

ORIGINAL ARTICLE

Biochemical changes of *Rosmarinus officinalis* under salt stress

Kiarostami\*, Kh., Mohseni, R., Saboora, A

*Department of Biology, Faculty of Science, Alzahra University, Tehran, Iran*

\*Email- [su\\_kiarostami@yahoo.com](mailto:su_kiarostami@yahoo.com)

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Plant growth parameters not affected by low concentrations of NaCl, but it decreased with higher concentrations. The content of photosynthetic pigments decreased at all salinity levels. The Na<sup>+</sup> content of leaves increased, whereas the K<sup>+</sup> content decreased with the progressive increase in NaCl concentration. Salinity increased proline and malondealdehyde contents. Stress induced by NaCl caused an accumulation of proline, total phenolic and antioxidant in rosemary plant.

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*Rosmarinus officinalis*, related to the lamiaceae family of plants. Rosemary is a native of the Mediterranean region of Europe and the Near East. Today rosemary is cultivated in nearly all countries as medicinal and ornamental plant. The herb rosemary has been used as a food spice and as a medicine since ancient times (Moss, et al 2003) Rosemary is used as an antispasmodic in real colic and dysmenorrhoea, in relieving respiratory disorders and to stimulate growth of hair. Rosemary extract relaxes smooth muscles and has choleric, hepatoprotective and antitumorogenic activity (Al-Sereiti et al 1999). It is also antiviral, inhibiting even

HIV protease at very low concentrations (Aruma et al 1996).

The potent antioxidant properties of rosemary extracts have been attributed to its phenolic compounds, mainly rosmarinic acid and diterpenes carnosic acid and carnosol (Sergi Munné-Bosch and Leonor Alegre 2002, Troncoso et al 2005). Rosemary is grown in many parts of the world including saline area. Salinity is one of the most important factor limiting plant growth and yield. About 25% of world's total area (including 15% of Iran's lands)

are saline. These areas contain about 33% of worlds and 50% of Iran's irrigated lands respectively (Kamkar et al 2004). In saline lands where salinity reduce agriculture area, the search for new crop cultivation is interesting in agricultural research .Salt tolerant plants can be used to produce economically important materials such as antioxidants and essential oils. Environmental condition and genetic influence plants secondry products (Aziz Eman et al 2008 , Peter, 2004). We investigated the effect of salt stress on rosemary in order to cultivation this plant at saline area and better landscape use in Iran.

## MATERIALS AND METHODS

**Sample preparation:** A pot experiment was conducted in Alzahra University ,Tehran, Iran. Rosemary plants were grown in greenhouse  $25 \pm 2^\circ\text{C}$  under 16 h photoperiod with supplementary light and irrigated three times a week. After two months the plants were treated with 50, 75, 100 and 150 mM NaCl. Treatments initiate with 20 mM NaCl at first week. Every week the pots were treated with 25 mM NaCl . Treatments were completed during three consecutive weeks and the pots irrigated with tap water after every three days. 7 day after the last day of salt treatment the plants were harvested and analyzed.

### Analysis of growth

Three plants per each replication were randomly harvested for growth measurement. Plants height and fresh weight were recorded. The fresh weight of shoots were measured immediately. The dry weight were measured by drying the plants at  $80^\circ\text{C}$  to give a constant weight.

### Photosynthetic pigment determination

Chlorophyll a, chlorophyll b and carotenoids were extracted in 80% acetone and were determined

spectrophotometrically by Lichtenthaler formula. ( Lichtenthaler 1994)

### Proline determination

Proline was quantified by using ninhydrin reagent and measured according to Bates et al (1973). Proline was extracted from 0.5 g of leaf in 10 ml of 3% sulfosalicylic acid. Two ml of extract was reacted with 2 ml acid- ninhydrin and 2 ml of glacial acetic acid for 75 min at  $100^\circ\text{C}$ . The reaction was terminated in an ice bath . The reaction mixture was extracted with 4 ml of toluene and vortexed. The absorbance of toluene layer was spectrophotometrically determined at a wavelength of 520 nm. Concentration was determined from a standard curve and calculated on a fresh weight basis ( $\mu\text{mol proline /g fw}^{-1}$ ).

