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Full Length Research Paper

Biochemical response of two *Atriplex* species (*Atriplex halimus* L. and *Atriplex canescens* (Pursh) Nutt.) under salt stress conditions

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Soil salinization is an environmental problem that many world regions must fight; it is one of the major plants abiotic stress factors. In fact, in addition to mineral imbalance and toxicity of certain ions, salinity causes, in consequence of osmotic pressure increase, water absorption decrease which leads to stress. In the current study, the behavior and response of two *Atriplex* species, *A. halimus* L. and *A. canescens*, (Pursh) Nutt. under effect of high salt concentrations (400 and 600 meq of NaCl + $CaCl_2$) and seawater at 25, 50 and 100% dilutions, were studied, using proline as metabolic marker in relation to resistance to salinity. Three-month-old *Atriplex* plants were subject to the various salinity levels. The amount of proline was determined from different plant organs after three weeks stress period. The results revealed a metabolic variability characterized by proline accumulation as function of the species, plant organ, and the concentration and nature of salt treatment. In general, the accumulation of proline within the plant occurs in proportion to the medium salt concentration. Indeed, on one hand, the recorded proline contents show that this accumulation is very important in *Atriplex canescens* (Pursh) Nutt, compared with *Atriplex halimus*. On the other hand, these contents are high in leaves of both species in presence of high NaCl + $CaCl_2$ and seawater concentrations.

Key words: Atriplex, salt stress, proline, tolerance, halophyte.

INTRODUCTION

The recorded global climate changes have certainly generated ecological mutations which, particularly contributed to intensification of desertification process, especially affecting arid and semi-arid regions. Within these regions, aridity has a permanently determinative effect due to the precipitation deficit (Munns et al., 2006) accompanied with high water loss by evapotranspiration (Epstein et al., 1980) and salt-rich water irrigation of culture, which is often non controlled (Yamguchi and Blumwald, 2005).

Salinity affects 100 million ha of the world's land area (Munns et al., 2006), which provide a third of the world's food supply. In Algeria, saline soils cover an area of 3.2 million ha (Hamdy, 1999). According to Le Houerou (2000), Algeria is among the most affected countries by salinization. The presence of salts in the soil affects plant physiological mechanisms and constitutes a major constraint to plant growth and hence limiting crop yield (Levigneron et al., 1995 (check the year publication as per reference section it is 1956); Ben Ahmed et al., 2008). Therefore, some species have disappear (Munns,
2002), and others are endangered (Charmard, 1993). With such disturbances and constraints, only plants.
called halophytes can resist and adapt, generating resistance and/or tolerance mechanisms towards these restrictions (Sambatti and Caylor, 2007). Contrary to glycophytes, halophytes naturally grow and develop on saline soils. However, during their development process different species show various degrees of tolerance salinity. In response to salt stress, halophytes induce tolerance mechanisms that contribute to adapt to osmotic

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and ionic stresses caused by high salinity (Lee et al., 2008). A large number of metabolites are involved in this adjustment (Munns, 2002). Among these organic molecules that are often associated with plant salt tolerance are nitrogenous molecules such as glycine and betain (Gerard et al., 1991) organic compounds such as soluble sugars (Ottow et al., 2005) and amino acids such as proline (Morant-Manceau et al, 2004). Many authors have reported its role as osmoticum (Taji et al., 2004; Turan et al., 2007). Proline accumulation is a common metabolic response of plants to face environmental constraints. Among these halophytes, the genus *Atriplex* is well adapted to harsh environmental conditions, and is characterized by high diversity.

This genus includes over 400 species. Most of them grow in regions with annual rainfall ranging from 200 to 400 mm/year (Abu-Zanati et al., 2004) and are dominant in many arid and semi-arid areas of the world (Nedjim et al., 2006). They are a useful material for the identification of physiological mechanisms and genes involved in resistance to abiotic stresses (Wang and Showalter, 2004). With this background the present work has been conducted with the objective to highlight one of the mechanisms of resistance to salinity through endogenous proline analysis for two species of *Atriplex*, *Atriplex halimus* L. and *Atriple canescens* (Pursh.) Nutt. subjected to an increasing salinity stress of seawater or combined salt solutions. These selected species are xerohalophytes of great agricultural and ecological interests, and allow adequate valorization of generally marginalized areas (Le Houerou, 2000; Chisci et al., 2001).

