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ORIGINAL PAPER

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THE EFFECTS OF COMMON AND NANO-ZINC FOLIAR APPLICATION ON THE ALLEVIATION OF SALINITY STRESS IN *Rosmarinus officinalis* L.

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ABSTRACT

Foliar application of micronutrients (both in common and nano-forms) to meet the nutritional demands of plants and even to overcome the stressful environments has gained great attention of agricultural systems. In our experiments, we tried to use the foliar nano-zinc and common zinc sources under salinity conditions and study their effects on some morpho-physiological traits of rosemary (*Rosmarinus officinalis*) as factorial experiment based on RCBD design. ANOVA results revealed the interaction effects of salinity and zinc foliar application on elemental content (K^+, Na^+ and Zn^{2+}), as well as essential oil yield of the plants. Carotenoids were influenced by the salinity levels. Soluble sugars content, flavonoids, H_2O_2 and MDA contents were influenced by individual levels of salinity and zinc foliar applications. Eventually, nano-zinc foliar spray was able to overcome the mild salinity effects on the plant growth and physiological parameters and it could be administered to the production systems and pioneer plant producers.

Key words: *Rosmarinus officinalis*, antioxidant enzymes, essential oil, total phenolics content

INTRODUCTION

Nowadays, due to the great incidence of water resources and soils salinity, the interest in yield improvement has gained a great attention of scientists and pioneer agricultural producers and even the small-scale production systems [Olfa Baatour et al. 2010, Hassanpouraghdam et al. 2011]. Salinity interferes the primary and secondary metabolism of plants by the huge impact on biochemical and physiological potential of plants at subcellular, cellular, tissue and organ level [Munns and Tester 2008, Hassanpouraghdam et al. 2011]. Plants have evolved diverse mechanisms to combat salinity defects [Munns and Tester 2008, Steffens 2014]. The mechanisms are: ionic balance including Na^+ accumulation in vacuoles to reduce its toxic effects in cytoplasm, to help in water absorption by making more negative cell osmotic potential, production of osmolites such as betaine, glycine, proline and soluble sugars to encourage water intake and to increase the plant relative water content (RWC) as well as retain high K^+/Na^+ ratio to prevent membrane damage [Ashraf and Ali 2008, Munns and Tester 2008, Iqbal et al. 2014]. Under salinity conditions, secondary stresses, like oxidative damage, arise leading to ROS molecules over-production, which are highly toxic and damage the cells by high-oxidation rates of proteins and membrane anchored lipids and eventually threatening

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the cell viability [Turan and Tripathy 2012, Steffens 2014]. Antioxidant enzymes such as peroxidases and superoxide dismutase (SOD) are the first barriers on the way to block ROS radicles [Turan and Tripathy 2012].

Using some micronutrients under saline conditions is another alternative to overcome the deterioration effects of ROS molecules [Munns and Tester 2008]. Zn is one of the most essential microelements influencing the growth, productivity and also mediates the plant responses under stressful environments. Zn is an un-substitutable co-enzyme for some of the key antioxidant enzymes and even has indirect roles in DNA and RNA multiplication [Song et al. 2015]. Foliar application is one of the optional methods to compensate the shortage of micronutrients [Vojodi Mehrabani et al. 2016]. However, just a small part of sprayed nutrients is able to penetrate the plants tissue and the majority of the nutrient is out-of-use mainly due to leaching, photo-degradation and hydrolization [Nair et al. 2010]. To increase the foliar application efficiency, the easiest way is to replicate the foliar sprays at the defined time-courses. But, the disadvantage of the frequent application is the environmental and especially soil and water resources pollution as well as the huge charges imposed that are not in favor of sustainable production systems.

Having the advantage of nutrients as nano-forms (size between 1−10 nm) is the most recently accepted alternative in main parts due to the higher solubility, high stability, slow release nature, high efficiency and the most important, more environment-friendly behavior of these compounds [Nair et al. 2010]. Shojaei and Makarian [2014] noted that Zn and nano-Zn foliar application of *Vigna radiata* plants under drought condition increased the pod number per plant, seed weight and biological yield.

Rosmarinus officinalis is a medicinal plant with many applications in pharmaceutical, hygienic and cosmetic industries and moreover, it has been used as a spice plant besides being a beautiful ornamental small shrub [Kiarostami et al. 2010]. Salinity stress adversely affects the growth, development, yield and quality of rosemary [Kiarostami et al. 2010]. In the present experiment, we aimed to assay the rosemary partial tolerance to the salinity stress foliar sprayed by zinc-oxide in common and nano-form. Some morpho-physiological traits were traced to have an idea on the cultivation distribution of this plant under salinity-faced environment and for more efficient use of the common production inputs.

