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Research Paper

Accumulation of antioxidant Enzymes, Proline, and ions in Atriplex Nummularia Subsp... seedlings as adaptive mechanisms to salinity

Science

KEYWORDS : Salinity, Antioxidant, Proline content, Ions, Chlorophyll

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ABSTRACT

The study aimed to evaluate NaCl concentrations which affect chlorophyll and carotenoid pigments as physiological parameters best reflecting the response of Atriplex nummularia seedlings to salt stress, and investigate after which level the antioxidant enzymes, Proline, and ions contents start to increase and as adaptive mechanisms to salinity. The experiment was conducted on plants grown at 0 , 40, 80 and 120 mM of NaCl. The results show that, the content of chlorophyll-a and b, total chlorophyll (a+b) and carotenoids decreased significantly with increasing sodium chloride concentration but no differences between 0 mM and 40 mM NaCl treatments, all enzymes activities increased significantly with the exception of Peroxidase(POD) with increasing NaCl level and Sodium(Na) increase ,potassium(K+) decrease with salinity increasing. In conclusion A. nummularia used many mechanisms to overcome salinity stress but increasing catalase(CAT) activity , proline content and ion accumulation are more observable mechanisms in this study.

Introduction

Naturally occurring salty soils cover about one billion hectares, while areas of secondary Stalinization involve nearly275 million hectares (Flowers and Flowers, 2005). The secondary Stalinization occurrence is caused by long cultivation periods and irrigation as well as deforestation (Brini et al., 2009). Salinization is rapidly increasing on a global scale and currently affects more than 10% of agricultural land, which results in a decline of the average yields of major crops greater than50% (Wang et al., 2009). Salinity affects plant growth due to low potential of soil solution (osmotic stress), specific ion effects (salt stress), and nutritional imbalance, or a combination of all these stresses (Ashraf and Harris,2004). Osmotic stress has an immediate effect on plant growth strongly affecting its rate. This effect is stronger than ionic stress(Munns and Tester, 2008). A high concentration of sodium ions results in physiological drought, inhibiting plant water uptake by the plant. The effects of both drought and salinity stresses cause cell dehydration, an increase in plasma membrane permeability, a decrease in photosynthesis rate and generation of oxidative stress, which inducing a decrease in yield (Li et al., 2010;Kalaji et al., 2011). The reduced rate of photosynthesis increases the generation of reactive oxygen species(ROS) (Apel and Hirt, 2004). Salinity stress limits plant development by negatively affecting various biochemical reactions and physiological processes such as photosynthesis, antioxidant metabolism, mineral nutrient homeostasis, osmolytes accumulation and hormonal signalling (Misra and Gupta, 2005; Khan et al., 2012). The primary effects of salt stress are caused by the existence of ions in rhizosphere limiting extraction of water by roots and reduced plant growth, while the secondary effects are caused by ionic disequilibrium resulting in inactivation of enzymes, nutrient starvation, ionic toxicity in tissues and oxidative stress. Salinity stress induces over production of reactive oxygen species (Nazar et al., 2011; Khan et al., 2012) that causes inhibition of photosynthesis and disturbance in mineral nutrient status (Turan and Tripathy,2012).For mitigation of adverse effects of salinity stress, several strategies have been adopted and efforts are made to explore mechanisms for salinity tolerance. The accumulation of compatible compounds (osmolytes) is related to improvement of plant tolerance to salt because of its ability to overcome osmotic and water stress and maintain nutrients homeostasis and ion compartmentalization (Nazar et al., 2011; Khan et al., 2012).Proline functions as an osmolyte for the intracellular osmotic adjustment and its accumulation plays a critical role in protecting photosynthetic activity in plants under salt stress (Silva-Ortega et al., 2008).Halophytes tolerate saline conditions, by use of halophytic plants in pasture and fodder production on saline soils is the only economically feasible solution available (Khan and Duke. 2001). *Atriplex* species (saltbushes) are dominant in many arid and semi-arid regions of the world, particularly in habitats that combine relatively high soil salinity with aridity (Ortiz-Dorda et al. 2005).The aims of this study are to investigate changes physiological traits , ions accumulation in shoot and response of biochemical processes to salinity stress , to assess the salt tolerance mechanism in this species grown wild in arid areas.

