



RESEARCH ARTICLE

Salt Stress Triggered Changes in Osmoregulation and Antioxidants in Herbaceous Perennial Inula Plants (Asteraceae)

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ABSTRACT

Global demand to cure ailments is a growing need. Inula genus extensively holds hundreds of species in warmer regions of Europe and Asia. It is being well-known for its phytochemical and pharmacological applications in industry thanks to its anti-inflammatory and antimicrobial interests. However, growth and production of Inula in the cutting-edge industry is commonly influenced by salt stress except for the halophyte species such as the *Inula crithmoides*. Salt tolerance level by means of changes in osmoregulation and antioxidant systems in an herbaceous perennial Inula plant has been biochemically evaluated here. Both salt stress treatments caused photosynthetic pigments' degradation, increase in the leaf levels of osmolytes, and induction of oxidative stress indicated by the malondialdehyde (MDA). Higher hydrogen peroxide (H₂O₂) amount was recorded in high salt concentration than low salt. High salinity caused an increase in ascorbate (ASC) and glutathione (GSH) contents besides target enzymes of Inula leaves. NaCl tolerance of Inula also was found comprehensible through the higher concentrations of proline and to a lesser extent, total soluble sugar. Salt tolerance mechanisms of this rich bioresource needs to be further studied in detail for herbal medicines in pharma sector.

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Introduction

Many geographic regions in the world has undergone changes due to increasing temperatures and fluttering precipitation regimes subsequently affects all aspects of life on earth. Excessive temperature and drought cause increase in soil salinity and significantly reduces product yield and quality. The effects of drought and temperature can be reduced by irrigation however; it leads to a further reduction in the already diminishing water resources. In any event, agricultural activities are the biggest factor in reducing the fresh water reserves in the world. Furthermore, irrigation increases soil salinity. Considering the fact that the world population continues to grow (UN's estimation is 8.6 billion people by 2030) and arable lands are shrinking, it is evident that, yield

should be increased at a high rate for nutritional and feeding purposes, for the reason that abiotic stress conditions pose a serious threat to agriculture. Plants cannot escape the conditions in which they exist and are victims of constantly changing conditions, yet they have evolved by developing biochemical and developmental signal cascades for the sustainability of growth, development and agricultural phytomass re-productivity.

Stress adaptation is ensured by the activation of the salt stress susceptible genes and the synthesis of various functional proteins result in restructuring of the relevant signaling pathways (Shinozaki and Yamaguchi-Shinozaki 2007). Reactive oxygen species (ROS) are generated in plants exposed to salt stress and are produced continuously. ROS is highly reactive and its overproduction has been shown to adversely affect

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membrane potential and the protection of biological macromolecules such as proteins, carbohydrates, lipids and DNA which ultimately leads to oxidative damage (Demiral and Turkan 2005).

Plants develop enzymatic and non-enzymatic defense systems to keep ROS at an optimal level that will not cause damage to the cell or remove redundant ROS from the cell and protect cells from further oxidative injury.

Ascorbic acid and glutathione are classified as non-enzymatic antioxidants, while primary enzymatic ones are superoxide dismutase (SOD), glutathione/ascorbate peroxidase (APX, GPX) and glutathione reductase (GR) (Foyer and Noctor 2009). Glutathione (L-gamma-Glutamyl-L-cysteinyl-glycine, GSH) stands as the most abundant free thiol in biological systems (Hayes and McLellan, 1999). GSH has vital roles in the cell, such as response and detoxification of oxidants or other chemicals and protection of the thiol-redox balance (Xiang C et al., 2001). ASC on the other hand is a well-known antioxidant as well that is involved in defense mechanisms against oxidative stress. Salt stress cause increased oxidation of the apoplastic ASC pool. Higher foliar ASC levels found in plants are able to tolerate oxidative stress with a better capacity (Potters et al., 2004). Analyzing the accumulation of uncharged and relatively stable reactive oxygen species (ROS), such as intracellular H_2O_2 , is an important factor to measure membrane damage (Maruta et al., 2012).

