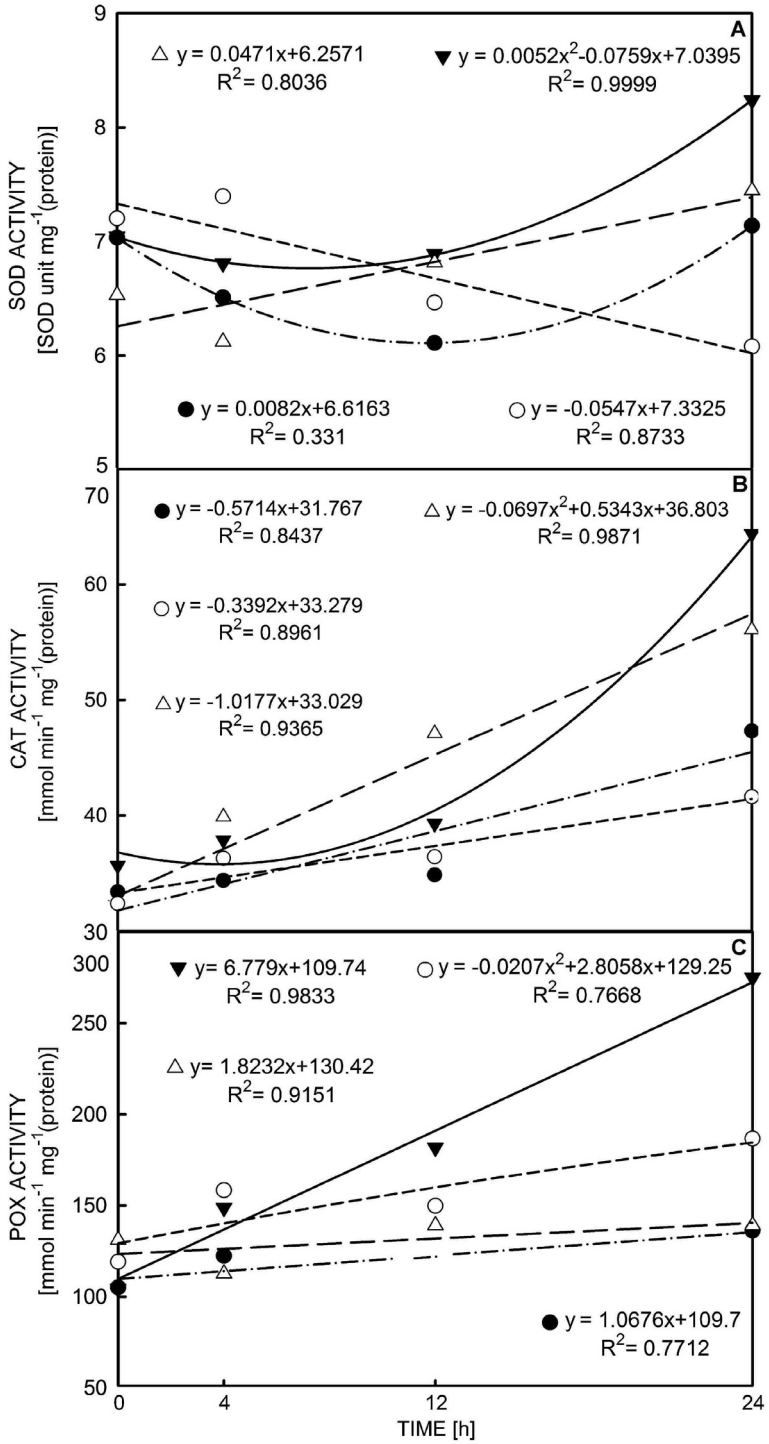

Fig. 4. Activities of SOD (*A*), CAT (*B*), and POX (*C*) in the roots of *Eichhornia crassipes* exposed to As in the presence and the absence of SNP (● - control, ○ - SNP, ▼ - As, Δ - As + SNP).

et al. 2013).

Under biotic and abiotic stresses, SNP can act as cell signal capable of triggering increases in activity of SOD and a consequent reduction in $O_2^{\cdot-}$ content (Arasimowicz and Floryszak-Wieczorek 2007). However, significant changes in the SOD activity in *E. crassipes* were not observed in the presence of SNP. This finding suggests that the observed reduction in $O_2^{\cdot-}$ content resulted from a direct antioxidant action of NO (Xiong *et al.* 2010). In fact, NO can interact with $O_2^{\cdot-}$ to form peroxynitrite ($ONOO^-$; Arasimowicz and Floryszak-Wieczorek 2007) which can be removed from a cell by thiolated compounds (Crow 2000) and CAT (Gebicka and Didik 2009). The action of NO as antioxidant was also observed in other plants subjected to As such as *Oryza sativa* (Singh *et al.* 2009) and *P. stratiotes* (Farnese *et al.* 2013).

Nitric oxide released from SNP acts as signalling molecule for CAT (Singh *et al*. 2009). In fact, a significant increase in CAT activity was observed after 4 and 12 h of exposure to As + SNP. Catalase maintained a low H_2O_2 content in the plants exposed to As + SNP similar to that in the control plants. After 24 h of exposure to the treatments, the CAT activity was higher in the plants subjected only to As. However, NO was able to accelerate a response of the plants to the pollutant, and this effect was more important than the activity of the enzyme in the final stage of the experiment, as can be proved by the lower H_2O_2 content in the treatment As + SNP. Moreover, it is likely that less H_2O_2 was generated in the plants exposed to As + SNP, as $O_2^{\cdot-}$ was eliminated without the participation of SOD. However, the POX activity was lower in the treatment As + SNP possibly due to an equilibrium between the activation of CAT and POX. This equilibrium is essential to keep ROS in acceptable levels and not to disturb cellular functions. This kind of response was also found in *Lycopersicum esculentum* (Miteva *et al.* 2005) and rice (Shri *et al.* 2009).

By reducing the deleterious effects of As, NO increased the tolerance index. The tolerance index represents a factor that allows analysis of the ability of a plant to tolerate stress conditions, and in combination with the bioaccumulation factor, it provides important information on the response of plants to pollutants (Hayat *et al.* 2010).

In conclusion, exogenous NO attenuated damage to *E. crassipes* triggered by As, and its beneficial effects primarily resulted in a direct elimination of ROS and a faster activation of the antioxidant enzymes. Thus, the plants exposed to As and SNP revealed few morphological changes, fewer cellular damage and a higher tolerance index than the plants exposed only to As. Furthermore, the absorption system remained active and the plants were able to absorb higher amounts of As and transport it to leaves as observed by the bioaccumulation factor. These results have important implications for phytoremediation and may be a basis for the development of new transgenic strategies to plants with a potential for phytoremediation of water contaminated with As since they indicate that NO is molecule able of increasing uptake and accumulation of the pollutant in tissues. The results also contribute to understanding cellular signalling mechanisms involved in the response to pollutants.

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