Material and methods

***Plant Material and Treatments*:**

Seeds of *Atriplex nummularia* were without any pretreatment under green house conditions included 32 and 17°C at day and night, respectively with 12 h daylight and were watered when needed. After occurrence of the first seven true leaves on the seedlings, they were transferred to 30 cm rim diameter and 40 cm deep plastic pots containing washed sand. Then the seedlings were treated with varying salinity using sodium chloride solutions with concentration 0, 20, 40 and 80 of m/mol chloride sodium chloride. (mM NaCl) for four months.

***Measurement of chlorophyll*:**

Chlorophyll *a* and *b* content were estimated after extraction of the pigments from fresh leaves by, N, N-Dimethylformamide, extra pure solution. Chlorophyll content was spectrophotometrically measured using Thermo Scientific GENESYS™ 10 Scanning UV/Visible Spectrophotometer with one cuvette position (Genesis 10-S, Thermo Fisher Scientific, Madison, USA). chlorophyll *a* and *b* were measured the wavelengths of 664 and 646 and carotenoid at measured at 480 nm., respectively for each sample.

Estimation of antioxidant enzymes activity:

***Extraction*:** Fresh Leaf samples (0.5 g) were homogenized in 3 ml extraction buffer (0.1 M potassium phosphate buffer (pH 7.0), 1mMEDTA, 0.05% Triton X-100) in a pre-chilled pestle and mortar, centrifuged at 15,000 g for 20 min at 4°C. kept in ice during the course of homogenization The supernatant was used for the estimation antioxidant enzyme activities.

***Catalase (CAT EC 1.11.1.6)*:**

Catalase(CAT) activity was measured spectrophotometrically according to the method of Aebi(1983). The disappearanceof H_2O_2 at 240 nm in a reaction mixture containing 50 m M sodium phosphate buffer (pH 7.0),20 mM H_2O_2 and 5 µl of supernatant. The decrease in the absorption was followed for 1min at 240 nm, and 1 m mol H_2O_2oxidized ml^{-1} min^{-1}. activity was expressed in EU mg^{-1} protein min^{-1}.

***Superoxide dismutase (EC 1.15.1.1)*:**

Superoxide dismutase (SOD) activity was done by the method of Dhindsa et al. (1981). The homogenate, transferred to centrifuge tubes, was centrifuged at 10,000 × g at 4oC for 10 min. SOD activity in the supernatant was assayed by its ability to inhibit the photochemical reduction. Absorbance of samples along with the

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the blank was read at 560nm, using the UV-Vis spectrophotometer, against the blank. The difference of % reduction in color between blank and the sample was then calculated. Fifty percent reduction in color was considered as one enzyme unit (EU) and the activity was expressed in EU mg^{-1} protein min^{-1}.

***Ascorbate peroxidase (EC 1.11.1.11*:**
Ascorbate peroxidase (APX) activity was done by the method used by Nakano and Asada (1981). A 0.05g of the fresh leaf material was ground in 4ml of extraction buffer containing 50 mM phosphate buffer (pH 7.2), 1% PVP (w/v), 1% Triton × The supernatant was collected and used for the assay. The activity of APX was determined by the decrease in absorbance of ascorbate at 290nm using the UV-Vis spectrophotometer. APX activity was calculated by using the extinction coefficient 2.8mM^{-1} cm^{-1} and expressed in Enzyme Units (EU) mg^{-1} protein min^{-1}.

Measurement of *Proline content*:
The proline content in the leaf samples was estimated by the method of Bates et al. (1973).A 0.05g of fresh needle material was homogenated in 3ml of 3% (w/v) Sulphosalicylic acid,6 N-orthophosphoric acid and 1% (w/v) ninhydrin solution. The homogenate was centrifugedat 10,000 xg for 10min. The half ml of the supernatant was taken in a test tube to which were added 2ml of acid ninhydrin and 2ml of glacial acetic acid. The resultant mixture was boiled at 100oC in a water bath. The reaction was then terminated by putting the test tubes in ice bath. Then, 6ml of toluene was added to each tube and mixed vigorously on a cyclomixer for 10–15 sec in order to facilitate quick diffusion/ movement of chromophores from the aqueous phase to non-aqueous phase. The toluene layer (upper) was separated from the mixture and the absorbance reads at 520nm on a UV-Vis spectrophotometer (Modal Lambda Bio 20, Perkin Elmer, USA), using toluene as the blank. The corresponding concentration of proline

was determined against the standard curve processed in the same manner by using L-proline (Sigma). The amount of proline has been expressed in μg g^{-1} f.w.