To alleviate the damage caused by salt stress, plants generally increase the synthesis of soluble sugars and proline to be able to enhance the efficiency of osmotic regulation. Proline regulates osmotic pressure in cytoplasm and preserve the structure of the cellular components, thereby increases the ability of the plant to resist salt stress (Bates et al., 1973). Soluble sugars can increase the concentration of cell sap and increase the stress tolerance of plants (Ashraf and Foolad 2007). Changes in photosynthetic pigments are also markers of salt stress tolerance.

The chlorophyll content normally shows a downward trend after salt stress in various plants (Karimi and Yusef-Zadeh 2013). The genus *Inula* which is a member of Asteraceae has ethnopharmacological importance. Especially its flower extracts containing rich flavonoids, phenolic acids and sesquiterpene lactones are a potential agent to relief many ailments (stomach ache, bruises, joint pain) in many countries all over the world used in the pharma industry (Wu et al., 2015). Studies by now are mainly focused on its phytochemical and pharmacological activities of root/flower extracts however, its salt stress tolerance has not been received significant attention even though its growth are greatly affected by abiotic stresses.

Here, we have analyzed how *Inula* plants (falls in Magnoliopsida class) response to changes at photosynthetic pigments, various osmolytes generally in plants (proline and soluble sugars), oxidative stress (MDA and H_2O_2), several enzymatic and non-enzymatic antioxidants under low and high salt treatments by using above mentioned knowledge. *Inula* plants under higher NaCl showed a better osmotic regulation and membrane protection, which led to a stronger tolerance.

Plant Growth Conditions and Stress Treatment

Inula seeds collected from nature (Hannover, Germany) and seedlings obtained by germination were selected based on the robust morphological traits and placed in the pots containing Miracle-Gro garden mix. Right after young plants were robust enough (after 3 weeks of germination) plants were watered twice a week with Hoagland containing NaCl at 100 (low salt) and 500 mM (high salt) final concentrations, or without salt for the control (non-stressed). All experiments were conducted in an environmental chamber, with following parameters: 16 h/8 h light/dark cycle at 23 ± 2 °C, $300 \mu\text{mol m}^{-2} \text{s}^{-1}$ of photon density of the leaf surfaces, and 50-80% relative humidity. Within the following 7 days, the plants were grown under salinity, and then they were harvested to conduct experiments. Leaf material was used to perform all kind of measurements and biochemical assays.

Enzyme Activity Assays

Inula leaves were powdered in liquid N_2 . Soluble proteins were extracted in 50mM phosphate buffer (pH 7.4), 1mM EDTA, 1% (w/v) PVP-40 besides 1% (v/v) Protease Inhibitor Mixture. The APX extraction buffer contained ascorbate (5mM). The homogenates were centrifuged at 12000g for 20min at 4 °C and the supernatant was collected for further enzymatic assays. APX activity was determined by rate of ascorbate oxidation at 290nm. The solution contained 50mM phosphate buffer (pH 7.4), 0.2mM H_2O_2 besides 0.5mM ascorbate in final reaction volume. Superoxide dismutase activity was detected with the help of the method of Dhindsa and Matowe (1981). The reaction product was spectrophotometrically measured at 560 nm. Guaicol peroxidase was monitored by the changes in absorbance at 470 nm as Urbanek et al., (1991) suggested (extinction coefficient $26.6 \text{ mM}^{-1} \text{ cm}^{-1}$ for tetraguaiacol). Glutathione reductase activity was determined with the oxidised glutathione (GSSG) and oxidation of NADPH (Foyer and Halliwell, 1976). Total proteins were calculated by Bradford protein assay.

Non Enzymatic Antioxidants Determination

Inula leaves were harvested (500mg), powdered in liquid N_2 and GSH and was measured spectrophotometrically at 412 nm as described by Griffith (1980). Ascorbate contents were determined at 265 nm with perchloric acid, NaH_2PO_4 (pH 5.6) and sufficient K_2CO_3 with 1 U ascorbate oxidase (Foyer et al., 1983).