MATERIALS AND METHODS

Plant material

The used seeds were collected from the station of El Mesrane, located 300 km south to Algiers. Two species were selected for the experimentation: *A. halimus* L. (native) and *A. canescens* (Pursh.) Nutt. (Introduced from North America).

Methods and culture conditions

The Ninety seed of each species were peeled manually and their bracts were removed. They were sterilized in a 5% sodium hypo-chlorite solution for 3 min and then rinsed abundantly with distilled water. Afterwards, they were placed to germinate in cells filled with mould and watered with distilled water every two days until plants reached the stage of five to six leaves. The obtained plants were then individually planted out into plastic pots filled with a mixture of sterilized sand and mould (2 v/v) and with a layer of gravel in the bottom to ensure drainage. The experiment was carried out in a semi-controlled greenhouse, temperatures was 20°C during the day and 15°C during the night, and relative humidity ranged from 60 to 70%. The 180 pots were watered every two days with Hoagland nutrient solution, (Hoagland and Arnon, 1938) diluted to 1/1000 and brought to 30% of field capacity for two months, and 60% for one month until stress application.

For both species, plants aged four months were divided into six lots, each lot contains fifteen repetitions. These plants have suffered a salt stress by watering with sea water diluted or pure, or with NaCl + $CaCl_2$ in nutrient solution at 60% filed capacity.
Stress is provoked as follows:

1. 45 plants were stressed with sea water, divided into three batches of 15 plants that are either treated with sea water diluted to 25 or 50% of the nutrient solution, or undiluted (100% sea water),
2. Two batches of 15 plants were treated with NaCl + $CaCl_2$ at 400 and 600 meq/L of the Nutrient solution,
3. Control plants (15) are watered with nutrient solution at 60% capacity retention of the substrate.

After the stress, which lasted eight days, the plants are removed, the aerial part is separated from the underground one. Leaves, roots and stems were weighed and then dried in an oven set at 80°C for 48 h. Dry matter was then weighed and placed in bottles closed with plasma plugs. Proline amounts were determined by the method of AOAC (1955) modified by Nguyen and Paquin (1971). They are expressed in µmoles of proline per 100 mg of DW^{-1} in reference to a calibration curve after reading the optical density at 505 nm, using a spectrophotometer.

Analysis of variance (ANOVA), was done using the ASSISTAT version 7.5 beta 2008 and mean comparison procedures, was performed to detect differences between treatments. Mean separation tests with Fisher's least significant difference (LSD) ($P < 0.05$).

RESULTS

Effect of seawater on the endogenous proline

The results show that among the two species, proline content was higher in organs of *A. halimus* L. supplied with sea water, in comparison with the controls (Table 1). Besides, the increase in salinity level, there was an increase, in proline content was noticed. This accumulation is higher in leaves especially when plants are stressed at 50 and 100%. The plants watered only with nutrient solutions have identical levels of proline in stems and leaves (0.51 and 0.53 µM.100 mg DW^{-1}). Whereas in roots, proline amount is about 0.35 µM.100 mg DW^{-1} The leaves had significantly higher proline than other organs ($P < 0.01$) in both control plants and those treated with different concentrations. These levels are 0.75, 0.91 and 1.1 µM.100 mg DW-1 respectively for plants stressed with sea water at 25, 50 and 100%. As for *Atriplex canescens* (Table 1), on one hand, that amount of proline increases from roots to leaves in non-stressed conditions. Leaves accumulated significantly lesser proline (0.54 100 mg DW^{-1}) compared to other organs. On the other hand, this content increased in leaves and stems as the degree of increased stress level. In leaves, the proline content changes rapidly in proportion to salt concentration and double under pure sea water supply. On the contrary, in the roots, this compound decreases significantly in plants stressed with 25% of sea water in comparison with the controls. This amount notably increases with 50% sea water treatment and then decreases again in the most concentrated conditions to

Table 1. Effect of salt stress (seawater) on proline content (µmole 100 mg^{-1} DW) in the organs of *Atriplex halimus* L. and *Atriplex canescens* (Pursh) Nutt.

