Arid Land Research and Management

ISSN: 1532-4982 (Print) 1532-4990 (Online) Journal homepage: http://www.tandfonline.com/loi/uasr20

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**To cite this article:** Ahlem Zrig, Jorge F. S. Ferreira, Foued Hamouda, Taieb Tounekti, Samy Selim, Soad Al Jaouni, Habib Khemira & Hamada Abdelgawad (2019): The impact of foliar fertilizers on growth and biochemical responses of *Thymus vulgaris* to salinity stress, Arid Land Research and Management, DOI: 10.1080/15324982.2018.1551817

**To link to this article:** https://doi.org/10.1080/15324982.2018.1551817

Published online: 12 Jan 2019.

ARID LAND RESEARCH AND MANAGEMENT
https://doi.org/10.1080/15324982.2018.1551817

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RESEARCH NOTE



# The impact of foliar fertilizers on growth and biochemical responses of *Thymus vulgaris* to salinity stress

Ahlem Zrig[a], Jorge F. S. Ferreira[b], Foued Hamouda[c], Taieb Tounekti[d], Samy Selim[e,f], Soad Al Jaouni[g], Habib Khemira[a,d], and Hamada Abdelgawad[h,i]

[a]Research Unit of Biodiversity and Valorization of Bioresources in Arid Zones, Faculty of Sciences of Gabès- City Erriadh, Gabes, Tunisia; [b]US Salinity Laboratory (USDA-ARS), Riverside, CA, USA; [c]Research Unit in Enterprise and Decisions, Higher Institute of Management, Gabes, Tunisia; [d]Centre for Environmental Research and Studies (CERS), Jazan University, Jazan, Kingdom of Saudi Arabia; [e]Department of Clinical Laboratory Sciences, College of Applied Medical Sciences, Jouf University, Sakaka, Saudi Arabia; [f]Microbiology and Botany Department, Faculty of Science, Suez Canal University, Ismailia, Egypt; [g]Department of Hematology and Youssef Abdulatif Jameel Chair of Prophetic Medicine Application (YAJCPMA), Faculty of Medicine, King Abdulaziz University, Jeddah, Kingdom of Saudi Arabia; [h]Laboratory for Molecular Plant Physiology and Biotechnology, Department of Biology, University of Antwerp, Antwerp, Belgium; [i]Department of Botany and Microbiology, Faculty of Science, Beni-Suef University, Beni-Suef, Egypt

**ABSTRACT**

Salinity reduces plant biomass and may lead to death when severe. To cope with the negative effects of this stress, plant species present specific physiological or biochemical responses. In this work, we hypothesized that spraying salt-stressed thyme leaves with $K^+$ and $Ca^{2+}$ could mitigate the negative effects of salinity on plant growth and metabolism. To test this hypothesis, we grew thyme plants under salinity stress for two and four weeks before applying foliar sprays. Also, to test the effect of stress relief, treated plants were allowed two weeks of recovery after four weeks of salt stress. In general, after two and four weeks of salinity stress, the leaf fresh weight of thyme plants was reduced by 31 and 43%, respectively. Salinity also decreased the relative water content, water, and osmotic potentials and led to ion imbalances and nutrient deficiencies. Salinity altered concentration of some essential oils, but leaf antioxidant contents remained fairly stable, except for a significant increase for plants under NaCl + KCl two weeks after treatment. Our results indicated that stressed plants accumulated significantly more soluble sugars and amino acids in comparison with the control. Foliar sprays with KCl and $CaCl_2$ reversed the negative effects of salinity on plant biomass and induced the accumulations of compatible solutes. Moreover, concentrations of some essential oils and gallic acid increased in sprayed plants, but these effects were dependent on the type and duration of the treatment. Overall, spraying leaves with $K^+$ and $Ca^{2+}$ was able to mitigate salinity stress in *Thymus vulgaris* even during the recovery period.

**ARTICLE HISTORY**
Received 8 October 2017
Accepted 20 November 2018

**KEYWORDS**
Amino acid; antioxidant activity; carbohydrate; essential oils; osmotic adjustment; Thyme

**CONTACT** Ahlem Zrig ahlem18zrig@yahoo.fr Research Unit of Biodiversity and Valorization of Bioresources in Arid Zones, Faculty of Sciences of Gabès- City Erriadh, Gabes, 6072, Tunisia.

## Introduction

Salinity may cause an imbalance in the nutrient status of plants negatively affecting plant growth and crop yield and thus, represents a worldwide threat to food security (Alizadeh et al. 2010). Under salinity stress conditions, the uptake of essential minerals by root is slow and their translocation to shoot is inefficient. For instance, salinity may cause a competition between $Na^+$ and $K^+$ and/or $Ca^{2+}$ and between $Cl^-$ and $NO_3^-$, which may lead to ion imbalances and nutrient deficiencies. $K^+$ contributes to many plant physiological processes such as photosynthesis, protein metabolism, and the maintenance of osmotic potential (Alizadeh et al. 2010; Tounekti et al. 2010, 2011). The cation $Ca^{2+}$ is also involved in cell membrane integrity, cell division, and cell elongation. Under salinity conditions, mineral application to soil decreased plant growth inhibition by increasing the production of secondary metabolites and by compensating the reduced levels of macronutrients (Singh, Ganesha Rao, and Ramesh 2007). Therefore, essential minerals fed to plants under stress may play a vital role in mitigating salinity stress on plant growth and function.

Salinity-induced growth reduction is accompanied by a decrease in relative water content (RWC) and altered water and osmotic potentials. Osmolyte accumulation in stressed plants usually occurs in conjugation with osmotic potential adjustment (OA) and may lead to stress alleviation (Hoque et al. 2007; Neocleous and Vasilakakis 2007; Ben Khaled et al. 2003). Salinity also increases the levels of reactive oxygen species (ROS), which in turn, modulate the antioxidant defense responses. Although ROS plays an important role in plants as signals, their over-accumulation leads to plant cell damage (Jaleel et al. 2007; Stanev 2010; Baser and Buchbauer 2010). To circumvent the deleterious effects of ROS and to minimize ROS-induced cell damage, plants have evolved a robust antioxidant defense system (Sairam and Tyagi 2004; Jaleel et al. 2007), in which a wide range of antioxidants (e.g. polyphenols, tocopherols, ascorbate, and glutathione) are involved. Furthermore, concentrations of some essential oil (EOs) and amino acids have been reported to increase during adaptation to various environmental stresses (Kowalczyk and Zielony 2008; Tounekti et al. 2011). Essential oils are considered as potential antioxidants in several medicinal plants, such as *Salvia officinalis* (Tounekti et al. 2011) and *Mentha suaveolens* (Kasrati et al. 2014). Thus, these compounds may play an important role in plant fitness under arid environmental conditions (Taiz and Zeiger 2004, Jordán et al. 2009). Some plant secondary metabolites, such as phenolic compounds, have redox properties, which allow them to act as reducing agents, hydrogen donors, and singlet oxygen quenchers. They also have metal chelation properties (Jordán et al. 2009).

Applying mineral fertilizers through foliar spray was reported as a good approach to increase plant yield under controlled stress conditions. Applying nutrients to the soil is not the most efficient method because some nutrients bind to soil particles and become unavailable (Dordas 2009) or the salt-stressed plant does not absorb enough water and, consequently, the essential salts (fertilizers) contained in that water solution. Therefore, foliar spray with nutrients is considered as an easy and efficient method to apply fertilizers that might help plants cope with environmental stresses. In this context, Kaya et al. (2002) reported the stimulating effects of $K^+$ and $Ca^{2+}$ on plant performance and defense against salinity stress. The growth promotion effects of $Ca^{2+}$ and $K^+$ feeding could be explained by their role in the regulation of ion transport and membrane

selectivity (Mumivand et al. 2011) and enhancing the osmotic adjustment via biosynthesis of sugars (Zhao, Oosterhuis, and Bednarz 2001) and amino acids (Takahashi et al. 2012).

Thyme (*Thymus vulgaris* L.) is an aromatic and medicinal plant belonging to the Lamiaceae family. It is widely used in medicine and in food preservation (Bassolé and Rodolfo-Juliani 2012). Its EO was reported to have biological activities including insecticidal (Ayvaz et al. 2010), larvicidal, antifungal, and antibacterial. The most active thyme EO ingredients are thymol and carvacrol. The best way to guarantee a stable supply of plant material for the EO industry is to increase cultivation of this species. However, the wider cultivation of thyme in semiarid regions requires the knowledge of its adaptation to environmental stresses, such as drought and salinity, and on the effect of these environmental constraints on its growth and metabolism (Lubbe and Verpoorte 2011), including the metabolism leading to EO biosynthesis and other compounds associated with plant survival under stress. In Tunisia, thyme species are grown wildly in calcareous soils and occur scattered in small populations in different bioclimatic zones extending from the sub-humid to the lower arid areas where the species becomes scarce. Recently, thyme was cultivated under greenhouse conditions and irrigated with low-quality water with elevated salinity. High salinity in irrigation water cause rapid reduction in plant growth and induce metabolic changes in response to salt stress. Also, it was reported that abiotic stress, such as salinity, can increase the accumulation of several secondary metabolites (Tounekti et al. 2011). It is expected that good management and adoption of suitable agronomic practices will improve water conservation while maintaining efficient crop production under saline irrigated conditions. The aim of the current study was to explore the effect of salinity stress and/or foliar spray of $Ca^{2+}$ and/or $K^{+}$ on thyme growth and its biochemical response to salinity stress. Our hypothesis was that increased $Ca^{2+}$ or $K^{+}$ availability, provided as foliar fertilizers, would mitigate the effect of salinity from irrigation water.