H_2O_2 , Proline, Sugars and Pigment Contents

Inula leaves (0.25 g) were homogenized in 5% trichloroacetic acid and 0.1 g of charcoal (activated) at 4 °C for H_2O_2 assay. The homogenate was centrifuged at $12,000 \times g$ for 15 min. 0.5 mL of 10 mM potassium phosphate buffer (pH 7.0) and 0.75 mL of 1 M KI were used to add in 0.5-mL of the supernatant which then was measured at 390 nm (Velikova et al., 2000). Proline concentration was determined by the

ninhydrin method of Bates et al. (1973) in dry leaf samples (0.1 g). Proline content was given as mg g⁻¹ DW using a standard curve. Dried inula leaf samples were powdered in 80% ethanol. The mix was filtered and the filtrate was centrifuged at 4°. Reducing sugar content was estimated by a color change at 600 nm (Ross, 1959). Total chlorophyll (chl) and Carotenoid (car) content of inula plants have been recorded by using Arnon's equations in 80% acetone (Arnon, 1949).

Statistical Analysis

The means of indicated replicates used for data analyses for all experiments conducted in this study. Significant differences between treatments were evaluated by SigmaPlot, version 11.0 software at 5% ($P \leq 0.05$) level.

Results

MDA and H₂O₂ Level

The concentration of MDA, a reliable biomarker of oxidative stress, was gradually increasing in parallel with the increment in salt concentration in the leaves (Figure 1). 100 and 500 mM NaCl treated leaves induced 1.6 and 2.6-fold higher than the control respectively. As with lipid peroxidation, H₂O₂ amount increased in both 100 and 500 mM NaCl treated groups. H₂O₂ was found 2.0 and 3.2 fold higher than control plants in 100 and 500 mM NaCl treatments respectively (Figure 1)

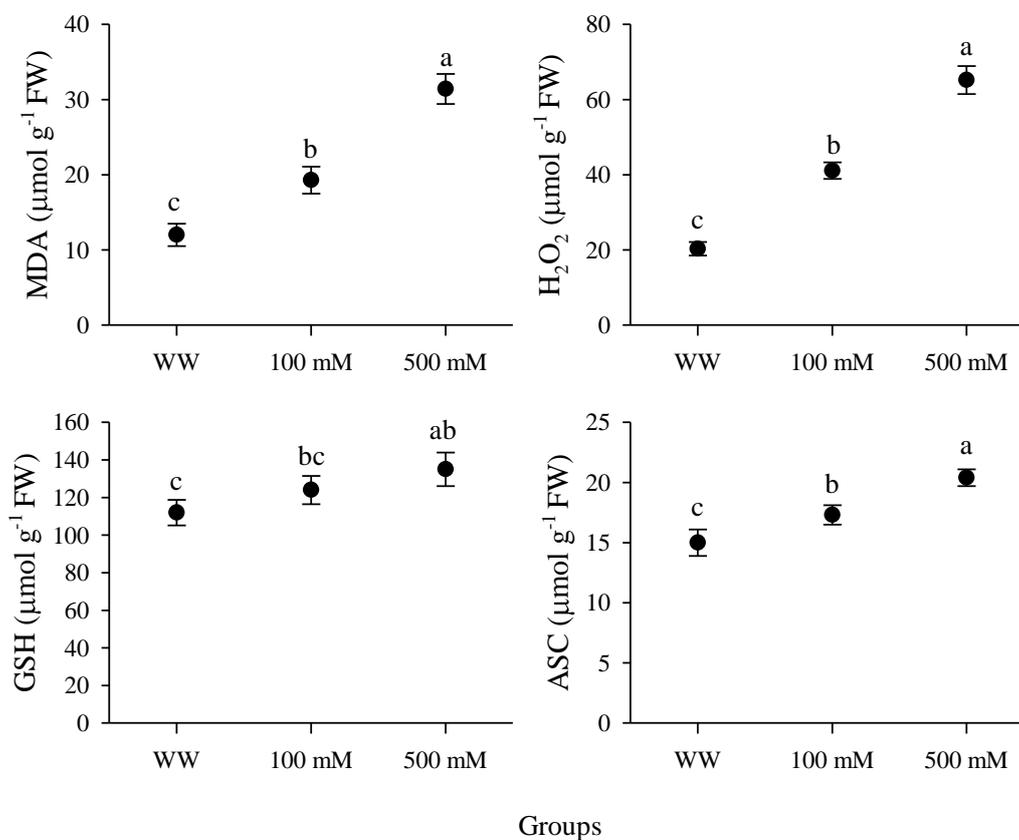


Figure 1 Changes of MDA, H₂O₂, GSH and ASC contents in Inula under different concentrations of NaCl 100 mM (low salt) and 500 mM (high salt) exposure for 7 days. WW: well-watered control, 100 mM: 100 mM NaCl and 500 mM: 500 mM NaCl. Data were shown as the means of \pm SD (N = 5). Different letters indicate significant differences between experimental groups ($p < 0.05$). Statistical analysis was carried out using one-way ANOVA followed by Tukey post-hoc test