MATERIAL AND METHODS

The experiment was conducted as factorial based on randomized complete block design with three replications at the Research Greenhouse of Azarbaijan Shahid Madani University, North-West Iran during the growing season 2016. The *Rosmarinus officinalis* seedlings were supplied by a local nursery and were transferred to the greenhouse during last week of April. The growing parameters were: ambient light intensity of about 450 µmo lm^{-2} s^{-1},16 : 8 hrs photoperiod and 25 : 20°C day-night temperature regime. The relative humidity was adjusted at about 65%. The pots were nourished with the half strength Hoagland’s nutrient solution for one month. To prevent salts accumulation at the growing area, the pots were washed with tap-water once a week. The salinity treatments included: 0, 50, 100 and 150 mM NaCl. To escape the sudden shock of stress, salinity levels were began from 25 mM, reaching to the defined levels by adding up 25 mM every 4 days. The first zinc foliar application was applied on 20 May, 2016. The second foliar application was two weeks later.

Preparation of zinc and nano-zinc solutions and the foliar applications. Micro-zinc (500 nm particle size) and nano-zinc (10−30 nm particle size) were supplied by the US-Nano Company (USA). The foliar applications were made at 5 and 10 mg L^{-1}.

Measurements. The plants at the beginning of flowering were harvested and the aerial parts and roots fresh and dry weight were recorded.

Carotenoid content. Total carotenoid contents of leaves were traced by the method of Prochazkova et al. [2001].

Total soluble solids (TSS). TSS of leaves was quantified by a portable refractometer (Erma, Japon) and the data were expressed as °Brix.

Total phenolics content (TPC). TPC of leaf tissues was determined using Folin-Ciocalteu method according to Kim et al. [2006]. The phenolics content was expressed as mg of gallic acid equivalent per gram of dry plant sample using the linear equation based on the calibration curve.

Total flavonoid content. Total flavonoids content was measured according to the method of Quettier-

-Deleu et al. [2000]. The data were expressed in mg of rutin equivalent per gram of plant dry weight.

Mineral analysis. Na^+ and K^+ contents were determined in the dried leaf samples by flame-photometry and Zn^{2+} was extracted and measured according to the method described by Honarjoo et al. [2013].

Determination of H_2O_2 and malondialdehyde (MDA) content. H_2O_2 content of the plant samples was determined as described by Heath and Packer [1968] and MDA was quantified according to the method of Amaranatharedd et al. [2015].

Essential oil extraction. The essential oil was extracted from 15 g of dry plant material by hydro-distillation during 3 h using a Clevenger-type apparatus and the oils were dried over anhydrous sodium sulfate [Vojodi Mehrabani et al. 2017]. Oil content was the amount in volume extracted from dry tissue expressed in per cents. The oil yield was calculated by multiplying the oil content by the aerial parts dry weight per hectare.

Experimental design and data analysis. The experiment was designed as factorial based on RCBD with three replications. LSD values were calculated at 1 and 5% probability levels.

RESULTS AND DISCUSSION

Leaf dry weight. The results are showing the independent effects of salinity treatments and foliar spray on the leaf dry weight. The highest plant leaf dry weight was recorded for the control and 50 mM salinity levels (9.3 g and 8.8 g), respectively (Tab. 1). With any increase in the salinity levels, the leaf dry weight of plants was negatively affected (Tab. 1). The idea is that rosemary is tolerable to 50 mM salinity levels without significant decline in its leaf dry weight. Olfa Baatour et al. [2010] reported that salinity stress drastically affected the leaf yield in *Majorana hortenses*. Reduced growth potential under saline-sodium growing environments is due to the high salt accumulation at the rizosphere area, nutrients imbalances in the soil solution, ionic competition, ionic-related specific effects and finally the adverse impact of all the above on the primary metabolism of plants [Munns and Tester 2008]. These events have impact on the photosynthesis rate, leaf area, stomatal conductance and CO_2 assimilation and ultimately reduce the plant yield [Aparicio et al. 2014, Das et al. 2015]. Zinc foliar spray had promotive effects on the plant yield and the highest yield was attributed to 10 mg L^{-1} of nano-zinc (Tab. 2). Our results are in-line with findings of Shojaei and Makarian [2015] on *Vigna radiata* and Torabian et al. [2016] in sunflower plants treated with nano-zinc. They noted that Zn and nano-Zn foliar application increased the IAA content in roots and hence, improved plants growth and productivity. Due to the smaller particle size of the nutrient's nano-forms, it is totally understandable that their absorption, translocation, accumulation and assimilation is more dynamic than that of the common forms. Moreover, the high absorption rate and higher specific surface area logically demonstrate the higher efficiency of nano-particles application compared to common forms.