## Materials and methods

### *Plant material, salt treatments, and leaf spray*

One-year-old *Thymus vulgaris* plants were grown in plastic pots containing dune-sand and perlite (2:1). The pots were covered with aluminum paper to reduce evaporation and contamination. Irrigation was done every 4 days with a complete nutrient solution (N, 1.8 mM; P, 0.35 mM; K, 0.64 mM; Ca, 1.0 mM; Mg, 0.35 mM; S, 0.35 mM; Fe, 0.03 mM; Zn, 0.4 µM, Mn, 5.0 µM; Cu, 0.1 µM and B, 0.02 mM). The treatments applied to potted plants were as follows: (i) control (nutrient solution alone); (ii) control + 100 mM NaCl; (iii) control + 100 mM NaCl, followed by foliar spray with 10 mM KCl; (iv) control + 100 mM NaCl, followed by foliar spray with 10 mM $CaCl_2$. The foliar spraying with $CaCl_2$ or KCl was done every 4 days on the entire plant canopy, while control plants were sprayed only with distilled water. To avoid osmotic shock, the NaCl concentration in the plant growth medium was gradually increased by 50 mM per day to achieve the target concentration of 100 mM. Treatments were applied for four weeks and to assess plant responses once stress was removed (recovery responses), salt-stressed plants (treatment ii) were irrigated with municipal water (low

salinity) for two weeks. Then, the leaves were collected, immediately frozen in liquid nitrogen and stored at −80 °C for biochemical analyses.

### Leaf ion concentration

After six weeks of experiment, before collection for ion analysis, plants were irrigated twice over the top with municipal water to remove excess salts from foliar sprays, then the leaves were collected and dried at 80 °C for 48 hours. Dry leaves (1.0 g) were extracted with 20 mL of 0.1 M $HNO_3$. After filtration, $Na^+$, $K^+$, and $Ca^{2+}$ concentrations were determined with an atomic absorption spectrometer (Avanta, GBC, Australia).

### Leaf relative water content and osmotic potential adjustment

Leaf relative water content [(RWC (%)] was measured and calculated as follows: RWC (%) = 100 × [(FW – DW)/(TW– DW)], where FW = fresh weight, DW = dry weight, and TW = turgid weight determined after soaking the leaf samples in distilled water for 24 hours at 4 °C in a refrigerator. Dry weight was measured after oven-drying the samples for 48 hours at 80 °C. Four leaves from the upper stem of each plant were used to measure RWC (%).

Predawn leaf water potential (Ψw) was also measured on four median leaves using Scholander pressure chamber (PMS, Albany, OR, USA, Gucci, Lombardini, and Tattini 1997). The osmolality of the expressed sap was measured with a vapor pressure osmometer (Wescor 5520, Logan, UT, USA). The osmolality values were converted to osmotic potential (Ψs) by the van't Hoff equation: Ψs = –ciRT, where ci = weight concentration of solute, R is a gas constant = 8,31 J.mol$^{-1}$. K$^{-1}$, and T is the temperature in °C (Nobel 1992). Hydrostatic pressure or turgor potential (Ψp) was calculated as the difference between osmotic potential (Ψs) and water potential (Ψw) values (Ψp = Ψw – Ψs).

Total osmotic potential adjustment (OA) was calculated as the difference in osmotic potential at full turgor between the control and salt-stressed plants (Martinez-Ballesta, Martinez, and Carvajal 2004). The osmotic concentrations of solutes were calculated by the van't Hoff Equation: Ψsi = −0.002479 (RDW) C, where Ψsi indicates the contribution (%) of solutes (individual Ψs); RDW is the dry mass relative to saturation (kg m$^{-3}$): RDW = DW/TW-DW; C is the molar concentration of solute (mol kg$^{-1}$); and 0.002479 m$^3$ MPa mol$^{-1}$, R is a gas constant at 25 C°T temperature. Also, it was assumed that the osmotic solutes exhibit ideal behavior (Alarcon et al. 1993).

### Extraction and analysis of carbohydrates

Sugars were extracted and analyzed using the method of Alasalvar et al. (2003). Approximately 100 mg of frozen leaves were extracted in 2 mL acetonitrile/water (1:1, *v/v*) for 2 min. The extract was kept in a water bath at 55–60 °C for 15 min and then filtered over a Whatman No. 541 filter paper. Sample filtrates were then used for the determination of the sugar contents using liquid chromatography (HPLC). The mobile phase (acetonitrile and HPLC-grade water at a ratio of 75:25 (*v/v*)) was used and column temperature and injection volume were set at 30 °C and 20 µL, respectively. Sugars

were quantified on the basis of peak areas and comparison with a calibration curve obtained with the corresponding standards (1–10 mg/100 mL of acetonitrile/water (1:1, *v/v*)) of sucrose, glucose, fructose, and starch. Sugars were expressed as milligrams per 100 g of leaf fresh weight (mg/100 g FW).

### Analysis of amino acids

Leaves were homogenized by using a MagNALyser (Roche, Vilvoorde, Belgium) for 1 min, at 7000 rpm, in 80% (*v/v*) aqueous ethanol (1 mL). Norvaline was used as an internal standard to determine the loss of amino acids during extraction. The homogenate was centrifuged at 14000 rpm for 20 min, the supernatant was evaporated under vacuum and the pellet was resuspended in chloroform (1 mL). Simultaneously, the residue was re-extracted with HPLC grade water (1 mL) using the MagNALyser and the supernatant was centrifuged (14000 rpm for 20 min) and mixed with the pellet suspended in chloroform. Then they were centrifuged for 10 min at 14000 rpm and the aqueous phase was filtered using Millipore micro filters (0.2 µM pore size) before assaying for free amino acids (FAA). Amino acids were derivatized by precolumn 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate (AQC) by using a Waters AccQ Tag$^{TM}$ reagent kit equipped with a Sinhaa BEH amide 2.1 × 50 column. The mobile phase A was eluent A diluted 1:10 with ultrapure water while B was 60% (*v/v*) acetonitrile in water (Eluent A contained: 140mM sodium acetate, 015 mM sodium azide, 0.25 mM EDTA, and 17 mM tri-ethyl amine dissolved in ultrapure water and its pH was adjusted to 4.95 with phosphoric acid). The injection volume was 5 µL, and UV detection of AQC amino acid derivatives was performed at 254 nm. Data were managed using Chromeleon chromatography software package (Salim and Ahmed 1992).

### Extraction and analysis of the essential oil

Extraction and identification of EOs were performed according to Zandi-Sohani (2011) by using hydrodistillation and GC/MS chromatography. EOs extraction was performed on frozen leaves collected from each plant. Extractions were performed under steam distillation at atmospheric pressure, using a modified Clevenger-type apparatus. The EOs were separated from the aqueous phase by adding chloroform. The dry EO components were redissolved in hexane 100% (*v/v*) and analyzed by GC/MS, as described by Tounekti et al. (2010). Analyses of EOs were performed with a GC/MSD HP 6890 Series II (Hewlett Packard, Wilmington, NC, USA) equipped with an on-line injection system and an HP 5973 A mass-selective detector. The instrument was equipped with an HP-5 (5% biphenyl-95% dimethylsiloxane copolymer) capillary fused-silica column (30 m × 0.25 mm; film thickness 0.25 mm). The initial oven temperature was held at 60 °C for 5 min, then programed to increase at a rate of 5 °C min$^{-1}$ to 220 °C, then held at 220 °C for 20 min. Helium was used as a carrier gas at a flow rate of 2 mL min$^{-1}$. The injection volume was 0.5 mL and the split ratio was 1:60. The mass spectra were obtained by electron impact ionization at 70 eV. The identification of the oil constituents was based on their GC-retention times and by matching their mass spectra with those of a Wiley-HP reference library in the instrument. The percentage composition of EOs was calculated from peak areas.

### Determination of polyphenols and flavonoids

Approximately 50 mg of fresh leaves were homogenized in 0.5 ml ethanol (80%) to extract polyphenols and flavonoids. After centrifugation, the pellet was washed twice with 0.5 ml 80% ethanol (*v/v*) and supernatants were combined. Total phenolic content was determined using a Folin–Ciocalteu assay according to Zhang et al. (2006), using gallic acid as a standard. Flavonoid content was estimated using the modified aluminum chloride colorimetric method (Chang et al. 2002), with quercetin as a standard.

### Determination of total antioxidant capacity (FRAP)

Antioxidants were extracted by homogenizing 150 mg of fresh tissue in 80% ethanol (1:5%, *v/v*). Total antioxidant capacity (TAC) was measured by using the ferric reducing/antioxidant power (FRAP) assay reagent (0.3 M acetate buffer, pH 3.6, 0.01 mM TPTZ (2,4,6-Tris (2- pyridyl)-s-triazine) in 0.04 mM HCl, 0.02 M $FeCl_3{\cdot}6H_2O$). The assay reagent was mixed with the extract and the mixture was read at 600 nm using a microplate reader (Synergy Mx, BiotekInstruments Inc., Vermont, USA), according to Benzie and Strain (1999). Trolox was used as the standard, and the results are given as µmoles Trolox equivalents $g^{-1}$ of fresh weight.