Antioxidant Substances

The effects of low and high salt exposure on the contents of antioxidant substances (ASC and GSH) in inula plants are shown in Figure 1. Salinity treatments caused an increase in endogenous ASC concentration. As compared to control, ASC increased by 15% and 36% in 100 and 500 mM NaCl treatments, respectively. Low salt (100 μM) treatment did not affect GSH concentration as much as high salt (500 μM) concentration. About 21% increment in GSH content was recorded in 500 mM NaCl treatment with regard to control. However, there was

also no statistical difference between the two NaCl treatments (Figure 1).

Osmolytes Accumulation

Levels of two types of osmolytes commonly used by plants: proline (Pro), and total soluble sugar (TSS), was measured in the leaves of inula plants subjected to NaCl stress. The proline content was increased in 100 mM and 500 mM NaCl and recorded 2.5 and 6.1 fold as to control (Figure 2). As compared

to control, the TSS content in leaves of inula plants was increased in 100 and 500 mM NaCl treatments. TSS was recorded 2.8 and 3.0 fold higher in 100 and 500 mM NaCl

treatments. However, no significant difference found between sugar contents of 100 and 500 mM NaCl treated plants.

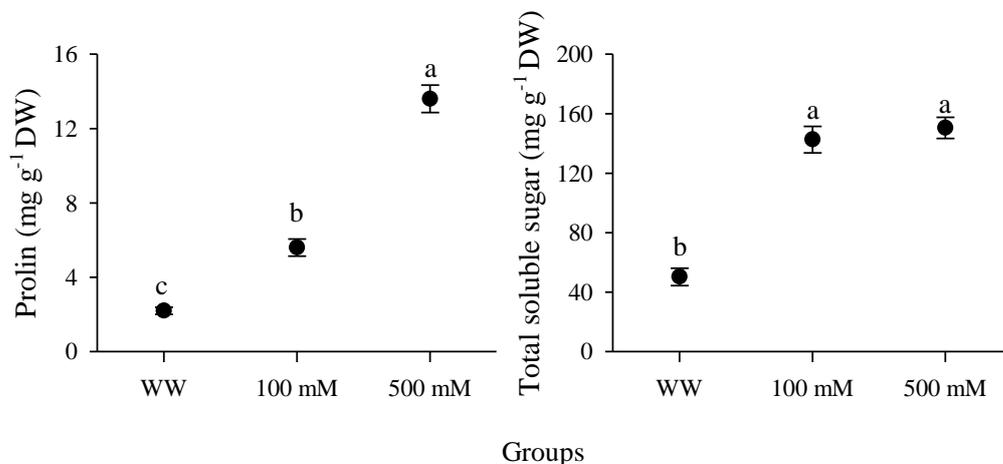


Figure 2 Changes of prolin and total sugar amounts in *Inula* under different concentrations of NaCl 100 mM (low salt) and 500 mM (high salt) exposure for 7 days. WW: well-watered control, 100 mM: 100 mM NaCl and 500 mM: 500 mM NaCl. Data were shown as the means of \pm SD (N = 5). Different letters indicate significant differences between experimental groups ($P < 0.05$). Statistical analysis was carried out using one-way ANOVA followed by Tukey post-hoc test.

Degradation of Photosynthetic Pigments

Total chlorophyll level show significant decrease in leaves of *Inula* plants subjected to 100 mM NaCl treatment but was disrupted by more than 50% in 500 mM NaCl treated plants (Figure 3). Both stress treatments caused significant

reductions in carotenoid concentrations. Concerning carotenoid levels in the leaves of *Inula* plants undergoing salt stress treatments, 1.3-fold decrease was detected at 100 mM NaCl, and a 47% decrease after 500 mM NaCl treatment as compared to controls (Figure 3).

