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Copper Effects on Growth, Lipid Peroxidation, and Total Phenolic Content of Rosemary Leaves under Salinity Stress

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ABSTRACT

Plant growth is often limited by low levels of soil micronutrients such as copper (Cu), especially in calcareous salt-affected soils of arid and semiarid regions. The aim of this work was to investigate individual and combined effects of salinity and Cu on growth, leaf relative water content (LRWC), cell membrane permeability, lipid peroxidation, and total phenolic content (TPC) of rosemary (*Rosmarinus officinalis* L.) in a hydroponic experiment. A factorial arranged experiment with three salinity levels (i.e., 0, 50, and 100 mM NaCl), and three levels of copper (i.e., 0, 0.5, and 1.0 µM Cu^{2+}, as $CuSO_4$) was performed. The salt stress led to a significant decrease in leaf relative water content especially at 0 µM Cu^{2+}. Salt treatments (50 and 100 mM) were found to increase electrolyte leakage and malonyldialdehyde content of rosemary; however, this increase was greater at 0 µM than 1.0 µM Cu^{2+}. Regardless of Cu treatments, salinity (50 and 100 mM) induced significant increases in TPC by 13 and 29%, respectively. The application of 1.0 µM Cu at the 50 mM NaCl treatments increased total phenolic content by 19% compared to 0µM Cu. Copper nutrition resulted in greater accumulation of phenolic compounds in plant roots and thereby decreasing lipid peroxidation under salt stress conditions.

Keywords: Cu, Medicinal plants, Rosemary, Salinity, Oxidative damage, Total phenolic content.

INTRODUCTION

Rosemary (*Rosmarinus officinalis* L., Lamiaceae) is an economically important herb known not only as a source of essential oils but also for its natural antioxidants such as polyphenolic compounds (Genena *et al.,* 2008). In the past few years, rosemary has been successfully cultivated in warm and dry climates of arid and semiarid regions (El-Rjoob *et al.,* 2008). However, as in the majority of cultivated plants growth and yield of rosemary can be affected by salinity (Baatour *et al.,* 2009) and nutritional disorders (Grattan and Greive, 1999). Salinity reduces plant growth due to osmotic stress and ionic cytotoxicity (Saleh and Maftoon, 2008; Silva *et al.,* 2008; Dashti *et al.,* 2010) as well as oxidative damages to macromolecules and cell structure (Neto *et al.,* 2006; Eraslan *et al.,* 2007).

Copper deficiency is a significant nutritional disorder of plants grown on alkaline-saline soils of arid and semi arid climates (Whitehead, 2000). Cu is known as an essential micronutrient for the function of copper-zinc superoxide dismutases (SOD) and catalase (CAT) which are the most important ROS scavenger enzymes. Copper plays an important role in the synthesis of

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phenolic compounds and its deficiency can decrease phenolics in the plants (Dicko *et al.,* 2006).

Changes in total phenolic content of medicinal plants have been reported to be influenced by salinity (Navaro *et al.,* 2006). Ksouri *et al. (*2007) reported an increase in TPC in *Cakile maritime* at 100 and 400 mM NaCl. Indeed, phenolic compounds participate in the defense against ROS which are inevitably produced when aerobic or photosynthetic metabolism is impaired by environmental stresses (Ksouri *et al.,* 2007).

In spite of its great importance, Cu nutrition of plants is much less investigated than other micronutrients such as zinc (Zn) and iron (Fe). On the other hand, there is a growing interest for the cultivation of rosemary in arid and semi-arid regions of Iran, where soils contain high levels of salts and low available Cu. The aim of this study was to investigate the effects of Cu on the biomass production, cell membrane damage, and total phenolic content of rosemary grown under saline condition.