***Ions determination*:**
Ion measurements were taken using 0.5 g of plant material that was boiled in 25 ml of water for 2 h at 100°C in a dry-heat bath. This hot-water extract was cooled and filtered using Whatman no. 42 filter paper, One ml of the hot-water extract was diluted with distilled water for ion analysis. Flame emission spectrophotometer JENWAY PFP7 model was used to determine the Na^+ and K^+ contents.

Results and discussion
The effect of salinity stress on chlorophyll and carotenoid
The content of chlorophyll-*a* and *b*, total chlorophyll (*a*+*b*) and carotenoids decreased significantly with increasing sodium chloride concentration although no differences between 0 mM and 40 mM NaCl treatments (fig. 2 and 3). This result confirms that,,the threshold level of salt stress is approximately 40 mM NaCl for most plants (Munns and Tester, 2008). The results of this study agreed with Khan (2003) who reported that saline stress slows down the production of photosynthetic pigments and Sharma and Hall (1991) mentioned saline stress induces degradation of β-carotene, which causes a decrease in the content of carotenoids. Our observation shows that increasing salinity affect negatively both chlorophyll and carotenoid so we can conclude that decreasing of carotenoid may lead to degradation of chlorophyll. Therefore Lima et al. (2004) explained degradation in carotenoid synthesis may imply degradation of chlorophylls.

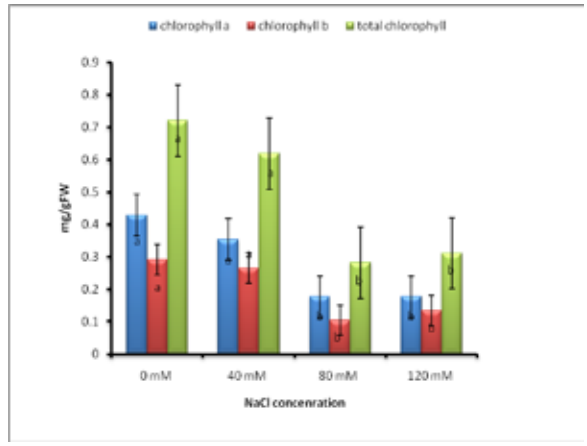

Fig. 1. . Effect of NaCl stress on chlorophyll content (expressed as mg/g fresh weight of leaf tissues) Bars represent mean ±standard error. Similar small letters within each salinity level are not significantly different ((P < 0.01)

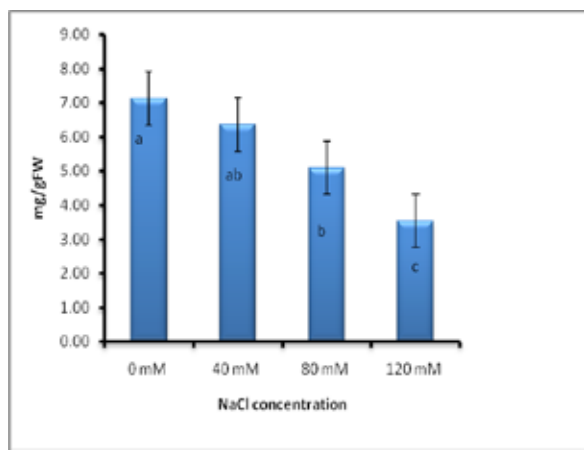

Fig. 2. Effect of NaCl stress on carotenoid content (expressed as mg/g fresh weight of leaf tissues) Bars represent mean ±standard error. Similar small letters within each salinity level are not significantly different ((P < 0.01)

Effects of salt stress on the activity Antioxidant enzymes
Effects of salt stress on the activity of SOD
The activities of SOD in different concentrations of NaCl had shown a ascending trend from 8.89 in control plant , 12.66 in plants treated with 20 mM NaCl , 14.42 in plants treated by 40 mM NaCl to reach the maximum activities 50.163 U g-1 FW in plants treated by 80 mM NaCl(Table 1). The increasing activities of SOD may be associated to salinity tolerance of this species to salinity stress which play a task to eliminate oxidative stress resulted from salinity stress as reactive oxygen species(ROS) as mentioned by Cheruth et al. (2009).