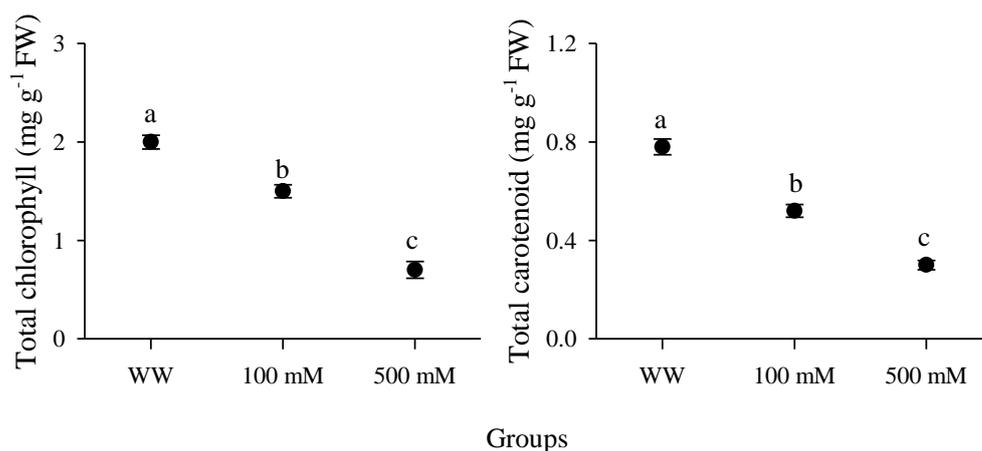


Figure 3 Changes in photosynthetic pigment contents of *Inula* under different concentrations of NaCl 100 mM (low salt) and 500 mM (high salt) exposure for 7 days. WW: well-watered control, 100 mM: 100 mM NaCl and 500 mM: 500 mM NaCl. Data were shown as the means of \pm SD (N = 6). Different letters indicate significant differences between experimental groups ($P < 0.05$). Statistical analysis was carried out using one-way ANOVA followed by Tukey post-hoc test.

Antioxidant Enzyme Activity

As an expected picture generally, a major increase of all enzyme activities was obtained in response to salt stresses. SOD activity of inula plants under these different NaCl concentrations were given in Figure 4. About 51% and 67% increment in SOD activity was recorded in 100 and 500 mM NaCl treatments comparing to their control. However, there was no remarkable difference between the two NaCl dosages. GPOX activity significantly increased with increasing NaCl levels.

GPOX activity was higher in 500 mM NaCl treated plants than that of 100 mM NaCl in terms of increasing ratio. For example, the activity was increased by 100% and 246% in 100 mM and 500 mM NaCl treatments as compared to control, respectively. Regarding APX activity, it increased by 240% under high salt (500 μ M) and in low salt (100 μ M) concentrations by 104% as compared to its control. At last, GR activity in Inula leaf extract, gave a concentration-dependent boost in response to salinity (about 118% higher than in its respective control) measured in the presence of 500 mM NaCl.