MATERIALS AND METHODS

Plant Growth

The experiment was conducted in the greenhouses of Yazd Agricultural and Natural Resources Research Center. Cuttings of rosemary (*Rosmarinus officinalis* L.) were collected from Yazd medicinal plants field. To avoid genetic variability, cuttings were obtained from one individual plant and grown in 10 liter pots filled with washed sand and kept for 3 months in a greenhouse under controlled conditions (16/8 h light/darkness, 24/18°C day/night temperature). Plants were irrigated twice a week with distilled water and Hoagland nutrient solution (Ballesta *et al.,* 2004), alternately. After 3 months, the plants were transferred to 5 liter pots containing the nutrient solution. Pots were covered with black plastic to inhibit light exposure of the solution. The nutrient solution used in this experiment contained (in µM): KNO_3 (3 mM), $Ca(NO_3)_2$ (2 mM), $NH_4H_2PO_4$ (0.5 mM), $MgSO_4$ (0.5 mM), KCl (50 µM), H_3BO_3 (25 µM), $MnSO_4$ (2.0 µM), $ZnSO_4$ (2.0 µM), $CuSO_4$ (0.5 µM), H_2MoO_4 (0.5 µM), Fe-ethylendiamino-di(o-hydroxyphenylacetic) acid (Fe-EDDHA) (20 µM). The pH of the nutrient solution was adjusted to 6.0 by adding HNO_3 0.1 mM. Treatments consisted of three levels of salinity (0, 50, and 100 mM) and three levels of Cu (0, 0.5, and 1.0 µM) which were applied by dissolving NaCl and $CuSO_4$ in the nutrient solution, respectively.

Leaf Relative Water Content (LRWC)

Leaf relative water content was measured using the method of Yamasaki and Dillenburg (1999). Individual leaves were sampled from the mid section of each plant and then weighed to obtain fresh mass (FM). In order to determine the turgid mass (TM), whole leaves were floated in distilled water inside a closed Petri dish. During the imbibition period, leaf samples were weighed periodically to reach a constant weight, after gently wiping water off the leaf surfaces with tissue paper. At the end of the imbibition period, leaf samples were placed in a pre-heated oven at 80°C, for 48 hours and then weighed (DM). All mass measurements were made using an analytical balance with a precision of 0.0001 g. The LRWC was calculated using the below equation:

$$LRWC= (FM-DM)/(TM-DM)\times 100$$

Cell Membrane Permeability (Electrolyte Leakage)

Electrolyte leakage was used to assess cell membrane permeability according to the method described by Lutts *et al.* (1996). Five leaf samples of two plants per replicate were taken from each treatment. Leaf samples were cut into 1 cm segments and placed in individual vials containing 10 mL

of distilled water after three washes with distilled water to remove surface contamination. These samples were incubated at room temperature (25°C) on a shaker (100 rpm) for 24 hours. Electrical conductivity of samples (EC_1) was measured after incubation. The same samples were then placed in an autoclave at 120°C for 20 minutes and the second measurement (EC_2) was done after cooling the solution to room temperature. The electrolyte leakage was calculated using EC_1/EC_2 ratio and was expressed as percent.

Lipid Peroxidation

Malonyldialdehyde (MDA) - a product of lipid peroxidation- content has been considered as an indicator of oxidative damage (Neto *et al.,* 2006). One hundred milligrams of fresh leaf samples were homogenized in 2.5 mL of 0.1% (w/v) tricholoroacetic acid (TCA). The homogenate was centrifuged at 3000×g for 20min. One ml of the extract was mixed with 2.25 mL of a 0.5% (w/v) thiobarbituric (TBA) acid solution containing 20% (w/v) tricholoroacetic acid. The mixture was heated at 95°C for 30 minutes and then quickly cooled in an ice-bath. The mixture was centrifuged at 3,000×*g* for 10 minutes and the absorbance of the supernatant was measured at 532 and 600 nm. After subtracting the non-specific absorbance (600 nm), the MDA concentration was determined by using molar extinction coefficient of 155 mM^{-1} cm^{-1} and the results were expressed as nmol MDA g^{-1} fresh weight (FW).

Total Phenolic Content

250 mg of fresh leaves were powdered with liquid nitrogen in a mortar and extracted with 10 mL of 80% methanol at 37°C for 3 hours in a shaking water bath (Tawaha *et al.,* 2007). After cooling, the extract was centrifuged at 3,500 g and stored at 4°C. Total phenolic content was estimated by the Folin-Cicoalteu colorimetric method as described by Singleton and Rossi (1965). Gallic acid was used as a standard phenolic compound. One mL of the filtered extracts was mixed with 2.5 mL of 10% Folin-Cicoalteu reagent. After 5 min, 2 mL of saturated sodium carbonate (75 mg L^{-1}) was added and the solution was incubated for 90 minutes at 30°C. The absorbance of the resulting blue-colored solution was measured at 765 nm. Quantitative measurements were performed based on a standard calibration curve of six points: 20, 100, 200, 300, 400 and 500 mg L^{-1} of gallic acid in 80% methanol. Total phenolic content of the plant was expressed as mg gallic acid equivalents (GAE) per gram of fresh weight.