### Statistical analyses

Variance of data was analyzed with the GLM procedure of SAS software (SAS Institute Inc., Raleigh, USA, 1996) for a Randomized Complete Block design with eight replicates. Where applicable, means were separated by Duncan's Test ($p \leq 0.05$). To normalize the data, EO data (percentages) were Log-transformed before analyses.

## Results

### Plant growth, leaf relative water content and osmotic potential adjustment (OA)

Salt stress (100 mM NaCl) decreased the fresh weight of thyme plants significantly by approximately 31 and 43%, 2 and 4 weeks after treatment, respectively ($p \leq 0.05$, Figure 1). However, no significant effects were observed in the subsequent two-week recovery period. The foliar application of both KCl and $CaCl_2$ on stressed plants significantly increased leaf biomass compared to plants under 100 mM NaCl, except for plants sprayed with $K^+$ during the recovery period (Figure 1). Particularly, spraying salt-stressed plants with $K^+$ increased leaf FW by 35 and 46% after 2 and 4 weeks, respectively compared to stressed, but not sprayed plants.

Plant water relation measurements showed that thyme plants were severely affected under salinity stress (Figure 1) and each parameter showed a characteristic time course of evolution in salinity-stressed plants. The RWC (%) decreased significantly and progressively under NaCl treatment alone at any time, including recovery, and with foliar $CaCl_2$ spray four weeks after treatment, while a slight decrease in RWC was observed for plants fed foliar KCl compared to control plants. The recovery treatment restored leaf turgor, as indicated by the increase in RWC in plants sprayed with both $CaCl_2$ and KCl (Figure 1).

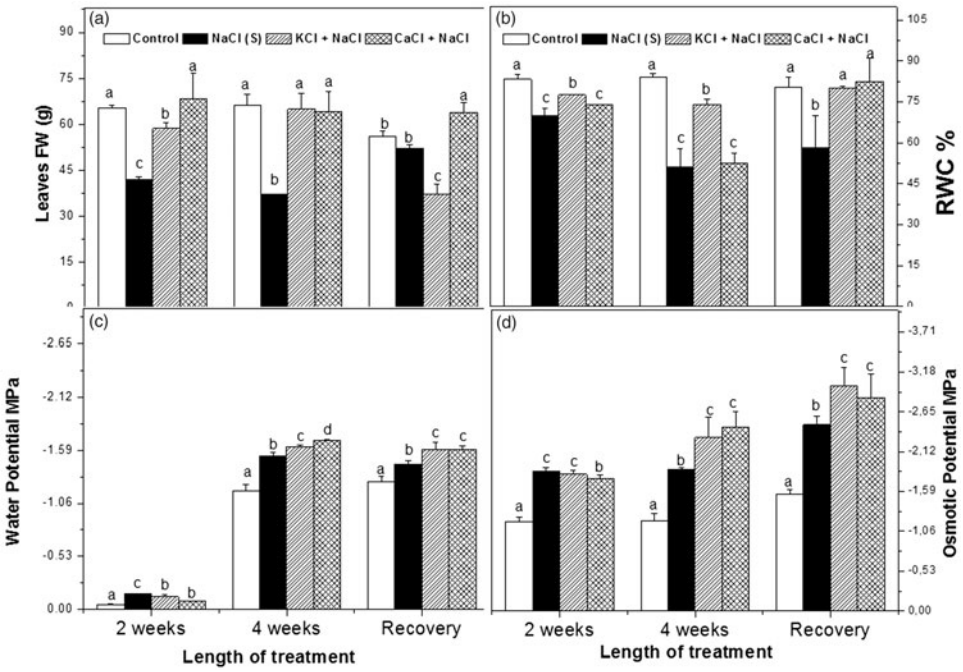


**Figure 1.** Biomass and water relations: Fresh weight, relative water content (RWC %), water and osmotic potential of the thyme leaves exposed to the following treatments: (1) 0 NaCl (control), (2) 100 mM NaCl, (stress (S)), (3) stressed leaves treated with KCl and (4) stressed leaves treated with $CaCl_2$ grown for two and four weeks of treatments and two weeks of recovery. Each bar represents the mean (± SE) of eight replicates and different letters indicate significant differences between treatments (Duncan test, $p \leq 0.05$).

The water potential ($\Psi_w$) of NaCl-treated plants decreased from −0.05 MPa to −0.15 MPa after 2 weeks and it reached −1.59 MPa after four weeks of treatment (Figure 1). Clearly, the strongest reductions were observed in NaCl-treated plants sprayed with $Ca^{2+}$ and $K^+$ with leaf $\Psi_w = -1.63$ MPa four weeks after treatment and also after the recovery period. NaCl-treated plants sprayed with $CaCl_2$ and KCl decreased the osmotic potential (−1.8 and −1.7 MPa, respectively) after two weeks as compared to control (Figure 1). Leaf osmotic potential drastically increased after four weeks of treatment, while in the subsequent recovery, the lowest osmotic potential was observed in salt-treated plants sprayed with KCl, which reached −2.84 MPa. Our results also showed that during salinity stress, osmotic adjustment (OA) increased in thyme leaves sprayed with $CaCl_2$ or KCl as compared to stressed plants (Table 1). Similarly, in the subsequent recovery, the OA in all treated plants increased compared to control plants.

### *Leaf ion concentration*

Salinity provided by the 100 mM NaCl treatment increased $Na^+$ concentrations in plant tissues 23-fold at two weeks, 6-fold at four weeks, and in 97-fold at recovery (Table 2). However, compared to plants under 100 mM NaCl, leaf Na accumulation at two weeks decreased 3.5- and 2.8-fold after leaf sprays of KCl and $CaCl_2$, respectively, and 2-fold

**Table 1.** Turgor potential (Ψp) and osmolality (OA) of thyme leaves exposed to the following treatments: (1) 0 NaCl (control), (2) 100 mM NaCl, (stress (S)), (3) stressed leaves with NaCl (S) and treated with KCl, and (4) stressed leaves with NaCl (S) and treated with $CaCl_2$, grown for two and four weeks of treatments and two weeks of recovery.

| | Treatments | $\Psi_p$ (PMa) | OA |
|---|---|---|---|
| 2 weeks | Control | 1.0 ± 0.01**b** | |
| | 100 mM NaCl (S) | 1.7 ± 0.03a | 0.68 ± 0.10b |
| | 100 mM NaCl + KCl | 1.6 ± 0.05**a** | 0.72 ± 0.06**a** |
| | 100 mM NaCl + $CaCl_2$ | 1.69 ± 0.05**a** | 0.70 ± 0.02**a** |
| 4 weeks | Control | 0.2 ± 0.05**c** | |
| | 100 mM NaCl (S) | 1.0 ± 0.02a | 0.76 ± 0.00c |
| | 100 mM NaCl + KCl | 1.0 ± 0.01**a** | 1.60 ± 0.00**a** |
| | 100 mM NaCl + $CaCl_2$ | 0.8 ± 0.12**b** | 1.15 ± 0.06**b** |
| Recovery | Control | 0.2 ± 0.02**c** | |
| | 100 mM NaCl (S) | 1.0 ± 0.08b | 0.85 ± 0.02c |
| | 100 mM NaCl + KCl | 1.7 ± 0.02**a** | 1.44 ± 0.01**a** |
| | 100 mM NaCl + $CaCl_2$ | 1.9 ± 0.03**a** | 1.29 ± 0.38**b** |

Each value represents the mean (±SE) of eight replicates; different letters indicate significant differences between treatments (Duncan test, $p \leq 0.05$).

**Table 2.** Ion concentrations in the leaves of thyme leaves exposed to the following treatments: (1) 0 NaCl (control), (2) 100 mM NaCl, (stress (S)), (3) stressed leaves with NaCl (S) and treated with KCl and (4) stressed leaves with NaCl (S) and treated with $CaCl_2$, grown for two and four weeks of treatments and two weeks of recovery.

| | Treatment | 2 weeks | 4 weeks | Recovery |
|---|---|---|---|---|
| Na (μeq.g$^{-1}$DW) | Control | 130.13 ± 9.84d | 198,9 ± 42,15b | 78.16 ± 9.41c |
| | 100 mM NaCl (S) | 2960.91 ± 285.44a | 3591,04 ± 69.54a | 1353,27 ± 115a |
| | 100 mM NaCl + KCl | 851.09 ± 188.29c | 2075,98 ± 92.64a | 1217,24 ± 92.5b |
| | 100 mM NaCl + $CaCl_2$ | 1065.50 ± 52.20b | 1091,70 ± 12.13b | 1196,50 ± 8,1b |
| K (μeq.g$^{-1}$DW) | Control | 693.09 ± 0.00a | 1083.63 ± 0.00a | 764.13 ± 18.57a |
| | 100 mM NaCl (S) | 435.42 ± 48.24c | 301.27 ± 36.59c | 424.04 ± 25.83c |
| | 100 mM NaCl + KCl | 625.31 ± 0.50a | 530.56 ± 292.86b | 806.20 ± 39.55a |
| | 100 mM NaCl + $CaCl_2$ | 476.21 ± 8.02b | 309.84 ± 42,98c | 652.42 ± 62b |
| Ca (μeq.g$^{-1}$DW) | Control | 1374.34 ± 0.00a | 914.27 ± 30.69a | 800.09 ± 19.07a |
| | 100 mM NaCl (S) | 1019.71 ± 94.64b | 707.13 ± 47.07b | 733.71 ± 33.83b |
| | 100 mM NaCl + KCl | 932.24 ± 7.703b | 747.44 ± 36.19b | 778.94 ± 66.29b |
| | 100 mM NaCl + $CaCl_2$ | 1232.41 ± 151.02a | 847.26 ± 40.59a | 875.05 ± 24.16a |

Values are the means ± SE of four replicates. Different letters indicate significant differences between treatments (Duncan test).

for both KCl and $CaCl_2$ after two weeks of recovery (Table 2). Leaf sprays had no effect on either $K^+$ or $Ca^{2+}$ accumulation in leaves, at any time after treatment, but leaves showed significant increases in both $K^+$ and $Ca^{2+}$ content when comparing plants under 100 mM NaCl and plants that received leaf spray treatments (Table 2). Salinity reduced leaf $K^+$ 37–72% at two and four weeks after treatment, respectively, but this reduction was only from 10–51%, at the same periods, when salt-stressed plants received foliar spray of KCl. Salinity also reduced leaf $Ca^{2+}$ 26 and 23% at two and four weeks after treatment, respectively, but this reduction was only 10 and 7%, at the same periods, when salt-stressed plants received foliar sprays of $CaCl_2$ (Table 2).