Table 1. Mean comparison for the effects of salinity and zinc foliar spray on leaf dry weight and some physiological traits of rosemary

NaCl levels	Leaf dry weight (g)	Carotenoid content (mg g^{-1} F_{wt})	TSS content (° Brix)	Flavonoid content (mg rutin g^{-1} D_{wt})
0	9.3^a	1.2^a	1.5^b	5.3^b
50	8.8^a	0.9^b	3.2^a	8.2^a
100	7.1^b	0.8^b	2.7^{ab}	2.3^c
150	6.5^b	0.6^c	2.9^a	1.8^d
LSD %	2.7	0.15	1.4	0.9

Similar letters in columns are non-significant based on LSD test

Table 2. Mean comparison for the effects of zinc foliar spray on leaf dry weight, TSS and flavonoid content of rosemary

Zn foliar application (mg L^{-1})	Leaf dry weight (g)	TSS content (° Brix)	Flavonoid content (mg rutin g D_{wt}^{-1})
0	9^c	1.4^c	5.3^b
5 (nano)	12^b	3.3^a	3.9^c
10 (nano)	15.6^a	3.9^a	8.5^a
5 (common)	13^b	2.6^b	6.8^b
10 (common)	12^b	2.9^b	8.3a
LSD %	2.3	1.6	1.0

Similar letters in columns are non-significant based on LSD test

Carotenoid content. Carotenoid content was affected by the salinity (Tab. 1). The highest one (1.2 mg g^{-1} F_{wt}) was attained by control plants. With any increase in salinity levels, carotenoid content was declined and the least amount (0.6 mg g^{-1} F_{wt}) was recorded with the highest salinity levels i.e. 150 mM. Carotenoids are the major isoprenoids produced by all the photosynthetic tissues and even by other plant tissues. These pigments have key roles in antioxidant pool of plants [Aparicio et al. 2014]. ROS molecules produced under stressful conditions impose the oxidative stress of chloroplast components such as thilakoid membranes, electron transport chain and on pigment contents and hence, adversely influence carotenoid content [Aparicio et al. 2014, Sub Ba et al. 2014].

Total soluble solid content (TSS). TSS content of leaves was affected by independent effects of salinity levels (Tab. 1) and by foliar application of zinc (Tab. 2). Nano-zinc foliar application at 5 and 10 mg L^{-1} concentrations positively influenced the TSS and the least amount for the criterion belonged to the control plants (Tab. 2). Zinc holds pivotal roles in the activity of key enzymes involved in carbohydrates metabolism; the major ones are carbonic-anhydrase, ribulose 1,5 bis-phosphate, carboxylases, oxidases and fructose 1,6 bis-phosphate [Hafeez et al. 2013]. Moreover, zinc has a role in IAA, DNA, RNA, proteins and chlorophyll biosynthesis as well as in starch formation and accumulation [Nahed et al. 2007]. The highest TSS content was measured at 50 and 150 mM salinity levels (2.9 °Brix) (Tab. 1). Results from the present experiment are concomitant with the findings of Farhoudi et al. [2011] and Najafi et al. [2010], who reported the increase in TSS amount in parallel with salinity levels. Under salinity environments, polysaccharides such as starch are degraded to produce soluble mono-saccharides helping the cell to maintain osmotic potential and to prevent dehydration damage [Parviz and Satyawati 2008]. Moreover, soluble sugars bind the side polar-branches of membranous proteins helping to maintain the membranes integrity and cell ultrastructure stability [Parviz and Satyawati 2008].

Leaf phenolics and flavonoid content. Flavonoid content was influenced by the independent effects of salinity and Zn foliar application treatments (Tabs. 1 and 2). However, the treatments had no significant effect on total phenolics. The greatest flavonoid content was traced at 10 mg L^{-1} of both nano-zinc and common Zinc source (Tab. 2). The highest recorded flavonoid content was determined at 50 mM NaCl treatment (Tab. 1). Kiarostamei et al. [2010] in their study on rosemary, reported that there was a positive relationship between total phenolics content and the antioxidant potential of plants and hence, the ROS scavenging potential.