### Cation analysis

The cations ( $\text{K}^+, \text{Na}^+$ ) were determined in dried ground leaves by Flame photometer (Jenway , Model PFP7) after acid digestion (Yash,p.K. 2000).  $\text{K}^+$  and  $\text{Na}^+$  contents were quantified based on a standard curve.

### Determination of malondealdehyde

Malondealdehyde (MDA) levels were measured by the method of Heat and Packer (1968)

0.5 g of fresh leaf was homogenized in 2.5 mL of 0.1% (w/v) trichloroacetic acid (TCA), and centrifuged at 1000 rpm for 5 min. For every 1 ml of supernatant 4 mL of 20% TCA containing 0.5% TBA was add. The mixture were incubated for 30 min at  $95^\circ\text{C}$ , chilled on ice, and centrifuged at 4000 g for 10 min. the absorbance of the supernatant was measured at 532 nm using a Philips PU 8620 spectrophotometer. Unspecific absorption at 600 nm was subtracted from the 532 nm values. The concentration of MDA was calculated by using a molar extinction coefficient of  $156 \text{ mM}^{-1} \text{ cm}$ .

### Phenolic compounds

phenolics were extracted by the method of Conde with some modification( Conde et al 1995).1 g of

dried powdered sample was extracted with 20 ml methanol 80 % at 70 °C in water bath for 3 h. After filtration the solvent was evaporated to dryness using a rotary vacuum evaporator and the residue re dissolved in 10 ml of water. The aqueous solution used for quantitative determination with UV/VIS spectrophotometer.

The reaction mixture was prepared by mix in potassium ferricyanid 83.4 mM 4- aminoantipiren 20.8 mM, NaHCO<sub>3</sub> 0.25 mM in a ration of 100:100:700 µL. 100 µL of sample was then added . Absorbance was measured using spectrophotometer at 510 nm and plotted against a standaed curve obtained from gallic acid (Faust et al 1967).

#### **Antioxidant assay**

For DPPH assay, the method of Oktay was adopted with some modifications .4mL of a 0.004 % solution of DPPH in methanol 80% was mixed with 1ml of various concentrations of methanolic extract (0.02–0.1 mg/mL). The reaction mixture was vortex and left in the dark at room temperature for 30 min. The absorbance of the mixture was measured spectrophotometrically at 517 nm. The ability to scavenge DPPH radical was calculated by the following equation:

$$(RSC\%) = (A_{\text{blank}} - A_{\text{sample}}) / (A_{\text{blank}}) \times 100$$

where (RSC %)= DPPH radical scavenging activity (%)

Ab<sub>S<sub>blank</sub></sub> is the absorbance of DPPH radical + methanol (1 ml DPPH and 3 mL methanol) , Abs<sub>sample</sub> is the absorbance of DPPH radical + sample extract

IC<sub>50</sub> Value was also calculated ( Oktay et al, 2003).

#### **Statistical analysis**

All data were analysed using SPSS software, when analysis of variance showed significant

difference between means, Duncan's multiple range test was applied to compare the means at p < 0.5.

## **RESULTS and DISCUSSION**

### **Growth analysiss**

The NaCl salinity reduced growth of rosemary plants and growth parameters decreased as salt stress increased. Growth inhibition is a common response to salinity ( Pardia 2005). The inhibitory effects of salt stress on growth parameters were also reported by other researchers using various plants ( Shaddad and Heikal 1982, Soussi et al 1998, Chaparzadeh et al 2004, Jenifer. and Franklin 2002 ).

Salt stress can lead to stomatal closure, which reduce CO<sub>2</sub> assimilation in the leaves and inhibit carbon fixation and lead to reduction in photosynthesis rate and plant growth.

### **Photosynthetic pigments**

Salinity caused a decrease in Chlorophyll and carotenoid content in rosemary plant.

Photosynthetic pigments content decreased progressively as salt stress increased. Salinity had more effect on Chl b content. The lowest pigments levels were observed in plants treated with 150 mM NaCl.

Salt stressed plants contained less carotenoid than the controls , however there was no significant difference in carotenoid content between various concentration of NaCl.

Similarly Munjal and Gosswami(1995), Weimburg (1975) Soussi et al (1998 ) , Xu ,Xinwen et al( 2008) and Jenifer and Franklin (2002) reported that salinity caused a decrease in chlorophyll and carotenoids level in other plants.

