Assessing of salt effect in regulation of growth and antioxidant defence in Arthrocnemum indicum under Arsenic stress alone or with salinity

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Metal pollution is one of the most acute environmental issues, aggravated by human activities. In the present study, authors intend to determine the tolerance traits of Arthrocnemum indicum to avoid As toxicity. In order to evaluate the mechanisms responsible for Arsenic (As) tolerance, plants were grown in semi-controlled conditions upon exposure to different As concentrations (0, 200, 500 and 800 µM) supplemented or not with NaCl (0, 200 mM). Growth parameters, micronutrient uptake, proline and glycine betaine contents and antioxidant enzymes activities (SOD, GPX and APX) were assessed. Plants demonstrated a good growth even after prolonged exposure to high metal concentrations. On the other hand, the GB and malondialdehyde content in the leaves of stressed plants increased significantly in all treatments. However, proline showed a steady level. Activities of SOD, GPX and APX varied according to the applied stress. Our results indicated that As induced oxidative stress in Arthrocnemum indicum and enzymatic and non-enzymatic antioxidants played significant roles in tolerance to As toxicity.

Keywords: Arsenic, combined stress, mineral uptake, antioxidant mechanisms

1. Introduction

The majority of the world's population is subjected to toxic chemical elements coming from rose anthropogenic pollution. The high doses of heavy metals in the environment of the Tunisian country could come from a diversity of industrial processes [1].

Heavy metal(loid)s altered the physiology of plants in various schemes by limiting growth, restricting the process of photosynthesis, perturbing the electron transfer chain of photosystem II, minimizing the biosynthesis of chlorophyll and disrupting the mineral status [2]. Among the heavy metal(loid)s, arsenic (As) is a highly toxic metalloid which leads to cellular toxicity and membrane damage. The latter is accompanied by an augmentation in
malondialdehyde levels evincing oxidative damage provoked by As toxicity. To counter this stress condition, plants are equipped with an antioxidant defence mechanism including enzymatic antioxidants and also non-enzymatic [3].

Halophytes are a group of plants which grow and reproduce in highly saline (> 200 mM NaCl) soil [4]. The physiological adaptation of halophytes to salt had played a vital role in survival in this habitat and in resolving adverse conditions [4]. It had been shown that halophytes were capable to face metal poisonous, evincing their possible use for remediation of metal-polluted soils. *Arthrocnemum indicum* is a stem succulent perennial halophyte from the family Chenopodiaceae [5]. However, little information about the phytoremediation potential of *A. indicum*. In our unpublished study, we demonstrated the accumulator potential of As. Here in this study, we will focus on the physiological response of plants subjected to As concentration of the metalloid single or combined with salt.

2. Materials and methods

2.1. Plant sampling and Experimental Setup

Cutting of *Arthrocnemum indicum* were irrigated with non-saline tap water for a period of six weeks (For more details see Sghaier et al. [6,7]. Then, young rooted cuttings were supplied by Hewitt nutritive solution [8] supplemented or not with NaCl (200 mM), three-time by a week) as described in Sghaier et al. [6,7]. plants were divided into groups of six plants and exposed to increasing concentrations of As alone or combined with salt (200 mM).

2.2. Leaf water status

The fresh weight (FW) was directed established, and the dry weight (DW) was estimated after plant material desiccation in an oven at 60 C for a week. Leaf water content (WC) was determined as:

\[ WC = 100 \times \frac{(FW - DW)}{FW} \]

2.3. Oxidative stress biomarkers

All enzymatic analyses were carried out at 4°C. For the extraction procedure, 500 mg of fresh leaves was added to 12 ml of sodium phosphate buffer (50 mM, pH 7.6) with 0.1 mM Na-EDTA. The mixture underwent centrifugation at 8923 rpm for 20 min, at 4°C, and the supernatant was undertaken for enzymes assays. Ascorbate peroxidase (APX) was determined as described by Tiryakioglu et al. [9] by recording the drop in absorbance upon oxidation of ascorbate at 290 nm. Guaiacol peroxidase (GPOX) was carried out by the method of Bergmeyer et al. [10] by recording the rise in absorbance upon the formation of guaiacol oxidation products at 470 nm (\( \epsilon = 26.6 \text{ mM}^{-1} \text{ cm}^{-1} \)). Superoxide dismutase (SOD) activity was assayed according to Marklund and Marklund [11] by noticing the decline of pyrogallol at 325 nm. Proteins were set according to Bradford [12].

2.4. Lipid peroxidation
MDA content was determined according to Heath and Packer [13], by using a mixture (20% trichloroacetic acid (TCA) and 0.5% thiobarbituric acid (TBA)). The absorbance of the supernatant was read at 532 and 600 nm in a Shimadzu UV-1603 spectrophotometer.