## *Essential oils*

The effects of salinity stress and of foliar sprays of $CaCl_2$ or KCl on EOs concentrations in thyme leaves were assessed based on the percentage of the 12 main EO constituents

(Table 3). At week 2, significant decreases were observed between control and NaCl treatments for α-pinene, g-terpinene, thymol, carvacrol, linalool, and borneol; at week 4, for α-pinene, camphene, p-cymene, g-terpinene, carvacrol, b-caryophyllene, and limonene. During recovery, between control and NaCl-stressed plants, there were significant decreases in the concentrations of camphene, g-terpinene, carvacrol, ß-caryophyllene, limonene, and borneol, while there were significant increases in α-thujone, α-pinene, p-cymene, and thymol. In control plants, the most abundant constituents of the EO in thyme were thymol (±30%), linalool (±21%), p-cymene (±15%), camphene = borneol (±6%), myrcene (4–9%). The highest increases in response to salinity were observed for myrcene both at weeks two and four and borneol at week 4 (Table 3). Regarding foliar applications of KCl and $CaCl_2$, significant increases in EO were only observed for myrcene at week 2 and thymol at week 4. During recovery, significant increases in response to KCl and $CaCl_2$ foliar applications were only observed for p-cymene.

The foliar application of KCl only increased p-cymene concentrations in stressed leaves by 32% compared to the control condition. However, the foliar supply of $CaCl_2$ increased the α-pinene and myrcene by 72 and 30%, respectively. KCl treatments caused an increase in the level of thymol and linalool contents by 35 and 34%, respectively as compared to stressed and controlled leaves after 30 days of stress. On the other hand, $CaCl_2$ foliar application in salt-stressed plants enhanced the accumulations of linalool (29%) and borneol (30%) as compared to control condition. In addition, the salinity caused a significant increase in p-cymene, myrcene, and α-pinene levels as compared to control in the subsequent recovery treatment. While the $CaCl_2$ foliar applications reduced the level of thymol in stressed-plants, but improved the accumulations of α-pinene, α-thujone, p-cymene, and myrcene.

### *Carbohydrate levels*

Compared to control, glucose levels increased after four weeks in both NaCl-only and in sprayed plants and only in 100 mM NaCl (NaCl) and KCl-sprayed plants during the recovery period. Fructose content increased significantly in $CaCl_2$-sprayed plants after two weeks and in NaCl and KCl-sprayed plants at four weeks and recovery. Sucrose content increased significantly in KCl- and $CaCl_2$-sprayed plants at two weeks and was significantly higher in KCl-sprayed plants at four weeks and recovery period, compared to the remaining treatments. Starch concentrations significantly increased, compared to control, in all treatments at two weeks, in sprayed plants, compared to control and NaCl and in NaCl and KCl-sprayed plants at the recovery period. (Figure 2). Compared to the control, NaCl treatment increased the concentration of glucose by 15, 69, and 71% at weeks 2, 4, and recovery period, respectively. Fructose levels increased significantly in response to high salinity by 34% after 4 weeks and by 36% in the recovery period. The foliar applications of $CaCl_2$ increased the concentration of glucose and fructose by 38 and 34%, respectively, only after two weeks of treatment. Similarly, the foliar applications of KCl increased significantly (71 and 63%) the concentrations of glucose after four weeks and after the recovery period. Besides, the fructose levels increased significantly (35 and 33%) after 4 weeks and after the recovery period when thyme leaves

**Table 3.** Essential oils composition (%) of leaves of plants of thyme exposed to the following treatments: (1) 0 NaCl (control), (2) 100 mM NaCl, (stress (S)), (3) stressed leaves with NaCl (S) and treated with KCl and (4) stressed leaves with NaCl (S) and treated with $CaCl_2$, grown for two and four weeks of treatments and two weeks of recovery.

| | Treatments | a-thujone | a-pinene | camphene | p-cymene | myrcene | g-terpinene | thymol | carvacrol | b-caryophyllene | limonene | linalool | borneol |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 2 weeks | Control | 2.3 ± 0.45a | 2.9 ± 0.43a | 6.8 ± 0.76ab | 12.0 ± 0.96ab | 4.3 ± 0.70c | 1.3 ± 0.11a | 32.8 ± 1.20a | 2.0 ± 0.12a | 3.5 ± 0.50a | 0.2 ± 0.03a | 21.2 ± 1.75a | 14.7 ± 0.54a |
| | 100 mM NaCl (S) | 2.8 ± 0.29a | 1.9 ± 0.07b | 9.5 ± 0.81a | 12.5 ± 0.14ab | 11.2 ± 1.73b | 1.02 ± 0.12b | 24.0 ± 0.84b | 1.8 ± 0.24ab | 4.9 ± 0.14a | 0.2 ± 0.00a | 18.05 ± 0.6b | 8.6 ± 1.26c |
| | 100 mM NaCl + KCl | 1.3 ± 0.33b | 1.7 ± 0.51b | 4.5 ± 1.06b | 14.7 ± 0.31a | 17.4 ± 1.07b | 1.3 ± 0.12a | 24.1 ± 0.20b | 1.3 ± 0.23b | 3.2 ± 0.98a | 0.1 ± 0.03b | 18.1 ± 0.15b | 10.8 ± 0.09b |
| | 100 mM NaCl + $CaC_2$ | 2.4 ± 0.33**a** | 3.0 ± 0.56a | 7.6 ± 0.61a | 11.1 ± 1.04b | 21.2 ± 0.32a | 1.1 ± 0.30b | 22.6 ± 3.37c | 1.7 ± 0.12ab | 2.4 ± 0.17b | 0.1 ± 0.03b | 16.9 ± 2.53c | 14.4 ± 1.79a |
| 4 weeks | Control | 1.7 ± 0.13ab | 2.2 ± 0.39a | 6.2 ± 0.50a | 15.1 ± 0.27a | 9.6 ± 1.13b | 1.1 ± 0.30a | 29.4 ± 3.22c | 3.4 ± 1.0b | 2.4 ± 0.44a | 0.1 ± 0.04a | 22.1 ± 2.41b | 5.7 ± 0.94c |
| | 100 mM NaCl (S) | 2.04 ± 0.48a | 1.52 ± 0.21b | 3.8 ± 0.28c | 8.7 ± 1.21b | 12.7 ± 2.53a | 0.3 ± 0.02b | 32.7 ± 4.86b | 1.1 ± 0.02c | 1.7 ± 0.04b | 0.06 ± 0.00ab | 24.2 ± 3.29b | 10.9 ± 0.03b |
| | 100 mM NaCl + KCl | 1.9 ± 0.04a | 0.8 ± 0.02c | 3.3 ± 0.05c | 6.3 ± 0.09c | 3.5 ± 0.02c | 0.22 ± 0.004b | 45.9 ± 0.27a | 4.3 ± 0.20a | 0.8 ± 0.02c | 0.04 ± 0.00b | 27.9 ± 2.17a | 11.9 ± 0.33b |
| | 100 mM NaCl + $CaCl_2$ | 1.6 ± 0.04c | 1.2 ± 0.47b | 3.2 ± 0.84c | 8.8 ± 1.07b | 5.9 ± 2.58c | 0.3 ± 0.05b | 34.7 ± 2.34b | 0.9 ± 0.08c | 1.3 ± 0.06b | 0.05 ± 0.01b | 26.0 ± 1.75a | 15.6 ± 1.05a |
| Recovery | Control | 4.6 ± 0.45b | 1.9 ± 0.148a | 7.2 ± 0.53a | 12.4 ± 0.89c | 15.2 ± 0.87b | 0.5 ± 0.04a | 24.0 ± 1.67b | 1.1 ± 0.08b | 1.7 ± 0.08b | 0.1 ± 0.00a | 18.0 ± 1.25a | 11.4 ± 0.62a |
| | 100 mM NaCl (S) | 7.2 ± 0a | 2.4 ± 0.29b | 4.7 ± 0.00b | 19.9 ± 1.05a | 16.1 ± 1.58a | 0.2 ± 4.83E-5b | 27.8 ± 0.3a | 0.6 ± 0.03c | 0.9 ± 3.67E-4c | 0.05 ± 0.00b | 16.2 ± 0.03b | 6.6 ± 0.01b |
| | 100 mM NaCl + KCl | 4.3 ± 0.34b | 2.11 ± 0.18b | 4.8 ± 0.13b | 20.9 ± 1.94a | 13.7 ± 0.75c | 0.3 ± 0.02b | 31.4 ± 3.81a | 0.8 ± 0.05c | 1.2 ± 0.12b | 0.05 ± 0.00b | 17.7 ± 0.55a | 10.6 ± 0.33a |
| | 100mM NaCl + $CaCl_2$ | 5.2 ± 0.73ab | 3.6 ± 0.41a | 9.1 ± 0.78a | 16.8 ± 0.95b | 17.9 ± 1.47a | 0.7 ± 0.13a | 18.9 ± 4.55c | 1.4 ± 0.20a | 2.5 ± 0.27a | 0.04 ± 0.01b | 12.7 ± 0.83c | 5.4 ± 0.91b |

Each value represents the mean (±SE) of eight replicates, different letters indicate significant differences between treatments (Duncan test, $p \leq 0.05$). Values are the means ± SE of eight replicates.