Species	Organ	Control	Seawater 25%	Seawater 50%	Seawater 100%	
Atriplex halimus	Leaves	0.53±0.19^{c}	0.75±0.28^{b}	0.91 ±0.26^{ab}	01.1 ±0.24^{a}	**
	Stems	0.51± 0.2^{b}	0.66±0.26^{ab}	0.73±0.32^{a}	0.77± 0.33^{a}	**
	Roots	0.35± 0.11^{c}	0.48±0.16^{bc}	0.54± 0.14^{b}	0.68± 0.25^{a}	
Atriplex canescens	Leaves	0.54±0.05^{c}	0.85±0.15^{b}	0.93 ±0.18^{b}	1.25± 0.19^{a}	**
	Stems	0.69±0.17^{b}	0.76±0.20^{ab}	0.80±0.17^{ab}	0.86±0.08^{a}	*
	Roots	0.75±0.15^{b}	0.68±0.21^{b}	0.90±0.18^{a}	0.78±0.14^{ab}	*

Each value represents an average of 15 samples ± SD. Values bearing the same letter in each line (parameters) are not significantly different at p <0.05. **= 1% significance level, *= 5% significance level.

Table 2. Effect of salt stress (NaCl + $CaCl_2$) on proline content (µmol 100 mg^{-1} DW) in the organs of *Atriplex halimus* L. and *Atriplex canescens* (Pursh) Nutt.

Species	Organs	Control	400 meq NaCl+ $CaCl_2$	600 meq NaCl+ $CaCl_2$	
Atriplex halimus	Leaves	0.53± 0.19^{b}	0.90±0.25^{a}	0.95±0.27^{a}	**
	Stems	0.51± 0.21^{b}	0.61±0.28^{b}	0.88±0.31^{a}	**
	Roots	0.35± 0.11^{b}	0.65±0.17^{a}	0.71±0.22^{a}	**
Atriplex canescens	Leaves	0.54 ±0.05^{c}	0.94 ±0.10^{b}	1.06 ±0.12^{a}	**
	Stems	0.69 ±0.17^{b}	0.74 ±0.09^{b}	0.92 ±0.09^{a}	**
	Roots	0.75 ±0.15^{a}	0.79 ±0.22^{a}	0.72 ±0.20^{a}	

Each value represents an average of 15 samples ± SD. Values bearing the same letter in each line (parameters) are not signif icantly different at p <0.05. **= 1% significance level, *= 5% significance level.

become comparable to those of control roots.

Effect of NaCl + $CaCl_2$ on endogenous proline

A.halimus plants under controlled condition, preferentially accumulates proline in the leaves and stems in similar amounts (0.53 and 0.51 µM.100 mg DW^{-1}, respectively), whereas in roots it was 0.35 µM. 100 mg DW^{-1} (Table 2). As for the plantlets treated with combined salts, the amount of proline increases gradually as stress level was increased. In leaves of *A. halimus* there was more accumulation of proline than the other organs in both control plants as well as in stressed ones. Under the control condition proline content was 0.53 µmoles.100 mg DW^{-1}, and it increased to 0.90 and 0.95 µmoles.100 mg DW^{-1} when it was treated with 400 and 600 meq NaCl + $CaCl_2$, respectively .

Besides, the amount of proline in roots is more than double (0, 65 and 0.71 µM.100 mg DW^{-1}) in comparison with the controls (0.35 µM. 100mg DW^{-1}). The highest proline content in the stem (0.88 0.35 µM. 100 mg DW^{-1}) was recorded with the stress at 600 meq.

When *A. canescens* was treated with NaCl + $CaCl_2$ with different concentration there was significant difference was noticed for proline content in leaves and stems (Table 2). The data revealed that, in absence of salts, proline is more concentrated in the roots followed by stems and leaves. In plants treated with different concentrations of salt, proline increased significantly in leaves and stems compared with the controls. It was noticed that the amount of proline changes regularly among leaves and stem under 600 meq of NaCl + $CaCl_2$ reaching a maximum value which is two times higher than the control.