Dry Weight Determination and Copper Analysis

At harvest, shoot and root were separated and washed twice with tap water and twice with distilled water. They were then dried in a thermoventilated oven at 70°C for 72 hours for dry weight determination. Copper analyses were carried out on a dry weight basis. Ground samples were dry-ashed at 550°C for 4 hours, mixed with hot 2M HCl, filtered, and then brought to a final volume of 50 ml with distilled water. Copper concentration was determined in the digest solution by atomic absorption spectrophotometery (Chapmann and Pratt, 1982).

Statistical Analysis

The experiment was set up in a factorial design with three levels of salinity and three levels of Cu in four replicates. Analysis of variance (ANOVA) was performed on all data using the SAS. Means were compared by the Lowest Significant Deviations (LSD) test at $P< 0.05$. Pearson's correlation between electrolyte leakage and MDA was also performed.

RESULTS AND DISCUSSION

The shoot and root dry weight (DW) of rosemary was significantly (P<0.05) decreased by salinity stress (Figures 1 and 2). The application of 50 mM NaCl in the nutrient solution (EC= 7 dS m^{-1}) caused only 7% reduction in the shoot and root dry matter production confirming that rosemary is a salt tolerant plant. Westerwelt (2003) also reported that rosemary could tolerate high levels of salinity (4-8 dS m^{-1}) in the soil. The adverse effects of salinity on shoot and root growth of rosemary were more evident at the lower dose of Cu (0 µM) in comparison with 0.5 and 1.0 µM Cu levels (Figures 1 and 2). These results suggested that Cu was protective against NaCl toxicity in plants. Copper deficiency decreases the rate of photosynthesis and increases the rate of respiration which result in limited water availability for cell expansion and growth (Olszewska *et al.,* 2008) especially under saline condition.

LRWC which directly reflects the water status of plants under stress condition (Saied *et al.,* 2005; Kaya *et al.,* 2007) was decreased in the leaves of rosemary grown under the 50 and 100 mM NaCl treatments (Figure 3). Salinity caused water deficit in rosemary leaves through an increase in soluble salts concentrations. Water deficiency decreases plant growth by limiting water availability for cell expansion and nutrient uptake (Yang et al., 2009). For example, leaf Cu concentration of rosemary plants grown in the 0.5 and 1.0 µM Cu treatments was decreased in response to salt stress (Figure 4). Similar results were obtained by Izzo *et al.* (1991) and Villora (2000) who reported that salinity decreased leaf Cu concentration of maize and zucchini plants, respectively.

Increasing salinity was associated with a

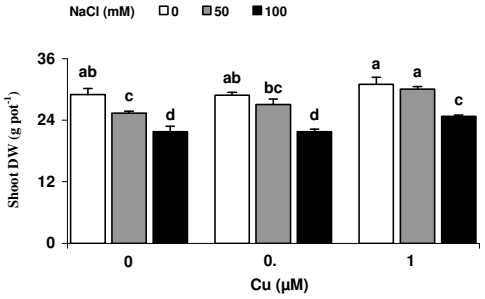

Figure 1. Effect of increasing Cu and NaCl in levels on shoot dry biomass (Mean±S.E) of rosemary (g pot^{-1}) (Different letters above bars indicate significant difference at P< 0.05).

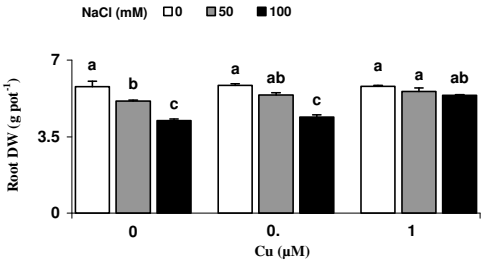

Figure 2. Effect of increasing Cu and NaCl in levels on root dry biomass (Mean±S.E) of rosemary (g pot^{-1}) (Different letters above bars indicate significant difference at P< 0.05).