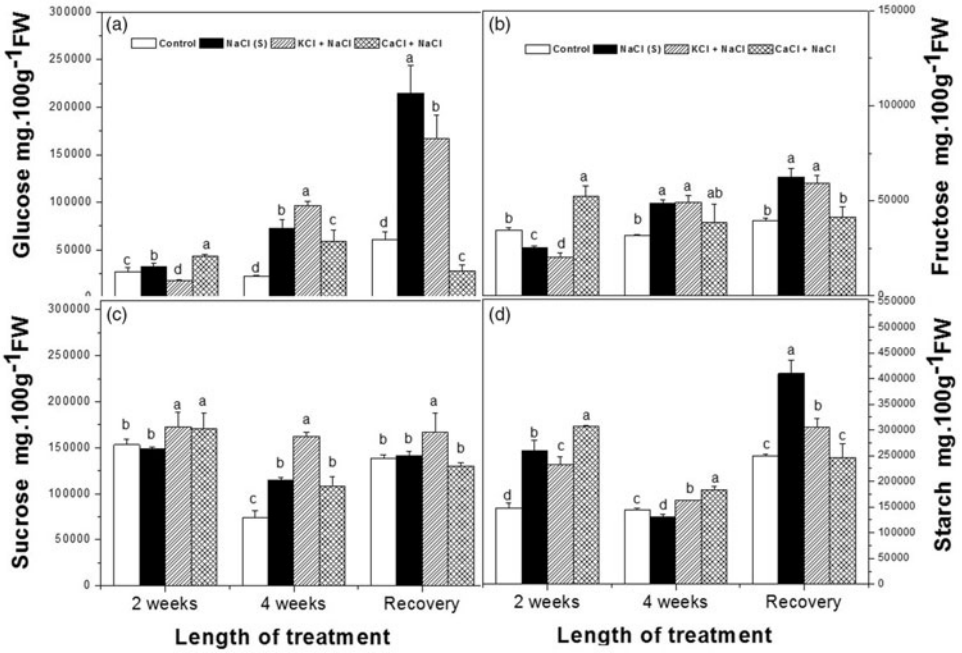


**Figure 2.** Sugars contents: Glucose, fructose, sucrose, and starch concentrations in thyme leaves exposed to the following treatments: (1) 0 NaCl (control), (2) 100 mM NaCl, (stress or S), (3) stressed leaves treated with KCl and (4) stressed leaves treated with $CaCl_2$ grown for two and four weeks of treatments, and two weeks of recovery. Each bar represents the mean (±SE) of eight replicates. Different letters indicate significant differences between treatments (Duncan test, $p \leq 0.05$).

were sprayed with KCl. Sucrose levels always increased significantly in response to leaf applications of both KCl and $CaCl_2$ at week 2, but only in response to KCl application at week 4 and after the recovery period (Figure 2). Leaf applications of $CaCl_2$ also caused a significant increase in carbohydrate level (except glucose) at all times evaluated, but the increase was not as high as that triggered by leaf application of KCl.

## *Amino acids*

Amino acid levels, except alanine, changed in salinity-stressed plants compared to the control treatment (Figures 3 and 4). Arginine content had a slight, but significant increase in NaCl and KCl applications at week 4, while all treatments, compared to control, increased arginine content during the recovery period. Asparagine content increased significantly in response to all treatments, except in response to $CaCl_2$ spray during the recovery period. Glutamic acid content only increased slightly, but significantly, in plants sprayed with KCl in week 4 (Figure 3). Glutamine content increased slightly, but significantly, in response to all treatments at all times, except for $CaCl_2$ application during the recovery time. Glycine content only rose slightly, but significantly, in response to NaCl and $CaCl_2$ applications. Ornithine content only responded to KCl applications at week 2 and slightly after the recovery period. Proline content increased significantly by 67, 83, and 38% in response to KCl applications at 2, 4, and recovery, respectively (Figure 4), in

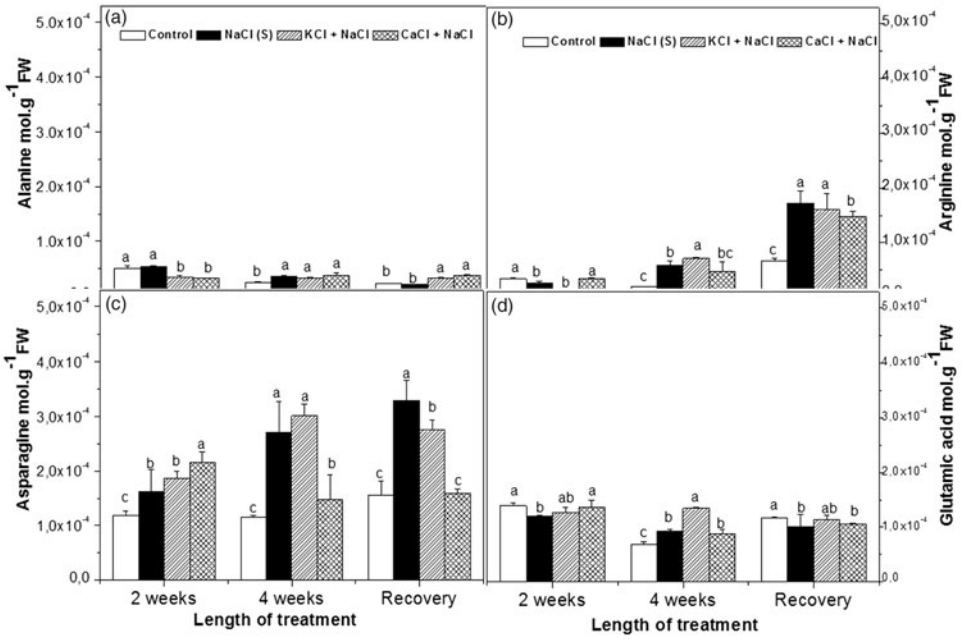


**Figure 3.** Concentration of alanine, asparagine, arginine and glutamic acid in thyme leaves exposed to the following treatments: (1) 0 NaCl (control), (2) 100 mM NaCl, (stress or S), (3) stressed leaves treated with KCl, and (4) stressed leaves treated with $CaCl_2$ grown for two and four weeks of treatments, and two weeks of recovery. Each bar represents the mean (±SE) of eight replicates. Different letters indicate significant differences between treatments (Duncan test, $p \leq 0.05$).

response to NaCl at week 4 by 64% and recovery period by 38% and to $CaCl_2$ applications at week 4 by 73%. Serine only increased in response to KCl applications at week 4 (Figure 4). Glycine, glutamine, and ornithine content increased in leaves from NaCl-stressed plants at week 2 by 20, 10, and 15%, respectively, while the level of serine was not affected by salinity. Interestingly, the levels of asparagine and proline remained remarkably high and increased further in response to treatments, throughout the experiment. In contrast, the levels of alanine, serine, and ornithine (except for K applications at week 2) remained unchanged in response to all treatments.

## *Cellular antioxidants*

The total antioxidant capacity (TAC) of thyme was evaluated by FRAP (Figure 5). After two weeks of salinity exposure, the highest TAC was detected in stressed plants sprayed with KCl, compared to control plants. Antioxidant capacities varied slightly and significantly in plants sprayed with $CaCl_2$, at both week 4 and recovery period, but FRAP values remained fairly similar.