DISCUSSION

Present investigation demonstrated the capacity for synthesis and accumulation of proline in *A. halimus* L. and *A. canescens* under the salinity stressed with sea water at 25, 50 and 100% and combined salts NaCl + $CaCl_2$ at 400 and 600 meq. Even in the absence of salts, both species synthesize and accumulate this amino acid with widely varying concentration. This accumulation varies from one organ to another, from one species to another, depending on the nature and intensity of stress.

Although the roles attributed to the accumulation of proline on stress are multiple, its role in plant adaptation to stress is still a subject of debate. The accumulation of

proline has been demonstrated in halophytes (Hu et al., 1992), subjected to salt stress such as in *A. halimus* (Bidai, 2001; Martinez et al., 2005; Ben Hassine et al., 2008), *Sesuvium portulacastrum* (Slama et al., 2007; 2008) and *Arabidopsis halophila* (Ghars et al., 2008) or subjected to drought stress (Ben Hassine et al., 2008). This ability of plants to the synthesis and accumulation of proline is also observed in many glycophytes, such as barley (Hassani et al., 2008), durum (Taji et al., 2004), tomato (Hernandez et al., 2000) and *Arabidopsis thaliana* (Ghars et al., 2008).

The results shown that in *A. halimus* stressed with sea water or combined salts (NaCl + $CaCl_2$), accumula-tion of proline occurs first in the root and moves towards the stems, and finally to leaves of both control plantlets and the stressed ones. This amino acid is mainly concentrated in leaves.

In the stems, the synthesis of proline is very slow. The recorded amount is almost the same under sea water stress at 50 and 100%, contrary to the salt-stressed plants with combined salts which show a high content at high salinity (600 meq). Indeed, this amino acid increases significantly under NaCl + $CaCl_2$ salinity. In roots also, the accumulation of this amino acid increases significantly with salinity.

In *A. canescens*, proline also accumulates in leaves when stressed by sea water at 25, 50 and 100% or treated with 400 and 600 meq of NaCl + $CaCl_2$. In treated plantlets, this accumulation begins in roots and moves towards stems and then leaves. However, in control plants, the direction is leaves, stems and finally roots. In *A. canescens*, highly saline environments (100% sea water and 600 meq) cause a decrease in endogenous proline content in roots. In leaves and stems, this amino acid increases with salt environment concentration.

Present investigation was in accordance with those of Bidai (2001) who reports that in *A. halimus* L. stressed with NaCl + $CaCl_2$ (400 and 600 meq) and sea water (50 and 100%), proline evolution occurs, and mainly increases towards the foliar parts of the plant.

Likewise, Slama et al. (2007) noticed a sharp increase in proline accumulation in leaves of a perennial halophyte, *S. portulacastrum*, stressed for 12 days with 100 mM NaCl, which is about three times higher than the control.

Ben Hassine et al. (2008) show that this amino acid accumulates in *A. halimus* seedling leaves having undergone a salt stress by adding NaCl 160 mM during 10 days. Ould El Hadj-Khelil (2001) noticed that in tomato plants under stress of 200 mM NaCl, the accumulation of proline is greater in young leaves than in the basal ones, and application of a second salt treatment generates a further increase in the amino acid. This increase is more important when leaf tissues are young and salinity is high.

Present investigation is in conformity with the earlier findings of De-Lacerda et al. (2001), who noticed that exposure of two genotypes of sorghum, one sensitive and the other tolerant, to 100 mM NaCl, causes an increase in proline in all plant parts of the two genotypes, especially in older leaves: 3rd and 4th leaves from the apex. Pakniyat and Armion (2007), confirm the accumulation of proline in leaves of some slat-resistant beetroot genotypes.

Changes in accumulation of proline in relation to different organs, and nature and intensity of the stress, reflects, in our experimental conditions, the resistance ability of the two studied *Atriplex* species to various culture media salinity levels. Our results confirm the spread and accumulation of this amino acid in stems, roots and leaves of *A. canescens* and *A. halimus* L., depending on the salts environment concentration. Di Martino et al. (2003) show that some plant species are capable of synthesizing organic compatible solutes, such as glycinebetaïnes and proline, and the kinetic accumulation depends on the stress intensity and its exposure duration.