**Table 1.** Salt stress effects on rosemary growth parameters and biochemistry

NaCl mM	0	50	100	150
Freshweight (g)	23.66 ±0.5 <sup>a</sup>	22.03± 0.25 <sup>a</sup>	19.5±0.29 <sup>a</sup>	13.26±.06 <sup>bc</sup>
Dry weight (g)	1.7±0.21 <sup>a</sup>	1.5±0.26 <sup>a</sup>	0.71± 0.2 <sup>b</sup>	0.33±0.4 <sup>b</sup>
Plant height(cm)	23.66±2.7 <sup>a</sup>	22.03±0.37 <sup>a</sup>	19.58±0.74 <sup>a</sup>	13.26±3.1 <sup>cb</sup>
Na <sup>+</sup> %	5.27±.13 <sup>c</sup>	5.7±.12 <sup>de</sup>	7.34±.17 <sup>c</sup>	10.44±.31 <sup>a</sup>
K <sup>+</sup> %	1.01±.05 <sup>a</sup>	0.843±.06 <sup>ab</sup>	0.76±0.07 <sup>bc</sup>	0.31±0.1 <sup>d</sup>
K <sup>+</sup> /Na <sup>+</sup>	0.19±.01 <sup>a</sup>	0.14±.01 <sup>b</sup>	0.1±0.01 <sup>c</sup>	0.3±0.1 <sup>c</sup>
Chla (mg/g fw)	16.64±0.8 <sup>a</sup>	17.83±0.3 <sup>a</sup>	11.77±0.8 <sup>b</sup>	7.73±1.1 <sup>c</sup>
Chl b(mg/g fw)	18.11±2.3 <sup>a</sup>	9.49±0.5 <sup>b</sup>	7.96±2.9 <sup>bc</sup>	3.57±0.53 <sup>c</sup>
Chl t(mg/g fw)	36.47±0.82 <sup>a</sup>	26.6±1.1 <sup>b</sup>	17.96±1.38 <sup>d</sup>	10.68±1.39 <sup>f</sup>
Chl b/ chl a	1.07±0.22 <sup>a</sup>	1.47±0.06 <sup>b</sup>	2.05±1.1 <sup>a</sup>	2.04±0.33 <sup>a</sup>
carotenoids(mg/g fw)	11.64±2.3 <sup>a</sup>	8.72±0.54 <sup>ab</sup>	8.85±2.91 <sup>ab</sup>	7.14±0.53 <sup>b</sup>
proline µmol /g fw	0.12±0.01 <sup>c</sup>	0.28±0.003 <sup>a</sup>	0.51±0.003 <sup>a</sup>	0.47±0.04 <sup>a</sup>
MDA(nmol/g)	0.00025±0.01 <sup>d</sup>	0.0018±0.02 <sup>d</sup>	0.00062±0.04 <sup>b7</sup>	0.00056±0.03 <sup>b</sup>
Phenolics(mg/g)	0.913±0.1 <sup>e</sup>	1.59±0.19 <sup>d</sup>	4.22±0.01 <sup>a</sup>	3.47±0.03 <sup>b</sup>
IC50 mg/ml	51.95±1.36 <sup>d</sup>	45.98±0.62 <sup>c</sup>	9.19±0.62 <sup>a</sup>	10.7±0.57 <sup>a</sup>

Decrease in chlorophylls level under salt stress may be due to reduction in pigment biosynthesis or enzymatic chlorophyll degradation (Xu ,Xinwen et al 2008 , Yang et al 2009)

The elevation of chl b/chl a ratio under saline condition suggested salinity had most adverse effect on Chlprophyll b content. Similar results obtained by Stoeva and Kaymakanova (2008) on *Phaseolus vulgaris* under sult stress.

The Chlprophyll level is an index of the photosynthesis (Xu et al 2008) and decrease in Chlprophyll level lead to reduction in growth parameters.

Slight reduction in carotenoids contents may be due to their protective role against reactive oxygen species. Salinity can lead to oxidative stress and causing significant decrease to photosynthetic systems. Carotenoids can protect photosynthetic system against reactive oxygen species generate

under salt stress (Parida and Das 2005, Parviz and Satava Waiz 2008).