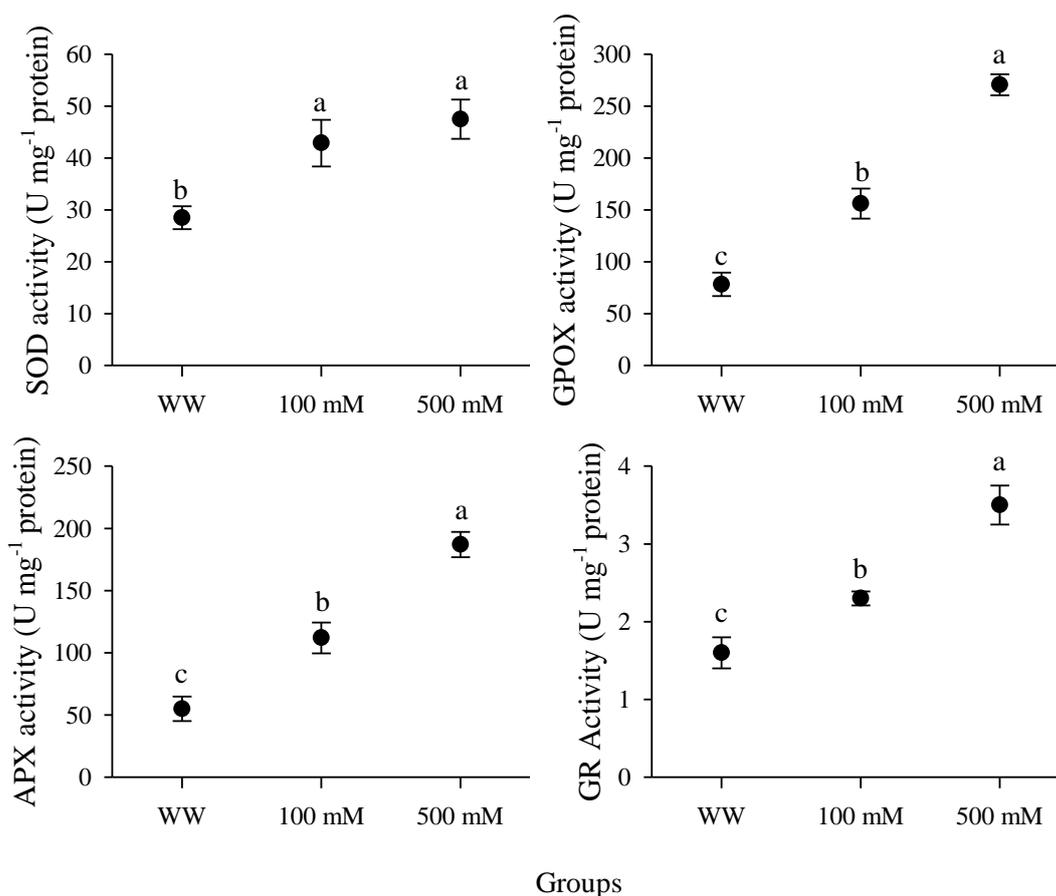


Figure 4 Changes of antioxidant enzymes SOD, GPOX, APX and GR activities of Inula under different concentrations of NaCl 100 mM (low salt) and 500 mM (high salt) exposure for 7 days. WW: well-watered control, 100 mM: 100 mM NaCl and 500 mM: 500 mM NaCl. Data were shown as the means of \pm SD (N=6). Different letters indicate significant differences between experimental groups ($P < 0.05$). Statistical analysis was carried out using one-way ANOVA followed by Tukey post-hoc test.

Discussion

It is a cornerstone in plant physiology that chlorophyll intactness in plants is directly related to plant health. Its concentration under salt stress is a sensitive indicator of the cellular metabolic state of the target plant. A decrease of chlorophyll concentration in many herbaceous and woody species under same saline environments has been reported by numerous researchers (Schiop et al., 2015; Taibi et al., 2016). A marginal reduction in chlorophyll content was detected in Inula plants subjected to two different concentrations of NaCl here in this study, was more obvious in the presence of high salt concentration. This situation appears to be a synergistic

effect on the specific enzymes inhibition (i.e. Rubisco or PEP carboxylase) related to chlorophyll synthesis and the acceleration of degradation by the chlorophyllase (Kumar et al., 2017). Carotenoids, besides their role as accessory pigments which called "light-harvesting", have functions on protecting chlorophylls from photo-oxidative injury (Katarina et al., 2014). Carotenoid level was also affected in Inula by salinity meaning direct correlation between carotenoids and chlorophylls lead chlorophyll loss might be due to the carotenoids degradation. Kumar et al., (2017) detected remarkable decrements in chlorophyll and carotenoid contents in oleander plants exposed to salts, monitored more clearly in the presence of higher saline conditions.

MDA is known as a reliable biochemical marker indicating oxidative stress damage. Different salt concentrations generated oxidative stress in *Inula* here, as supported by the increased amounts of MDA and endogenous H₂O₂. The increments found in the amount of H₂O₂ and lipid oxidation have shown that oxidative stress occurs as a secondary stress in *Inula* plants. Salinity leads high levels of ROS in plant cells due to malfunction of electron transport chain and accumulated photo reducing power (Hossain and Dietz 2016). This excessive electro-chemical energy can be destroyed via the Mehler reaction, which generates ROS, such as H₂O₂, besides membranes damages, mirrored in elevated EL and MDA levels (AbdElgawad et al., 2016).