2.5. Osmo-compatible solutes

Free proline was extracted from blended samples in aqueous 3 % sulphosalicylic acid, underwent centrifugation at 10,000 rpm for 15 min, and the proline in the supernatant was determined using ninhydrin according to Bates et al. [14].

GB was determined as described by Grieve and Grattan [15]. After dilution with 2 N H₂SO₄ followed by dissolving of the pellet in 9 mL of 1,2-dichloroethane, the final step was the reading of the absorbance at 365 nm.

2.6. Metal concentration

Fresh plant material was blended in HNO₃: H₂SO₄: HClO₄ (10:1:0.5; v/v/v) for 2 h 30 min at 110°C. Total concentrations of Na, K, Ca, and Mg were determined by atomic absorption spectrometry (Perkin Elmer PinAAcle 900T, USA) (see details in Sghaier et al. [6, 7]. The blanks, used to adjust the atomic absorption spectrometer to zero, were handled in the same manner as described above.

2.7. Statistical analysis

As the data missed normality and homogeneity, the statistical analysis was based on non-parametric tests. The Kruskal Wallis test was performed using Statistica software (Statasoft).

3. Result

3.1. Biomass production

The shoots growth of A. indicum subjected to 200, 500 and 800 µM of As was reduced. The root growth was restrained at low doses of As and the highest reduction was at 800 µM As. However, salt application (200 mM) enhanced the shoot growth by 2-fold in plants treated with 200 µM As and 500 µM As as compared to those treated with single As. The root FW remained unaffected by salinity treatments except at 200 µM As + salt where an improvement in roots biomass was noticed. This augmentation was about 2.5-fold as compared to those treated at the same concentration of As alone (Fig. 1a).

The water content (WC %) of A. indicum shoots and roots remained unaffected in all the treatments, except in combined treatments of high As, where a decrease in WC % was observed as compared to control in shoots while an increase in roots was noted at 200 µM +salt (Fig. 1b).
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Fig. 1 Dry weight (DW) (a) and Water content (WC) (b) in *Arthrocnemum indicum* under different concentrations of As (0, 200, 500, 800 μM) in the absence or presence of 200 mM NaCl. Different lower case letters represent statistically significant differences (p<0.05).

3.2. macronutrient uptake

Na+ was found in high amounts in control and in NaCl-treated plants but increased in plants exposed to mixed treatments in shoots and roots respectively. When plants were subjected to As at low doses (200 μM), NaCl uptake declined in roots and shoots compared to other doses or untreated plants. Na content in shoots and roots was higher for plants exposed to NaCl in the presence of HM than in other treatments. In addition, Na content was more predominant in the roots than in the shoots (Fig. 2).
Fig. 2. Na⁺ uptake in *Arthrocnemum indicum* under different concentrations of As (0, 200, 500, 800 μM) in the absence or presence of 200 mM NaCl in the nutrient solution. Different lower case letters represent statistically significant differences (p<0.05).

Our data showed that K content in the shoots and root significantly rose with increasing As doses. Moreover, as compared to As alone, K content declined in combined treatments in shoots and roots (Fig.3b). The accumulation of Ca and Mg in shoots and roots remained constant as compared to the control in both solitary and mixed treatments of As. No variations were noticed, unless, some exception was noted: a huge augmentation of Ca levels at 800 μM As were registered and a decline in the endogenous content of Mg at 200 μM As in the shoots and in the roots. The level of Ca and Mg significantly dropped in shoot and root under elevated As in mixed treatments, as compared to the control (Fig.3 a,c).
Fig. 3. Calcium (a), Potassium (b), Magnesium (c) ions concentrations in Arthrocnemum indicum under different concentrations of As (0, 200, 500, 800 μM) in the absence or presence of 200 mM NaCl. Different lower case letters represent statistically significant differences (p<0.05).
3.3. MDA content and Antioxidative response

The MDA content was significantly increased by 4-fold at low doses and 7-fold in high arsenic. Leaves of plants exposed to NaCl treatment even exhibited lower MDA concentration than control plants, the ROS accumulation declined in combined treatments of arsenic and salinity as compared to single As and it remained unchanged at all doses (Fig. 4a).

The antioxidative enzyme activity was differentially altered by different treatments of salinity and arsenic. The highest value for SOD activity was registered in A.indicum exposed to salinity with a rising of 2-fold as compared to the control. An augmentation in SOD was recorded at low doses of As. The SOD activity was declined by 1.21-fold in elevated arsenic treatment comparing to the control. Moreover, the addition of salt with elevated arsenic doses enhanced the SOD activity by 2.4-fold as compared to 800 μM As (Fig. 4b).

The activity of APX augmented in low doses of As where the highest rising around 2-3-fold was recorded as compared with control. However, it remained up to the control level in all other treatments of salt and arsenic (Fig. 4c).