MDA content. MDA content was affected by independent effects of salinity and foliar spray as well (Figs. 1−2). Salinity stress increased the MDA content of plants and the least MDA content was recorded in control ones (19.16 µmol g^{-1} F_{wt}) (Fig. 1). The results are relatively the same as those from Najjar-Khodabakhsh and Chaparzadeh [2016] for watercress and Farhoudi [2011] for rapeseed. In many plant species, high NaCl concentrations cause growth retardation and may result in plant death because of drastic changes in ion and ROS homeostasis and in altered gene expression [Li et al. 2014]. Farhoudi [2011] demonstrated that membranes deterioration and the subsequent MDA production in response to the lipids breakdown can be considered as a suitable criterion for the plant responses to the salinity stress. Negative correlation between MDA content and plant growth parameters was reported as well [Farhoudi 2011]. The highest MDA content was recorded in control plants (24.16 µmol g^{-1} F_{wt}) and the lowest amount was quantified in plants treated with 10 mg L^{-1} of both nano-particle and common zinc-oxide (Fig. 2). Salt stress and Zn shortage decrease the photosynthesis rate, stomatal conductance, respiration, chlorophylls *a* and *b* and cytokinin content of leaves but, in contrast, increase the amounts of MDA, H_2O_2 and electrolyte leakage [Sub Ba et al. 2014]. Zinc as an important essential micronutrient, increases the antioxidant capacity and also stabilizes the cell membranes as well as due to the great involvement in SOD enzyme structure and inhibiting NADPH oxidase, it plays a pivotal role in cell membranes integrity [Prasad 2010, Hafeez et al. 2013].

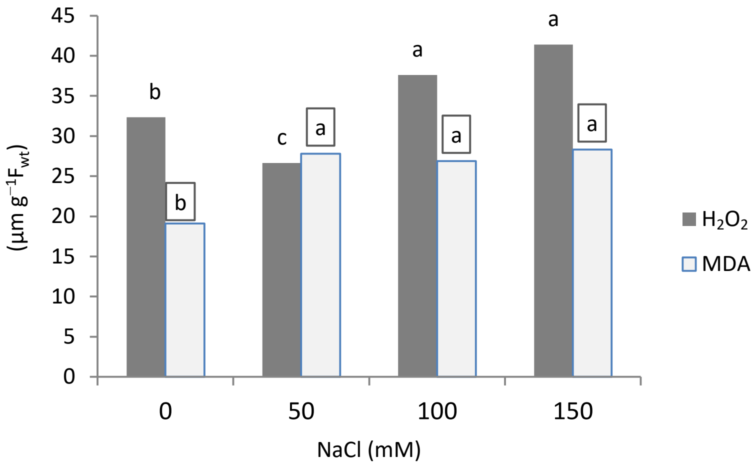

Fig. 1. Effects of NaCl salinity stress on H_2O_2 and MDA content of rosemary. Similar letters in the columns are non-significant based on LSD test

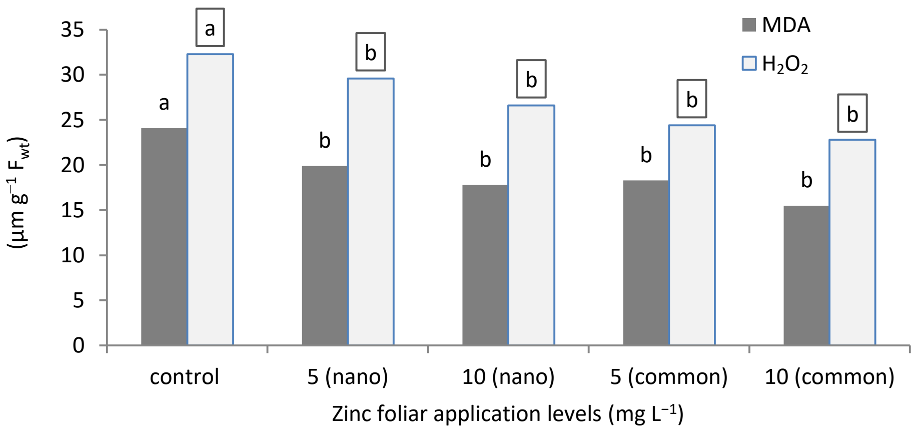

Fig. 2. Effects of common and nano-form zinc-oxide foliar application on H_2O_2 and MDA content of rosemary. Similar letters in columns are non-significant based on LSD test