## *Total polyphenols and flavonoids*

Under salinity stress, a significant increase in total polyphenol content, compared to the control, was observed at week 2 for NaCl and KCl foliar application treatments, while

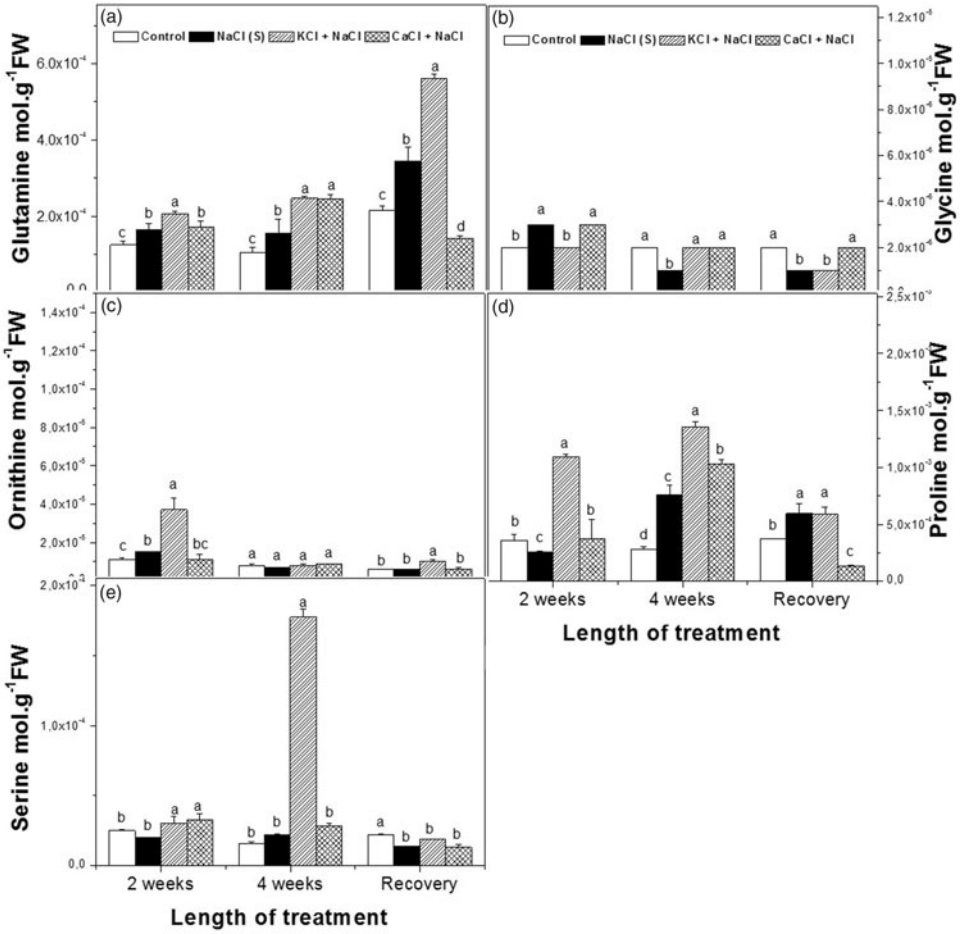


**Figure 4.** Concentration of glutamine, ornithine, serine, glycine, and proline in thyme leaves exposed to the following treatments: (1) 0 NaCl (control), (2) 100 mM NaCl, (stress or S), (3) stressed leaves treated with KCl and (4) stressed leaves treated with $CaCl_2$ grown for two and four weeks of treatments and two weeks of recovery. Each bar represents the mean (±SE) of eight replicates; different letters indicate significant differences between treatments (Duncan test, $p \leq 0.05$).

at week 4, all treatments had significantly higher total polyphenol content. At the recovery period, only NaCl-stressed plants had significantly higher polyphenol content (Figure 6). Flavonoid contents were slightly, but significantly higher in leaves at week 2 in response to NaCl and in salt-stressed plants treated with both foliar sprays, while at week 4, flavonoid contents were significantly higher only in salt-stressed plants sprayed with $CaCl_2$. During recovery, flavonoid contents were slightly, but significantly higher in salt-stressed plants treated with both foliar spray treatments (Figure 6).

### *Correlations of primary and secondary metabolite contents with TAC level*

Positive correlations (from 0.5 to 1.0) between total antioxidant capacity (TAC) were found with glucose content, the concentrations of essential oil components linalool and

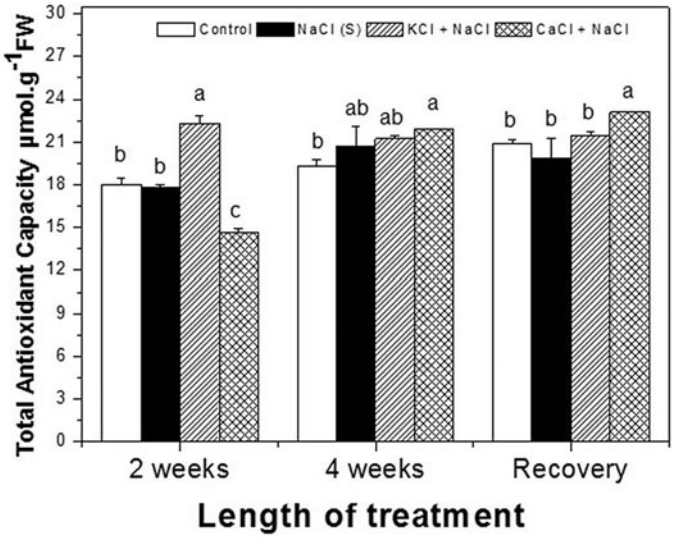


**Figure 5.** Total antioxidant activity (TAC) levels in thyme leaves exposed to the following treatments: (1) 0 NaCl (control), (2) 100 mM NaCl, (stress (S)), (3) stressed leaves treated with KCl and (4) stressed leaves treated with $CaCl_2$ grown for two and four weeks of treatments, and two weeks of recovery. Each bar represents the mean (±SE) of eight replicates. Different letters indicate significant differences between treatments (Duncan test, $p \leq 0.05$).

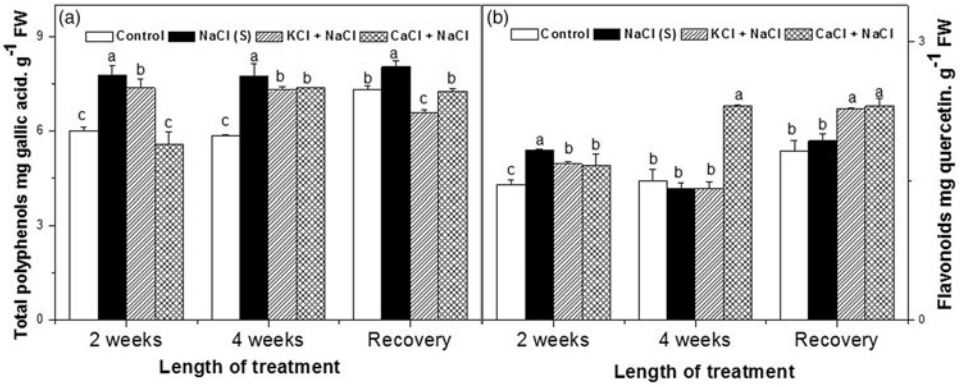


**Figure 6.** Concentrations of total polyphenols and flavonoids in thyme leaves exposed to the following treatments: (1) 0 NaCl (control), (2) 100 mM NaCl, (stress (S)), (3) stressed leaves treated with KCl and (4) stressed leaves treated with $CaCl_2$ grown for two and four weeks of treatments and two weeks of recovery. Each bar represents the mean (±SE) of eight replicates. Different letters indicate significant differences between treatments (Duncan test, $p \leq 0.05$).

thymol and of the amino acids arginine and proline, while concentrations of polyphenol and flavonoid levels correlated either with thymol and alanine concentrations and with alanine content, respectively. There was also a positive correlation between TAC (FRAP) and total polyphenol content, while there was a lower correlation ($\leq$0.5) between flavonoid and polyphenol contents or FRAP (Figure 7).

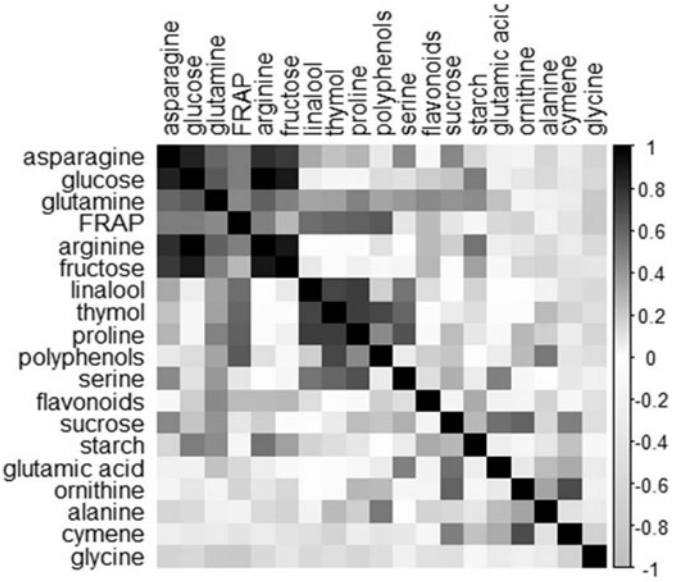


**Figure 7.** Correlations between antioxidant activities (FRAP) secondary metabolites and primary metabolites. Pearson's correlation coefficient ($n = 64$ observations) for each pair of parameters in combination is presented. The gray and black colors represent positive ($0 < r < 1$) and negative ($-1 < r < 0$) correlation, respectively.