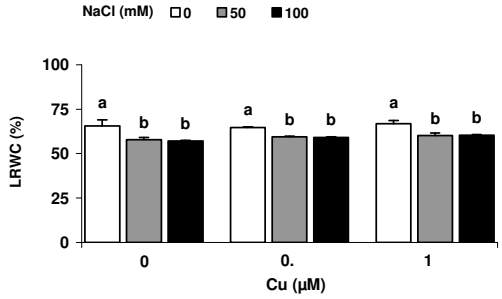

Figure 3. Effect of increasing Cu and NaCl in levels on leaf relative water content (Mean±S.E) of rosemary (%) (Different letters above bars indicate significant difference at P< 0.05).

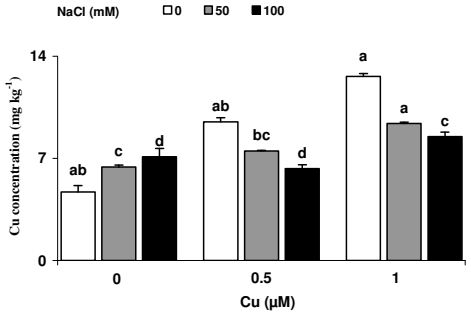

Figure 4. Effect of increasing Cu and NaCl levels on leaf Cu concentration (Mean±S.E) of rosemary (mg kg^{-1}) (Different letters above bars indicate significant difference at P< 0.05).

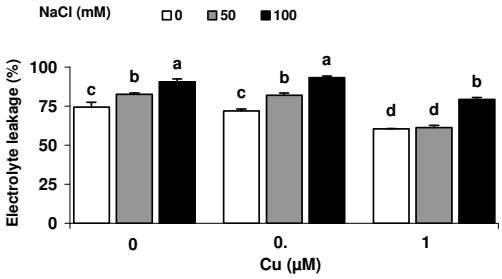

Figure 5. Effect of increasing Cu and NaCl levels on membrane permeability (Mean±S.E) of rosemary (%) (Different letters above bars indicate significant difference at P< 0.05).

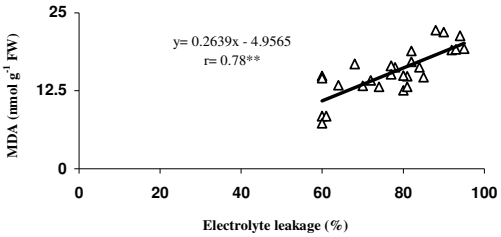

Figure 6. Relationship between electrolyte leakage and malonyldialdehyde (MDA) in the leaves of Rosemary (n= 36).

Table 1. Effects of salinity and copper concentration on the malonyldialdehyde content of rosemary leaves.

NaCl (mM)	Cu (µM)			
	0	0.5	1	Mean
	MDA (nmol g^{-1} FW)			
0	16.5±0.275 c	13.5±0.558 e	8.0±0.655 f	12.7±3.75 C
50	17.4±1.337 c	13.5±1.091 e	14.3±0.768 de	15.0±2.04 B
100	21.8±0.458 a	19.1±0.094 b	15.0±0.150 d	18.6±2.99 A
Mean	18.6±2.55 A	15.4±2.89 B	12.4±3.33 C	
		Mean square		
NaCl		80.46 **		
Cu		85.28 **		
NaCl×Cu		9.44 **		

** Significant at *P*< 0.01.
Means followed by the same letter (small letters for means and capital letters for means of rows and columns) are not significantly different at 5% level of probability using LSD test.

significant (P< 0.05) increase in the electrolyte leakage (Figure 5) and lipid peroxidation (Table 1) of rosemary leaves. Salinity can cause oxidative damage to cell membrane and lipids (lipid peroxidation), leading to an increase in electrolyte leakage (Amor *et al.,* 2005; Kaya *et al.,* 2007; Arvin and Donnelly, 2008). Supporting this idea, the electrolyte leakage was positively correlated with the MDA content, indicating that the membrane injury induced by salt stress is a result of oxidative damage (Figure 6). The increase in electrolyte leakage and lipid peroxidation by salinity became more severe at 0 µM Cu^{2+} than 0.5 and 1.0 µM Cu^{2+}. Since copper is directly involved in both gene expression and protein synthesis, it seems that copper deficiency inhibits the activities of CAT, resulting in extensive oxidative damage to membrane lipids (Marschner, 1995). Leaf Cu concentration was increased in response to Cu application (Figure 4) and thus lower electrolyte leakage and MDA content at 1.0 µM Cu^{2+} treatments could be attributed to a higher activity of CAT (Data not published). These findings are in agreement with those of Henryka (1999) who found that Cu inhibits lipid peroxidtion in root nodule of yellow lupine plants.