GPX activity remained unaffected in salt and low concentration of arsenic as compared to control and maintained up to the control in all treatments of single As. Whereas, GPX activity was significantly increased by 4.2 and 3.4-fold after the application of the salt and in high arsenic doses in combined treatment As + NaCl (Fig. 4d).
Fig. 4. MDA content (a), enzymatic activity: APX(b), SOD (c) and GPX (d), Proline (e) and glycine betaine (f) content in the leaves of *Arthrocnemum indicum* under different concentrations of As (0,200,500, 800 μM) in the absence or presence of 200 mM NaCl. Different lower case letters represent statistically significant differences (p<0.05).

The GB content of the shoot showed a gradual increase with a rising concentration of As (Fig. 5a). The plants treated with salt and arsenic showed an augmentation in GB content as compared to those supplied with a single As (Fig. 5a).

However, in plants treated with single As, there were no significant changes in proline content. The proline content was unaffected in salt stress (Fig. 5b). Similarly, under combined treatments of arsenic and salt treatments the levels of proline remained the same as the control (Fig. 5b).

Fig. 5. glycine betaine (a), and Proline (b) content in the leaves of *Arthrocnemum indicum* under different concentrations of As (0,200,500, 800 μM) in the absence or presence of 200 mM NaCl. Different lower case letters represent statistically significant differences (p<0.05).

**Discussion**

*A. indicum* shoots and roots of fresh phytomass declined under single As, suggesting that the As treatment imposed severe negative impacts on nutrient metabolism. Arsenic interacted with thiol groups of enzymes inducing metabolic inhibition [16]. As has been proposed to attribute growth inhibition mediated to cell cycle arrest and inhibition of DNA synthesis and repair mechanisms [17]. Decreased plant growth could be a result of water deficiency and nutrient imbalance as reported in *Acanthus ilicifolius* and *Salvadora persica* [18, 19]. Otherwise, As had the potential to generate oxidative stress through ROS which hampered cellular biomolecules [16]. Thus, the ROS production under elevated As treatment could be a reason for the development inhibition [17].

The Addition of NaCl to the As minimized the toxic effects of As on the plant [20]. It was signalled that the improved plant growth by NaCl addition was not due to the beneficial
effect of NaCl on growth, since the addition of NaCl did not recover the biomass of *Carpobrotus rossii* in the absence of metals [21]. Our results indicated that salinity improved As tolerance in *A.indicum*. It was reported that the cross-tolerance of salinity and arsenic could be allocated by some kind of cross-talk system between arsenic and salinity [17].

In the present study, the shoot water content remained unaltered in all the treatments. In accordance with our result, *Sueada maritima* maintained a steady WC under salinity and high As-stress [20]. RWC% was maintained under both salinity and As stress conditions in *S. persica* which suggested that the water uptake was not affected even at high salt and arsenic treatments in this plant [19]. *A.indicum* was well adapted to high concentration salinity and drought conditions. This property might allow *A.indicum* to cope with toxic levels of As to some extent by preserving osmotic balance and keeping the same water status under stress conditions. Further, synchronization of absorption and transport of different mineral ions was necessary for preserving the osmotic potential [22]. The presence of As did not significantly alter the uptake of Na except at low doses of As. Similarly, in *S. persica* no variation in the endogenous content of Na was noted which suggested that As did not alter the minerals uptake system [19]. The unaffected level of Na in the presence of As has also been reported in *S. maritima* and was accompanied by an unchanged level of WC in the shoot which hypothesized that Na might be sequestered in vacuolar tissue under saline conditions by keeping shoot turgor [20].

Interestingly, Na and K ions owing very similar ionic radius and hydration energy [23]. Hence, under saline conditions, Na penetrated the cell via K channels situated in cell membranes causing an increase in Na content and a decrease in the concentration of important nutrients such as K [23]. K is a crucial macronutrient engaged in the activation of more than 50 enzymes, chlorophyll biosynthesis, and cytoplasmatic pH homeostasis [23]. Due to these important physiological functions of K in plant cells, preserving a proper amount of K under salt stress was pivotal. Therefore, the ability of the plants to limit K loss, preserving of higher K and lower Na levels were associated with the salinity resistance of plants [24]. The K accumulation in the shoot and root of *A.indicum* was not affected under salinity, As and combined treatments (at low doses of As).