**Table 4.** $K^+$, $Ca^{2+}$, and $Na^+$ contribution to Osmotic adjustment (OA).

| Treatments | | Ions contributions (%) | | |
|---|---|---|---|---|
| | | $Na^+$ | $K^+$ | $Ca^{2+}$ |
| 2 weeks | Control | 8.18 ± 0.36a | 4.42 ± 0.25a | 8.18 ± 0.36a |
| | 100 mM NaCl(S) | 3.63 ± 0.14c | 0.05 ± 0.01d | 3.63 ± 0.14c |
| | 100 mM NaCl + KCl | 3.14 ± 0.37c | 2.00 ± 0.07c | 3.14 ± 0.37c |
| | 100 mM NaCl + $CaCl_2$ | 5.34 ± 0.02b | 2.25 ± 0.19b | 5.34 ± 0.02b |
| 4 weeks | Control | 22.77 ± 3.18a | 2.09 ± 0.04b | 22.77 ± 3.18a |
| | 100 mM NaCl (S) | 14.79 ± 1.98b | 1.23 ± 1.98c | 14.79 ± 1.98b |
| | 100 mM NaCl + KCl | 12.22 ± 1.28c | 16.58 ± 2.3a | 12.22 ± 1.28c |
| | 100 mM NaCl + $CaCl_2$ | 6.74 ± 0.42c | 3.24 ± 0.05b | 6.74 ± 0.42c |
| Recovery | Control | 4.32 ± 0.24a | 4.13 ± 0.22b | 4.32 ± 0.24a |
| | 100 mM NaCl (S) | 1.54 ± 0.09b | 14.33 ± 1.8b | 1.54 ± 0.09b |
| | 100 mM NaCl + KCl | 1.68 ± 0.18b | 20.94 ± 2.5a | 1.68 ± 0.18b |
| | 100 mM NaCl + $CaCl_2$ | 1.95 ± 0.03b | 14.88 ± 1.1b | 1.95 ± 0.03b |

Plants of thyme exposed to the following treatments: (1) 0 NaCl (control), (2) 100 mM NaCl, (stress (S)), (3) stressed leaves with NaCl (S) and treated with KCl, and (4) stressed leaves with NaCl (S) and treated with $CaCl_2$, grown for two and four weeks of treatments and two weeks of recovery. Each value represents the mean (±SE) of eight replicates; different letters indicate significant differences between treatments (Duncan test, $p \leq 0.05$).

## *Contribution of primary and secondary metabolites to leaf osmotic adjustment (OA)*

The contribution of inorganic solutes to leaf osmotic adjustment (OA) is presented in Table 4. Spraying salt-stressed thyme plants with KCl and $CaCl_2$ did not contribute to OA in leaves at week 2, whereas $Na^+$ contributed to OA during the experimental period. Furthermore, $K^+$ ions accounted for most OA in the leaves of stressed thyme plants when sprayed with KCl at week 4 and after the recovery period by 16.58 and

**Table 5.** Glucose, fructose, sucrose, proline, glutamine, as well as asparagine contribution to osmotic adjustment (OA). Plants of thyme exposed to the following treatments: (1) 0 NaCl (control), (2) 100 mM NaCl, (stress (S)), (3) stressed leaves with NaCl (S) and treated with KCl and (4) stressed leaves with NaCl (S) and treated with $CaCl_2$, grown for two and four weeks of treatments and two weeks of recovery.

| | Treatments | Contributions to OA leaf (%) | | | | | |
|---|---|---|---|---|---|---|---|
| | | Glucose | Fructose | Sucrose | Proline | Glutamine | Asparagine |
| 2 weeks | Control | 2.89 ± 0.83b | 4.10 ± 0.23b | 0.01 ± 3.22E-4b | 0.44 ± 0.04b | 0.09 ± 0.008b | 18.21 ± 0.72b |
| | 100 mM NaCl (S) | 2.72 ± 0.22b | 2.01 ± 0.10c | 0.006 ± 0.001c | 0.14 ± 0.00c | 0.07 ± 0.01b | 15.94 ± 2.85b |
| | 100 mM NaCl + KCl | 1.46 ± 0.19c | 1.45 ± 0.27c | 0.019 ± 0.002a | 0.59 ± 0.05a | 0.11 ± 0.00a | 15.94 ± 2.85b |
| | 100 mM NaCl + $CaCl_2$ | 4.390.2a | 5.19 ± 0.384a | 0.004 ± 3.361E-4c | 0.35 ± 0.16a | 0.08 ± 0.00b | 26.67 ± 0.87a |
| 4 weeks | Control | 10.95 ± 2.82c | 12.81 ± 4.08c | 0.04 ± 0.006d | 0.84 ± 0.03c | 0.52 ± 0.03b | 37.21 ± 6.75c |
| | 100 mM NaCl (S) | 45.30 ± 3.69a | 31.5 ± 5.4a | 0.104 ± 0.002b | 2.58 ± 0.03a | 0.52 ± 0.02b | 65 ± 2.12b |
| | 100 mM NaCl + KCl | 29.93 ± 1.48b | 19.09 ± 1.76b | 0.16 ± 0.02a | 1.91 ± 0.03b | 0.59 ± 0.01a | 77.5 ± 1.76a |
| | 100 mM NaCl + $CaCl_2$ | 13.25 ± 1.24c | 8.80 ± 3.20c | 0.05 ± 0.018c | 0.76 ± 0.09d | 0.29 ± 0.02c | 41.5 ± 2.47c |
| Recovery | Control | 8.98 ± 0.66b | 4.43 ± 0.04a | 0.01 ± 4.57E-4a | 0.32 ± 0.01a | 0.12 ± 0.00b | 20.74 ± 2.89a |
| | 100 mM NaCl (S) | 9.83 ± 1.77a | 3.37 ± 0.5c | 0.003 ± 5.64E-4b | 0.16 ± 0.01c | 0.10 ± 0.006c | 18.17 ± 2.16b |
| | 100 mM NaCl + KCl | 9.55 ± 2.46a | 3.54 ± 0.44b | 0.008 ± 0.001a | 0.26 ± 0.05b | 0.16 ± 0.01a | 18.27 ± 3.29b |
| | 100 mM NaCl + $CaCl_2$ | 0.98 ± 0.13c | 1.7 ± 0.4b | 0.003 ± 8.31E-5b | 0.04 ± 0.00d | 0.04 ± 0.00d | 9.07 ± 1.01c |

Each value represents the mean (± SE) of eight replicates; different letters indicate significant differences between treatments (Duncan test, $p \leq 0.05$).

20.94%, respectively. The contribution of glucose and fructose was mainly higher in salt-stressed thyme plants at week 4 (Table 5), while the contribution of both sugars decreased after the recovery period. Proline was the amino acid that accounted the most for the OA in leaves of salt-stressed thyme at week 4. However, glutamine and asparagine accounted for the most OA in KCl-treated leaves at week 2 of salt-stress treatment. Thymol and cymene contributed to OA in leaves of salt-stressed thyme plants only at week 4 (Table 5), while linalool contributed to OA of salt-stressed plants sprayed with $Ca^{2+}$.

## Discussion

It was previously indicated that proper fertilization of stressed plants with minerals improved plant's performance and stress tolerance (Kaya et al. 2002). Mineral fertilization could regulate ion transport and membrane selectivity (Mumivand et al. 2011) and enhanced leaf osmotic adjustment by stimulating the biosynthesis of osmolites such as sugars (Zhao, Oosterhuis, and Bednarz 2001) and amino acids (Takahashi et al. 2012).

Although thyme responses to salinity stress have been reported (Munns 2002), using mineral fertilization to alleviate stress effects requires detailed knowledge of plant growth, physiology, and biochemistry. Foliar spray of $K^+$ and $Ca^{2+}$ were reported to improve plant growth and reduce environmental stress effects (Mumivand et al. 2011, Kaya et al. 2002). Since mineral fertilizer application requires only basic technology and is an environmentally friendly process, the use of this approach can be considered as practical to reduce environmental stress effects. In this work, we evaluated KCl or

**Table 6.** Essential oil contribution to osmotic adjustment (OA) in thyme plants exposed to the following treatments: (1) 0 NaCl (control), (2) 100 mM NaCl, (stress (S)), (3) stressed leaves with NaCl (S) and treated with KCl, and (4) stressed leaves with NaCl (S) and treated with $CaCl_2$, grown for two and four weeks of treatments and two weeks of recovery.

| | | Contributions to OA leaf (%) | | |
|---|---|---|---|---|
| | Treatments | Thymol | Linalool | $_P$-Cymene |
| 2 weeks | Control | 0.03 ± 0.003b | 0.03 ± 0.008c | 8.11 ± 0.08b |
| | 100 mM NaCl (S) | 0.01 ± 0.002c | 8.01 ± 0.15a | 7.75 ± 2.87b |
| | 100 mM NaCl + KCl | 0.04 ± 0.005a | 0.03 ± 0.004c | 6.44 ± 1.75b |
| | 100 mM NaCl + $CaCl_2$ | 0.03 ± 0.015b | 7.13 ± 0.55b | 12.31 ± 1.10a |
| 4 weeks | Control | 0.037 ± 0.004d | 0.03 ± 0.006c | 15.12 ± 0.76c |
| | 100 mM NaCl (S) | 0.23 ± 0.000-b | 0.07 ± 0.006b | 37.49 ± 0.52b |
| | 100 mM NaCl + KCl | 0.37 ± 0.006a | 0.27 ± 0.04a | 44.67 ± 4.03a |
| | 100 mM NaCl + $CaCl_2$ | 0.12 ± 0.004c | 0.08 ± 0.03b | 16.26 ± 2.97c |
| Recovery | Control | 6.59 ± 1.41c | 0.007 ± 0.01a | 14.35 ± 0.60a |
| | 100 mM NaCl (S) | 0.01 ± 0.0008.a | 0.01 ± 0.0003a | 9.22 ± 0.56c |
| | 100 mM NaCl + KCl | 0.013 ± 0.002a | 0.006 ± 4.38Ea | 11.71 ± 3.46b |
| | 100 mM NaCl + $CaCl_2$ | 0.002 ± 0.0002b | 0.001 ± 0.0002a | 7.73 ± 0.18c |

Each value represents the mean (±SE) of eight replicates. Different letters in the same column indicate significant differences between treatments (Duncan test, $p \leq 0.05$).

$CaCl_2$ foliar sprays and the changes associated with their use in thyme plants under salinity stress. The parameters evaluated included shoot biomass, water relation, concentrations of mineral ions, primary (sugars and amino acids), and secondary metabolites (EOs, polyphenols, and flavonoids).