In contrast to the reduced growth of rosemary, total phenolic content in the leaves was greatly enhanced by salinity (Table 2). The reduction in growth induced by salt stress may have resulted in a new pattern of resource partitioning providing

Table 2. Effects of salinity and copper concentration on the total phenolic content of rosemary leaves.

NaCl (mM)	Cu (µM)			
	0	0.5	1	Mean
	TPC (mg gallic acid g^{-1} FW)			
0	14.45±0.050 f	14.69±0.120 d	17.58±0.106a	15.57±1.51 C
50	16.65±0.021 e	16.52±.0150 d	19.75±0.064 a	17.64±1.59 B
100	20.18±0.035 c	20.12±0.097 b	20.21±0.031 a	20.17±0.07 A
Mean	17.09±2.50 B	17.11±2.40 B	19.18±1.22 A	
	Mean square			
NaCl	47.7 **			
Cu	13.0 **			
NaCl×Cu	3.1 **			

** Significant at $P< 0.01$.
Means followed by the same letter (small letters for means and capital letters for means of rows and columns) are not significantly different at 5% level of probability using LSD test.

additional carbon skeletons for phenolic biosynthesis. Regardless of copper concentration, the highest contents of TPC were found in the leaves of plants grown at the 100 mM NaCl treatment. Similarly, in pepper and *Cakile maritime* plants, Navarro *et al.* (2006) and Ksouri *et al.* (2007) found that salinity induced significant increases in TPC. Copper nutrition effect and its interaction with salinity on TPC were significant (Table 2). At the 50 mM NaCl treatment, plants exposed to 0.5 and 1.0 µM of Cu, accumulated TPC by 12% and 37% greater than those grown in the Cu-free treatment. At the 100 mM NaCl treatment, these values changed to 12% and 15%, respectively. The increase in TPC by Cu became less distinct when TPC was also increased by salinity. This indicated that salinity was a more effective factor than Cu nutrition in the increase of TPC. Dicko *et al.* (2006) also reported that Cu increased TPC in sorghum by increasing biosynthesis of phenolic compounds.

CONCLUSIONS

The results showed that salinity induced oxidative stress, resulting in lipid peroxidation and increase in cell membrane permeability to toxic ions (e.g. Na^+ and Cl^-) which in turn reduced the plant growth. Copper nutrition reduced lipid peroxidation and membrane permeability while it increased total phenol content of salt stressed plants. It seems that copper nutrition may effectively ameliorate salt-induced oxidative damage in rosemary.

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تأثیر مس بر رشد، پراکسیداسیون چربی و کل محتوای فنلی برگ رزماری در شرایط تنش شور

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چکیده

کمبود عناصر ریزمغذی مانند مس موجب کاهش رشد گیاهان در خاک‌های شور و آهکی مناطق خشک و نیمه‌خشک می‌شود. هدف این پژوهش بررسی اثرات اصلی شوری و تغذیه مس بر رشد، رطوبت نسبی برگ، نفوذپذیری غشای سلولی، پراکسیداسیون چربی و ترکیبات فنلی برگ رزماری رشد یافته در کشت محلول بود. این پژوهش به صورت آزمایش فاکتوریل با سه سطح شوری (صفر، ۵۰ و ۱۰۰ میلی‌مولار کلرید سدیم) و سه سطح مس (صفر، ۰/۵ و ۱ میکرومولار) در قالب طرح کاملاً تصادفی انجام گرفت. شوری ۵۰ و ۱۰۰ میلی‌مولار موجب کاهش رطوبت نسبی برگ رزماری به خصوص در سطح صفر میکرومولار مس شد. افزایش شوری موجب افزایش معنی‌دار نفوذپذیری غشای سلولی و پراکسیداسیون چربی گردید که این افزایش در سطح صفر میکرومولار مس بیشتر از سطح ۱ میکرومولار بود. صرفنظر از غلظت مس، افزایش سطح شوری به ۵۰ و ۱۰۰ میلی‌مولار به ترتیب موجب افزایش ۱۳ و ۲۹ درصدی غلظت ترکیبات فنلی برگ رزماری شد. به نظر می‌رسد که مس تأثیرات اکسیداتیو منفی را از طریق افزایش غلظت ترکیبات فنلی، حفظ نفوذپذیری غشای سلولی و کاهش پراکسیداسیون چربی بهبود بخشیده است.