Effects of salt stress on the activity of Catalase (CAT)
The results of this study showed that the activities of Catalase (CAT) under salinity stress were significantly increased gradually with the increasing concentrations of NaCl compared to control, in higher NaCl concentration (80 mM) the enzymes activity was 3 times compared to control (Tble. 1). These results confirm the finding of Mallik et al. (2011) CAT activity increased significantly in response to the NaCl treatment . the rule of this enzyme was stated by Ho et al. (1998) CAT may possibly decompose hydrogen peroxide into water and oxygen to eliminate the peroxide in plants, and the higher activity of CAT was, the stronger salt tolerance mechanism.

Table 1: Effect of salt stress treatments on enzymes activities (EU g^{-1} FW)

Treatment	Superoxide dismutase (SOD)	Catalase (CAT)	Peroxidase(POD)	Ascorbate Perioxdisase(APX)
0 mM NaCl	8.893 [d]	136.723 [d]	8.617 [a]	0.530 [b]
40 mM NaCl	12.661 [bc]	158.740 [c]	8.740 [a]	0.790 [a]
80 mM NaCl	14.420 [b]	287.810 [b]	8.433 [a]	0.730 [a]
120 mM NaCl	50.163 [a]	379.286 [a]	9.697 [a]	0.827 [a]
P value	< 0.0001	< 0.0001	0.959	0.0020

Means with the same letter are not significantly different

Effects of salt stress on the activity of Peroxidase (POD)

Although the activities of Peroxidase (POD) were not affected under salinity treatments but the results show an increasing trendas salinity increase. This confirms pervious findings of Mandhania et al.(2006) who reported peroxidase (POD) could remove H_2O_2 in plants to prevent the cell membrane from oxidation by H_2O_2. consequently, improving the salt tolerance of plants .From the results of this study we note that SOD, CAT and POD activities increase in high salinity level (120 mM NaCl). This enzymes acting tother to reduce oxitive stress, this finding agreed with, Cheruth et al. (2009) who concluded superoxide dismutase (SOD) could catalyze disproportionation reaction of two superoxide radicals to generate O_2 and H_2O_2, and then the H_2O_2 was catalyzed and removed by POD and CAT, which was the response started first in the resistance to oxidative stress of plants (Cheruth et al. 2009).

Effects of salt stress on the activity of ascorbate peroxidase (APX)

Activities of ascorbate peroxidase (APX) significantly increased with increasing levels of salinity from 0.530 to 0.79 , 0.73 and 0.83 U g-1 FW in leaves of plants subjected to 40 mM, 80 mM, and 120 mM NaCl respectively. Many studies reported that activities of ascorbate peroxidase (APX) increase in *A. portulacoides* (Benzarti *et al.* 2012), *Salicornia brachiata* (Parida and Jha 2010) and *B. parviflora* (Parida *et al.* 2004) under salinity stress (Jithesh *et al.*2006). our findings indicate that APX activities playing a role in detoxification under salinity stress. Tolerate salinity stress by increasing activities of SOD, APX and POD activities salinity stress, various studies have also shown that genetically engineered plants containing higher levels of ROS scavenging enzymes, such as SOD, APX, and POX, have improved tolerance to abiotic stresses

The effect of salinity stress on proline contents

The effect of salinity stress on proline contents was highly significant The amount of proline increased with increasing NaCl concentration in irrigation water. the proline content estimated from fresh leaf tissues and was found higher (138.71, 110.35 and 29.63 µg/g FW) in treatment 120 ,80 and 40 mM NaCl respectively a proximately by 8 ,4 and 2 times compared to control plants (fig. 3).Our observations indicate that the increasing salinity stress leads to increasing in proline content These results agree with the earlier study of Najafi et al.(2006) who reported accumulation of free proline in in *Pisum sativum* under salinity stress and also agree with the recent study of Ashfaque et al.(2014) who found accumulation of proline in *Triticum aestivum* under salinity stress. The accumulation of proline in this study may improve plant tolerance to oxidative stress resulting from salinity. Hoque et al. (2007) demonstrated positive correlation between proline accumulation and enhancement of antioxidant enzyme activity in tobacco cells submitted to salinity stress. Proline plays a protective function against salinity stress in plants . It acts as a compatible osmolyte, enzyme protectant, free radical scavenger, cell redox balancer, cytosolic pH buffer and stabilizerfor subcellular structures (Kishor et al., 2005; Verbruggen and Hermans, 2008).