Cellular antioxidant system and its activation is crucial for sustaining redox balance which is a need for salt adaptation or tolerance response in plants (Nikalje et al., 2018). The activities of the target antioxidant enzymes (SOD, GPOX, APX and GR) induced in parallel with increasing salinity. SOD is known as one of the most powerful antioxidants in the system (Sales et al., 2013). We determined that the total SOD activity in the leaves of *Inula* was greatly increased in both NaCl concentrations. As indispensable antioxidative enzymes, GPOX and APX catalyze H₂O₂ to water and oxygen reactions under stress (Gong et al., 2014). Here, high dose salt treatment induced a greater increase in both GPOX and APX activities. APX and GR also support cellular redox state, by catalyzing H₂O₂ to water and oxygen coupled to ascorbate oxidation, and the reduction of glutathione (GSSG) to GSH by a cofactor (NADPH). In our experiments, most likely that the activation of these enzymes the plants was counteracting higher ROS levels. The results also supported a well-known notion that this enzyme system is a defense arsenal under high salinity. Our results are in conformity with findings that different concentrations of table salt induced SOD, CAT and APX activities in *Nitraria tangutorum* (Yang et al., 2010).

Besides appreciable contribution of the APX, GPOX and GR enzymes in counterbalancing the damages caused by ROS, supporting capabilities of metabolites (ascorbate and glutathione) is creditable as well. Considering, cellular GSH and ASC reductants involved in the defense against ROS it was not a surprise to monitor GSH and ASC increases in leaves of stressed *Inula* plants. Present findings form a well correlation between GSH and GR activity in *Inula* exposed to different salinity. Changes in GR are likely to modulate total glutathione pool. Indeed, GR activity boosted with increasing NaCl levels. The fact that NaCl stress further induces activity of GR in *Inula* plants, might show involvement of different metabolic routes as well as maintaining high GSH levels in the plant tissues. It is also possible that other antioxidant molecules modulate/compensate for fluctuations in the glutathione amount (Kaur and Bhatla, 2016). Our data indicated that different salt stress treatments elevated the level of ASC gradually. Higher endogenous ASC is critical for sustainable antioxidant capacity due to its role in preserving APX activity (Zhou et al., 2009). The results suggested that the increases in GSH and ASC were essential for tolerance to salt stress-induced oxidative stress.

Proline and soluble sugars are for osmotic adjustment in a cell, thus can improve growth under stresses (Singh et al., 2015). In our study, the accumulation of proline showed a

linear increase under two NaCl concentrations but this increase was higher in high salt (500 µM) in leaves of *Inula*. Similar to our data, two Mediterranean halophytes (*Plantago crassifolia* and *Inula crithmoides*) showed that high proline accumulation contributes to osmotic balance under 450-600 mM NaCl (Pardo-Domènech et al., 2016). Increased proline level under salinity in the present work, can be because of the up-regulation of proline synthesis and degradation enzymes simultaneously. Indeed, proline accumulation under stress is either because of the upregulation of proline biosynthesis gene expressions (*P5CS*, *P5CR*) or because of down-regulation of the target genes in its degradation pathway (PDH silencing) (Marco et al., 2015). Sugar accumulation is an osmotic balance pathway as well permitting plants to maintain their storage reserves (Smeekens, 2000). By detecting total soluble sugar increase in parallel with the salt stress, showed that both solutes helped buffering the redox potential of the cell and protected the cellular structures against NaCl. *Inula* plants may adopted some mechanisms such as synthesizing more osmolytes to rapidly adapt to that NaCl levels. The increase in osmolytes might also be related to the the ion content status of the tissue. Nikalje et al, (2018) have also proved that both proline and TSS were positively correlated with Na increase and thus possible that pathways such as protection of integrity of membranes and/or improved stability of ion transporter proteins or channels might contribute to salt tolerance as well.

Conclusion

Inula leaves exhibited well organized responses to higher NaCl related to the induction of high osmolytes (proline and TSS) and antioxidant systems to ease NaCl damage. Especially, activation of GPOX, APX and GR and non-enzymatic antioxidants (GSH and ASC) accumulation contribute to fight the injurious effects of oxidative stress as expected. Although *Inula* is not mainly categorized as a halophyte genus, non-halophyte species as used in this study are nevertheless quite robust against high salt and species grown in this niche are suitable for use in pharmaceutical industries thanks to their NaCl tolerance capacity.

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