H_2O_2 amounts. H_2O_2 content was influenced by independent effects of salinity and zinc foliar application (Figs. 1 and 2). With salinity, H_2O_2 content was increased; the highest amount was attributed to 150 mM NaCl treatment (41.4 μmol g^{-1} F_{wt}). The least H_2O_2 content was recorded in the control (Fig. 1). Zinc foliar application significantly reduced H_2O_2 accumulation and the highest amount under foliar sprays was found in untreated control plants (Fig. 2). Sub Ba et al. [2014] demonstrated that zinc application ameliorated the salinity effects by reducing the H_2O_2 over-production. As mentioned before, the yield (dry weight) reduction may be due to ROS over-dosage and their deteriorative effects. H_2O_2 over-accumulation and MDA over-expression go to the cell membranes unstability and the growth and productivity was influenced, correspondingly. Das et al. [2015] reported that Na^+ accumulation under salinity environments deteriorates the cell membranes in plants. In contrast, peroxidases have dominant role in scavenging ROS produced by the action of salinity stress and Na^+ accumulation in agronomic crops [Sofo et al. 2015]. Peroxidases are assembling of ascorbate and glutathione reductase cycle enzymes scavenging H_2O_2 to produce H_2O. Several scientists consider the activity of these enzymes as the key factors to protect plants against diverse stressful environments [Sub Ba et al. 2014, Sofo et al. 2015].

Table 3. Mean comparison for the interaction effects of salinity and zinc foliar spray on yield and some physiological traits of rosemary

NaCl (mM)	Foliar spray with common zinc-oxide and nano-zinc form (mg L^{-1})	Leaf Zn^{2+} content (mg kg^{-1} D_{wt})	Leaf Na^+ content (mg kg^{-1} D_{wt})	Leaf K^+ content (mg kg^{-1} D_{wt})	Essential oil yield (L ha^{-1})
0	0	39^{def}	871^{i}	1670^{c}	5.4^{cde}
0	5 (nano)	81^{a}	998^{bcdef}	1962^{a}	6.4^{cde}
0	10 (nano)	98^{a}	860^{i}	1350^{d}	15^{a}
0	5 (common)	70^{b}	860^{i}	1232^{f}	9.9^{bc}
0	10 (common)	97^{a}	900^{bcd}	1324^{d}	11.9^{ab}
50	0	62^{c}	1002^{b}	1322^{d}	5.7^{cde}
50	5 (nano)	48^{c}	900^{i}	1223^{f}	7.5^{bcde}
50	10 (nano)	58^{c}	1009^{bc}	1062^{e}	7.9^{bcde}
50	5 (common)	46^{c}	930^{def}	1405^{d}	5.7^{cde}
50	10 (common)	54^{bc}	1060^{bcde}	1290^{d}	7.2^{bcde}
100	0	44^{c}	942^{cdef}	1423^{d}	3.2^{de}
100	5 (nano)	40^{ce}	970^{cdef}	1291^{f}	4.7^{cde}
100	10 (nano)	55^{b}	957^{cdef}	1388^{d}	8.4^{bcd}
100	5 (common)	42^{ce}	1180^{bcdef}	1639^{c}	4.7^{cde}
100	10 (common)	59^{b}	1080^{b}	1628^{c}	7.9^{bcde}
150	0	37^{e}	1399^{a}	1365^{d}	2.4^{e}
150	5 (nano)	22^{g}	1184^{a}	1373^{d}	3.73^{de}
150	10 (nano)	48^{ce}	978^{b}	1241^{f}	5.1^{cde}
150	5 (common)	35^{fg}	986^{bc}	1406^{d}	2.5^{e}
150	10 (common)	28^{fg}	926^{def}	1153^{e}	4.8^{cde}
LSD %		6.23	14.4	12	4.4

Similar letters in columns are non-significant based on LSD test

Essential oil yield. Essential oil yield was significantly affected by the interaction of salinity and Zn foliar application (Tab. 3). The highest oil yield was recorded in control plants foliar sprayed with 10 mg L^{-1} of nano-zinc and control × 10 mg L^{-1} common zinc-oxide (Tab. 3). Salimi et al. [2016] reported that in *Achillea millefolium*, the oil content was the highest at 150 mM salinity level. Seemingly, secondary metabolites production is an evolutionary mechanism to overcome the oxidative stresses within the plants. Ghohari et al. [2013] emphasized a close correlation between primary and secondary metabolism in *Ocimum basilicum* and noted that for reaching the highest secondary metabolites pool, plants have to be grown under suitable production systems. Mineral nutrition and environmental cues influence the essential oil biosynthesis in plants. Zinc influences the photosynthesis and carbohydrate metabolism. CO_2 and glucose are the main sources for the terpenoids biosynthesis. Zinc nutrition has inevitable action on the biosynthesis, accumulation and yield of essential oils [Nahed et al. 2007].