Ca figured as the crucial ion which equilibrated the membrane structure and roles [24]. Osmoprotectant in shoot under As stress in the absence of salt might be intervened via K and Ca ions, as their content was significantly elevated in plants treated with single As as compared to As combined with salt. In fact, in mixed treatment, Na proceeded as the major ion, controlling the osmotic potential and mitigating the toxic effects of As [25]. In contrast to our result, a decline in Ca concentration in the combined treatment had been reported in *Tamarix gallica* [6,7]. and *Atriplex atacamensis* [25] which could be due to the lower availability of Ca and limited absorption and transport of Ca to the tissue under salinity [25]. The Mg content of the shoot reduced significantly under 800 µM As + salt. Other studies reported various attitudes concerning Mg uptake, for instance, the absorption and transport of Mg were altered due to the application of NaCl in *S. persica* [19, 24]. Another trend had also been reported by Panda et al. [20] in *S. maritima* roots’, the Mg content diminished after the exposure to the salt with arsenic as compared to single arsenic.
A significant augmentation in the lipid peroxidation level was observed under high As treatment. In fact, the ROS raising was damaging but, simultaneously might act an important role in signaling cascades, causing the expression of stress-sensitive genes [26]. So As led to the synthesis of ROS in A. indicum which could be a basic signal for incitement of several metal resistance approaches. Further, salt addition declined the MDA level at low as well as elevated As stress as compared to single As. Similar to our result, Vromman et al. [25] reported a decline in the MDA doses in As with salt. Our results also suggested that NaCl supplementation increased the membrane stability and improved the cross-tolerance to As and salinity.

Hence, in order to avoid oxidative injuries, plants possess a well-defined defence system for ROS scavenging that involves enzymatic and non-enzymatic antioxidants. In fact, enzymes activities were stimulated under lower As concentrations and reduced under elevated As stress. An increase in antioxidants enzymes activities at lower As stress might be due to the activation of plant defence mechanisms under metal stress [2]. However, the reduction in the activities at elevated doses could be caused to the disruption of the homeostatic balance of ROS [23]. In this line, SOD activity was elevated in control plants of S. maritima and a reduced activity under salinity and As treatments were registered [20]. Whereas, the activity was maintained at a steady level in all the treatments demonstrating an elevated activity of SOD as compared to the threshold for scavenging O$_2$•$^-$ produced by salinity and As-stress [25]. the constant SOD activity in all the treatments indicated that the activity of SOD was not altered by the stress conditions. While the decline in the activity might be attributed to the utilization of this enzyme to sequester O$_2$•$^-$ radical [25]. Moreover, our data showed the activity of APX increased with increased As stress and its activity remained at steady control in the presence of elevated doses of As and unaltered when As was added to the salt. However, prior studies had also documented the increased, decreased, and unchanged levels in the APX activity in response to heavy metals stress [25]. Increased expression of APX has also been reported in Kosteletzkya pentacarpus under Cd stress [27]. In S. maritima, the APX activity decreased with salinity but remained the same as in control with As [20].

whereas the GPX activity remained unchanged under As-stress and increased under salinity. This result indicated that salinity and As stress induced generation of H$_2$O$_2$ which was scavenged either by APX or GPX, thereby maintaining the appropriate level of H$_2$O$_2$ for stress signalling. Otherwise, Kofronová et al. [28] reported that Spartina densiflora faced oxidative stress in its habitat and harmonized its antioxidative system depending on the level of metal. Antioxidant enzymes were not the only way to take off the most reactive ROS. Hence, assaying antioxidant molecule levels, e.g., proline and glycine betaine were required to have a complete scenario of the scavenging capacity [28].

Proline also played an important role in the cell wall architecture as a precursor of hydroxyproline presented in high amounts in cell wall protein [29]. Proline level was unaffected after exposure to single As or combined with salt. In accordance with our agreement, the proline level in S. maritima plants imposed to single As treatments were at the same level of control. This could be caused by the decrease in the activity of proline biosynthetic enzymes or augmented activity of proline degrading enzymes, inducing a steady level of proline. In most plant species, P5CS was encoded by two distinct genes which were differently controlled based on the stress occurrence [27].
Further, our results showed a noteworthy accumulation of glycine betaine, in the leaf of *A.indicum*. The increased accumulation of GB during As stress functionally maintained minimum hydration within the cells and protected the cells from severe oxidative injury [29]. GB had a role in heavy metal chelation, maintenance of pH, intracellular ionic transfer regulation and cellular homeostasis [27]. So, the augmentation of GB in *A. indicum* could reduce the As induced injury. An augmentation in GB levels was observed in the rice seedlings exposed to As stress [29]. Similarly, the NaCl along with CdCl2 raised the Cd resistance potential of the halophyte *Bruguiera cylindrica* [30]. Taken together, both these examples demonstrated that the combined application of NaCl and metal stress in halophytes rise the metal resistance of the halophytes more than when it was supplied individually.

**Conclusion**

The results of this study could conclude that *A.indicum* was able to survive under high As concentration. The supplementary salt improved the tolerance capacity of the plant to resist the As toxicity which indicated that As and salinity cross-tolerance was achieved by the efficient enzymatic and non-enzymatic antioxidative defence system and maintained the suitable level of ROS and protected the membrane integrity under stress conditions.

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**Compliance with ethical standards**

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