### *Effect of foliar spray of KCl or $CaCl_2$ on the growth of salt-stressed plants*

In general, salinity stress reduces plant biomass (Ben Taarit et al. 2010, Tounekti et al. 2011) and this inhibitory effect has been attributed to nutritional imbalance and to a decrease in photosynthesis (Munns 2002). The substitution of $K^+$ by $Na^+$ due to their competition for entry into plant root cells may lead to nutritional imbalances, which could explain the decrease in $K^+$ and $Na^+$ concentration either by excessive $Na^+$ absorption or by deficient $K^+$ absorption. Thus, foliar spray of KCl or $CaCl_2$ could alleviate $K^+$ and $Ca^{2+}$ mineral deficiencies by delivering these minerals directly to the leaves and maintaining necessary $K^+$and $Ca^{2+}$ concentrations in leaves. Nutrient balance can be associated with a protecting effect of cell membranes against the adverse effects of salinity (Kaya et al. 2002). Our results are in agreement with the findings of others working with different plant species who found that nutrients were absorbed by the leaves when applied onto the shoot. Under saline conditions, the lack of selectivity for $K^+$ and $Ca^{2+}$ observed in this present investigation may have caused a nutrient imbalance.

### *Effect of foliar sprays of KCl or $CaCl_2$ on water relation and osmoprotection in salt-stressed plants*

In addition to salinity effects on ion balances, salinity also may induce osmotic effects and/or ionic imbalances through either nutritional deficiency or through excess $Na^+$ ions. The inability of plants to adequately hydrate their tissues under saline conditions

causes water stress. Tounekti et al. (2010, 2011) indicated that salinity rapidly decreased RWC (%) and water potential. The main water potential gradient between growing regions of the shoot and the xylem was achieved through osmotic gradients generated by $Na^+$ (Araújo et al. 2006, Tester and Davenport 2003). Plants may also increase $\Psi_w$ to maintain suitable leaf turgor as an adaptive strategy against stress (Chartzoulakis et al. 2002; Azevedo-Neto et al. 2004). Osmotic adjustment helps to maintain plant turgor to sustain plant growth (Gonzalez and Ayerbe 2011). In our study, spraying leaves of salt-stressed plants with KCl or $CaCl_2$ favored the osmotic adjustment in leaves. Treated plants also showed the lowest decrease in Ψs and Ψw, mainly at week 4 and during the recovery period. Sprayed plants seemed to adjust their osmotic potential better than NaCl-stressed plants mainly during the recovery period, which may have helped plants survive salinity stress and increase their growth after exposure to salinity stress and this ability appeared to be enhanced by the foliar application of KCl or $CaCl_2$.

Accumulation of organic solutes such as free sugars and proline is an adaptive mechanism to counterbalance salinity-induced osmotic stress and to protect cellular structure (Hasegawa et al. 2000, Zhu 2001, Bohnert and Jensen 1996). Our results agree with those who reported that increases in carbohydrate levels contributed to the osmotic cell adjustment (Lee et al. 2008; Shahba, Alshammary, and Abbas 2012) and that sugars accounted for 50% of the total osmotic potential in plants grown under salinity stress (Rathinasabapathi 2000). Interestingly, leaves sprayed with KCl or $CaCl_2$ improved the accumulation of soluble sugars, which could have significantly contributed to OA under salinity stress. In agreement, $K^+$ enhanced the osmotic adjustment of cotton plants under salinity stress by stimulating the biosynthesis of the components of non-structural carbohydrates (Zhao, Oosterhuis, and Bednarz 2001). Also, it was reported that salinity and mineral fertilization by foliar spray enhanced the biosynthesis of amino acids such as proline, ornithine, asparagine, and glutamine and that such concentration increases could overcome the salinity stress (Pagter et al. 2009). Proline, in addition to its role as a compatible osmolyte, may provide some protection against photoinhibition by restoring photosynthetic activity (Szabados and Savourè 2009). Proline also protects the structure of proteins and maintains their structural stability (Rajendrakumar, Reddy, and Reddy 1994), while also believed to play a role as an antioxidant (Reddy 2001, Parida et al. 2004). In agreement with the above, plants fertilized with $K^+$ may accumulate more amino acids (Takahashi et al. 2012). Several studies also confirmed that there was a relationship between amino acids metabolism and $K^+$ (Cuin and Shabala 2007).

### *Foliar sprays of KCl or $CaCl_2$ and the antioxidant status of stressed plants*

*Thymus vulgaris* is rich in phenolic and flavonoids, compared to other medicinal plants (Gendy et al. 2014, Hosseinzadeh et al. 2015), which confer the plant a high antioxidant capacity (Hazzit et al. 2009). Salinity and other environmental stresses are known to induce an antioxidant defense response in a number of plants (Muthukumarasamy et al. 2000). A strong positive correlation between the increase in total antioxidant capacity (FRAP) of stressed plants and the increase in total polyphenol content was observed in salt-stressed thyme plants. This indicates that the polyphenols produced by thyme leaves

have a strong antioxidant capacity and that both FRAP and polyphenols levels reflected similar responses to salinity stress. Similarly, the increased antioxidant activity of stressed plants was attributed to the induction of phenolic content (Siddhuraju and Becker 2007). Ben Taarit et al. also reported an increase in total phenolic content in *Salvia officinalis* plants under moderate salinity levels. In addition, the antioxidant capacity assessed by FRAP was amplified by foliar application of KCl at week 2, while total polyphenol content increased due to NaCl stress and foliar KCl at week 2 and due to NaCl and both foliar sprays of KCl and $CaCl_2$ at week 4. At recovery, foliar spray with $CaCl_2$ increased FRAP values, while total polyphenol content increased only with NaCl treatment. Flavonoid concentration increased in response to foliar $CaCl_2$ at both weeks 2 and 4 and in response to both foliar KCl and $CaCl_2$ during recovery. These responses during recovery suggest that although irrigation with distilled water may have flushed salts from the pot mixture, it did not eliminate the salts that already accumulated inside plant tissues during weeks 2 and 4.

Furthermore, we recorded that soluble sugars and proline, postulated to act as antioxidants, were positively correlated with TAC. Previous studies have reported such function for the monosaccharides in *Aloe vera* (Kang et al. 2013). Proline could also reduce the effect of salinity stress in thyme plants by indirectly increasing cell antioxidant concentrations as well as by adjusting cell osmotic potential (Vendruscolo et al. 2007). Thymol and linalool concentrations were also correlated with TAC measured by FRAP and may have been involved in antioxidant capacity enhancement to prevent toxic effects of $Na^+$ in leaves. Similarly, the strong antioxidant profile of EO of thyme plants was reported in a recent study (Stanisavljević et al. 2011).

### *Essential oils in response to salt-stress and foliar spray in thyme leaves*

Generally, the contents of phenolic substances are influenced by a large number of biotic and abiotic factors, agro-technical processes, and climatic conditions such as drought and salinity (Jordán et al. 2009). In agreement, the oil content of *Salvia officinalis* remained unchanged (0.47–0.48%) in control plants, but increased from 0.65–0.76% in salt-stressed plants (Hendawy and Khalid 2005). Oil composition of thyme plant in response to cultivation, using fertilizers/mineral treatments is not well studied (Jamali et al. 2014). Mineral fertilization could affect the EO composition (Figueiredo et al. 2008) and this impact of minerals can depend on the type and period of the treatment. In our study, mineral application for a short-term was not enough to increase monoterpene level, similar to previously reported results for *Salvia sp.* (Tounekti et al. 2011). The role of mineral treatments in enhancing EO levels has also been reported in several medicinal plants such as *Satureja hortensis* (Alizadeh et al. 2010), *Rosmarinus officinalis* (Tounekti et al. 2011), and *Salvia officinalis* (Tounekti et al. 2010). In our study, the monoterpenes were the most prevalent group with thymol (21–45%) as the dominating compound, followed by linalool (21.2%) and p-cymene (12%). This chemical composition of the essential oil had been previously reported in thyme (Asbaghian et al. 2011). A similar chemical signature was also reported in other members of the Lamiaceae family, such as peppermint grown under osmotic stress (Charles, Joly, and Simon 1990).

## Conclusions

Our results suggest that supplementary foliar application of KCl and $CaCl_2$ improved salinity tolerance of thyme plants and reduced salinity effect on plant growth by possibly improving mineral availability in leaves. Mineral fertilization largely increased the concentration of organic solutes, such as glucose and fructose, for osmotic adjustment. Salinity and foliar sprays also led to the accumulation of the EO components thymol, myrcene, and borneol; the amino acids asparagine and proline; and of polyphenols. These overall increases suggest that these primary and secondary metabolites may act, isolated or synergistically, to mitigate the toxic effects of salinity caused by either $Na^+$, $Cl^-$, or both. However, the interaction between salinity stress and the biosynthesis of primary and secondary metabolites is complicated and difficult to isolate in different species. Although the results achieved in our work may not be immediately transferable to other species under salinity stress, they add to the arsenal of information on how plants react to salinity stress by synthesizing different metabolites that may help them adjust the osmotic imbalance triggered by salinity, while maintaining cell membrane integrity and helping plants adjust to stress and resume their growth and development. Our results with the foliar application of KCl and $CaCl_2$ may be used to help improve the cultivation of thyme species in semiarid areas of elevated salinity.

## ORCID

Foued Hamouda http://orcid.org/0000-0003-4902-5164

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