#### **Ionic content**

Salinity caused decrease in K<sup>+</sup> content and K<sup>+</sup>/Na<sup>+</sup> ratio in treated plants however Na<sup>+</sup> content increased at all salt levels.

Under salinity stress high NaCl uptake competes with the uptake of K<sup>+</sup> and lead to reduction of K<sup>+</sup>/Na<sup>+</sup> ratio and Na<sup>+</sup> toxicity. Decrease in K<sup>+</sup> accumulation under salt stress has been reported in other plants, including wheat, Sorghum, beat, barely and rice (Zaho and Ren 2007).

Na<sup>+</sup> accumulation in salt stressed plants lead to low water potential, Change in essential ion uptake and ionic imbalance and reduces leaf expansion photosynthetic rate and limit growth (Zaho and Ren, 2007, Sairam et al 2002 ,Jenifer et al 2002, ), Greenway and Munns 1980, Sharma 1997) The

increase of Na<sup>+</sup> uptake in shoots of salt-treated plants did not drastically affect their growth status.

It appeared that rosemary is moderately salt tolerant (Tounekti et al 2008). This tolerance is apparently due to the ability of these plants to accumulate Na<sup>+</sup> in their leaves and to maintain turgor and osmotic adjustment.

#### **Proline**

Proline content of salt stressed plants was higher than of the control although salt stressed plants having no significant increase in proline content. It is possible that the level of salt in the medium was not sufficient to increase proline. High accumulation of proline in leaves are important adaptive mechanism of salt tolerance. Proline acts as an osmolyte and reduces the osmotic potential, thus reducing toxic ion uptake (Hare et al 1998)

Under salinity rosemary plant accumulated Na<sup>+</sup> to maintain leaf turgor, Although they need synthesis of organic solute especially proline. Proline and Na<sup>+</sup> accumulation is a mechanism used for maintaining turgor and reducing the adverse effect of salt stress (Bandeh – hagh et al 2008).

Proline may be act as radical scavenger and protects cells against salt induced oxidative stress (Hong et al 2000). Since total phenolic content and antioxidant activity of salt stressed plants increased, it seems that the phenolic antioxidant system of rosemary reducing the effect of salt stress and proline content did not significantly increased due to salt stress.

#### **Malondealdehyde**

MDA content in rosemary plants was poorly increased under saline condition.

MDA is regarded as a marker for evaluation of lipid peroxidation that increases with environmental stress. Under normal physiological conditions low concentrations of lipid peroxidation products are found in tissues and cells. In the presence of

oxidative stress more lipid peroxidation products are formed due to cell damage

The increase in lipid peroxidation may be due to the incapability of antioxidants to scavenger reactive oxygen species results from salt stress. The present results were agreed with the results of Ben Amor et al (2005), and Chaparzade et al 2004). A little increase in MDA level may be as a result of an effective antioxidant protection system in rosemary plant.

#### **Phenolic compounds and antioxidant activity**

Antioxidant activity and total phenolic content increased in salinized plants. Leaf phenolic content was significantly increased at 50 mM NaCl, and decreased at 150 mM NaCl. IC<sub>50</sub> in 100 mM NaCl-treated plants was 83% lower than that observed in control plants. Phenolic compounds are commonly found in plants and have different biological effects. Plants can synthesize and accumulate phenolic compounds in response to stress (Dixon and Paiva, 1995, Mamdouh et al 2002)., Rosemary is a good source of phenolic antioxidant compounds. The antioxidant activity of phenolic compounds can play an important role in neutralizing ROS (Zheng and Wang, 2001). Reactive oxygen species contribute to various environmental stress including salinity.

We observed a correlation between the phenolic content and antioxidant activity. The close correlation between antioxidant activity and phenolic content has been demonstrated by the other workers (Liu et al 2007, Verzellon et al 2007, Naciye et al 2008).

Rosemary leaf contains phenolic acids (2-3% rosmarinic, chlorogenic, and caffeic). Rosmarinic and carnosic acid were reported as major antioxidant active phenolic compounds in rosemary plants (Almela et al., 2006). An increase in total phenolic content and antioxidant activity of rosemary under salinity can reduce oxidative stress. It can be interesting for production of antioxidant compounds in rosemary plants under salt stress. These results

confirm by others (Riadh et al 2007 , Riadeh et al 2007).

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