The effect of salinity stress on ions accumulation

Salinity significantly affected content of plants Na^+ (P<0.0001), K^+ (P<0.0001), potassium decreased significantly with increasing salt concentration (fig.). While the sodium content of plants increase as salinity stress increase by 2, 3, and 3.3 times in 4o, 80 and 120 mM NaCl compared to control (fig.4). Also sodium potassium ratio increase as salinity increase. Halophytes have the ability to accumulate ions, such as Na^+ in the vacuole so that the cytoplasm is maintained at substantially low ion concentrations, thereby avoiding inhibition of metabolic processes (Munns, 2002). Halophytes utilize internal inorganic ions such as Na^+ and Cl^- for osmotic adjustment, by sequestering them in vacuoles with associated synthesis and accumulation of organic / compatible solutes in the cytoplasm (Flowers and Colmer 2008; Hussin *et al.* 2013).The linear increase in shoot Na^+ and Cl^- ions in *L. stocksii* seedlings reported in an earlier study (Zia *et al.* 2008) also supports this assumption. Similar results have been reported for co-occurring species such as *S. fruticosa* (Hameed *et al.* 2012).Halophytes survive salinity by sequestering salts in vacuoles and accumulating organic osmolytes in their cytoplasm (Flowers and Colmer 2008; Nedjimi 2014).the results of this study in with pervouis finding such as *Atriplex nummularia* is among a group of halophytes that complete their life cycle at high salinity levels and have the ability to accumulate high concentrations of micronutrients much greater than the required (Ramos et al., 2004). But because of the toxic effect of ions, salt sensitive metabolic processes take place in the cytoplasm, while the salt necessary for osmotic adjustment is stored in vacuoles, so the ion compartmentation is the key salt tolerance mechanism (Flowers and Flowers, 2005).Ion uptake and its root to shoot transport in plants grown under salinity usually reflects their tolerance to salt stress. Increasing the NaCl content in soil causes an increased uptake of Na^+ by plants. Sodium ions are competitive for K^+, so their uptake noticeably decreases (Turan et al., 2010; Babu et al., 2012).

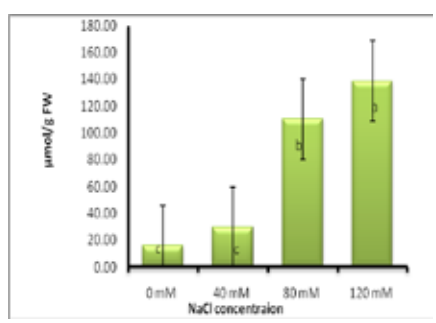

Fig. 3. Effect of NaCl stress on proline content (expressed as mg/g fresh weight of leaf tissues) Significant difference between means at the (P < 0.01) level determined by (ANOVA).

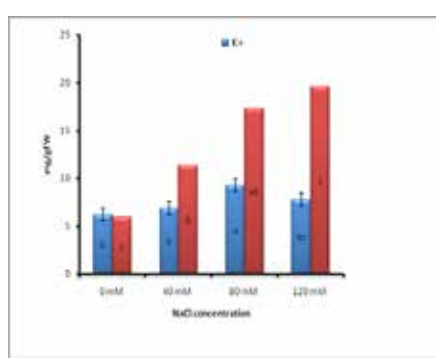

Fig. 4. Effect of NaCl stress on the concentration of ions (K and Na) mg g^{-1} FW (expressed as mg/g fresh weight of leaf tissues) Significant difference between means at the (P < 0.01) level determined by (ANOVA).

Conclusion

We can conclude that the NaCl concentration in the range 80–120 mM affect chlorophyll and carotenoid contents *A. nummularia* negatively as physiological response. The salt tolerance mechanisms of *A. nummularia* at seedlings stage are increasing antioxidant enzyme activities to over oxidation effect and accumulating proline as a compatible osmolyte, enzyme protectant, free radical scavenger, cell redox balancer, cytosolic pH buffer and stabilizerfor subcellular structures and ion for osmotic adjustment, and avoid ion toxicity by sequestering them in vacuoles.

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