Many studies show that proline migrates to the leaves especially in berseem (Ben Khaled et al., 2003) and in some cultivars of wheat (Goudarzet and Pakniyat, 2009). However, in other species, proline would be located in roots (such as corn) (Rodriguez et al., 1997).

According to De-Lacerda et al. (2003) and Kavi et al. (2005), significant amounts of proline accumulation in higher plants, are normally a response to salinity to protect the cell by balancing the osmotic pressure of the cytosol vacuole and the external environment. This accumulation is due to the compartmentalization of the amino acid, from which results the expression sites of plant resistance to salt stress (Belkhodja and Benkablia, 2000). In addition, the transport of this amino acid from the source (synthesis organ) to the site of resistance appears to be an important factor in the plant acquisition of resistance to salinity (Paquin et al., 1986).

Different opinions were put forward on the role of proline in resistance to salt stress. Stewart and Lee (1974) reported that, there was a neutralizing effect in the compartments of the cell vis-à-vis the ionic compounds, especially in the vacuoles. According to Qian et al. (2001), its accumulation contributes to the acquisition of resistance through the osmotic adjustment controlled by proline. It could also intervene in the regulation of cytoplasm pH (Denden et al., 2005) or as nitrogen and carbon reserve plants use after the period of stress (Hare et al., 1999; Zerrad et al., 2006). It has been reported that this compatible osmolyte stabilizes cell membranes by interacting with phospholipids, and functions as a collector of hydroxyl radicals, a source of energy and nitrogen (Kavi et al., 2005).

In regard to the influence of different organs, this study shows that whatever the nature of stress, stems, roots and leaves of *A. canescens* synthesize and accu-mulate higher nitrogen compound than in *A. halimus* when treated with NaCl + $CaCl_2$ with different concen tration. In

controls, the proline content is identical in leaves of the two species, but it is higher in stems and roots of *A. canescens*. These results are in accordance with those noticed by Glenn et al. (1992, 1994, 1996).

According to Glenn and Brown (1998), tolerance to water deficit and salt stress in *A. canescens* is related to a common mechanism of absorption of Na^+, which is used directly for osmotic adjustment.

As proline is one of organic osmolyte that accumulates in higher plants in response to environmental stresses (Hanson et al., 1977; Dix and Pearce, 1981). Numerous studies have shown a positive relationship between the accumulation of proline and stress tolerance of plants, but some believe that increasing the concentration of this compound under stress is a consequence and not an adaptive response to stress, then, as a sign of metabolic disruption (Dix and Pearce, 1981).

Nanjo et al. (2003) noticed a negative relationship between salt tolerance and accumulation of proline. The accumulation of proline is a consequence of cell homeostasis disturbance and/or an increase in the use of photosynthesis products for the proline biosynthesis to the detriment of plant growth. Huber (1974) has also demonstrated that salt generate inhibition of pyrroline-5-carboxylate dehydrogenase, an enzyme involved in the degradation of proline and improves pyrroline-5-carboxylate.

Conclusion

In the light of this investigation, *A. halimus* L. and *A. canescens* (Pursh) Nutt. the presence of abiotic stress causes an accumulation of proline. However, quantitative variation depends upon the species, the organ and type of the provoked stress. This variation is probably related to the role of this amino acid at the cellular level and its involvement in osmotic adjustment. On the other hand, we found that proline is accumulated mainly in leaves at higher contents in *A. canescens* (Pursh) Nutt.

According to our findings, the two Atriplex species are well adapted to arid environment and are able to grow in the presence of different salinity concentrations ranging from 150 to 600 mM NaCl, which is higher than sea water salt concentration. Present investigation also indicated that it is possible to improve the ability of species to grow under high saline condition and resist it by inducing an accumulation of proline. This element appears to contribute to osmotic adjustment of *Atriplex*. Thus, it appears that both species use the same tolerance strategy towards salt stress carboxylate reductase involved in proline synthesis.

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