Leaf Na^+, K^+ and Zn^{2+} content. The highest values for K^+ content were recorded for control plants not-faced with salinity but, foliar treated with 5 mg L^{-1} of nano-zinc. The least K^+ amount was measured for 50 mM salinity × 10 mg L^{-1} nano-zinc and 150 mM salinity × 10 mg L^{-1} common zinc oxide (Tab. 3). Similar results were reported by Vojodi Mehrabani et al. [2017] in *Lavandula Stoechas* L. plant. The major reason for this is most probably due to the antagonistic behavior between Na^+ and potassium and the fact that sodium may proceed K^+ in absorption rate. Aparicio et al. [2014] reported the low K^+/Na^+ content of 'Picual' olive cultivar under saline environments due to the high efficiency of Na^+ intake by plants. Those scientists clearly reported that high K^+ content of plants under salinity conditions could be a reliable factor for the salinity tolerance consideration in plants. Retaining the suitable K^+ amounts inside plants is crucially essential for the cells viability. K^+ accumulation within roots helps in the regulation of osmotic potential of root cells and aids for the translocation of solutes within xylem and moreover, is essential for the water equilibrium inside plant and hence greatly reduces the drought effects induced by the salinity [Munns and Tester 2008].

The highest value of Na^+ content was in 150 mM NaCl salinity treatment × control non-sprayed and with 150 mM salinity × 5 mg L^{-1} nano-zinc (Tab. 3). The reverse correlation between the leaf dry weight in rapeseed and Na^+ accumulation has been reported by Farhoudi [2011] as well. Therefore, more K^+ intake and the elevated peroxidase activity prevented leaf dry weight reduction. The intensified Na^+ accumulation in plants tissues is one of the primitive responses to salinity stress [Aparicio et al. 2014]. Munns and Tester [2008] had the idea that increased Na^+ absorption and accumulation in the cytoplasm under salinity conditions have huge toxicity to cell metabolism and also could replace K^+ specific sites. Substitution of K^+ by Na^+ in the specific sites in cytosol adversely affects the cell metabolism and eventually plant productivity [Das et al. 2015]. The prevention of plant growth under salinity condition is likely due to the reduced osmotic potential of the plant, reduced water availability or the hyper-accumulation of Na^+ and Cl^- in the plant tissues [Munns and Tester 2008, Das et al. 2015]. The highest Zn^{2+} content was recorded with no salinity treatments × 5 and 10 mg L^{-1} nano zinc and 10 mg L^{-1} common zinc oxide (Tab. 3). Ghohari et al. [2013] reported that Zn foliar spray increased the biomass of basil plants. It seems that Zn positive effects on vegetative growth is because of its crucial role in IAA biosynthesis, photosynthesis and N metabolism [Hafeez et al. 2013]. Considering above, the increased growth of rosemary plants in our experiment is seemingly due to IAA production and its related effects on growth parameters. Zn has essential roles in protein biosynthesis, cell membranes integrity and the tolerance of plants to the environmental stresses [Hafeez et al. 2013, Vojodi Mehrabani et al. 2016].

CONCLUSIONS

Salinity is one of the major stressors affecting the plant growth and productivity. The most important with the salinity stress are its long-lasting effects. Unlike some other stresses which affect just some growth phases of the plants, salinity stress influences all the growing periods of plants by reducing the osmotic potential and interferes the absorption of nutrients like K^+, Ca^{2+} and NH_4^+ and finally reduces the plant growth and productivity. Salt absorption and ionic toxicity drastically influence the cell ongoing metabolism and physiological processes. Results from the present experiment clearly showed negative effects of salinity on plant yield, carotenoids and soluble solids content. Salinity increased peroxidases and MDA content, as well. Zinc foliar spray improved flavonoids, total soluble solids and essential oil contents. In total, rosemary is capable of tolerating the salinity levels up to 50 mM and that nano-zinc foliar application was capable of enhancing the plants yield and productivity under saline-sodium conditions.

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