

Changes in growth, oxidative metabolism and essential oil composition of lemon balm (*Melissa officinalis* L.) subjected to salt stress

Cristine Bonacina¹, Cláudia Borsari Trevizan¹, Juliana Stracieri², Tiago Benedito dos Santos³, José Eduardo Gonçalves^{4,5}, Zilda Cristiani Gazim¹, Silvia Graciele Hülse de Souza^{1*}

¹Programa de Pós-graduação em Biotecnologia Aplicada à Agricultura, Universidade Paranaense - UNIPAR, Praça Mascarenhas de Moraes, 4282, Umuarama, PR, Brazil

²Universidade Estadual de Maringá - UEM, Estrada da Paca s/n, 87502-970, Umuarama, PR, Brazil

³Universidade do Oeste Paulista - Unoeste, Rodovia Raposo Tavares s/n, 19067-175, Presidente Prudente, SP, Brazil

⁴Mestrado em Tecnologia Limpas, UniCesumar, Av. Guedner, 1610, Maringá, PR, Brazil

⁵Instituto Cesumar de Ciência, Tecnologia e Inovação - ICETI, Maringá, PR, Brazil

*Corresponding author: silviahulse@prof.unipar.br

Abstract

Agricultural crops are severely affected by salinity. Recent studies have shown that salt stress signaling components affect plant metabolism and stimulate the accumulation of organic osmolytes and antioxidant substances. This work aimed at assessing the growth and development of lemon balm, *Melissa officinalis* L., subjected to salt stress, as well as its antioxidant response, essential oil composition, and essential oil yield (%). A completely randomized experimental design was conducted under greenhouse conditions, where 30-days-old lemon balm plants were treated with different NaCl concentrations (0, 50, 100, 150 and 200 mM) for sixty days. The highest concentrations (150 mM and 200 mM NaCl) caused significant reductions in shoot height, shoot fresh mass, relative water content, water activity and chlorophyll production due to changes in osmotic activity. The activities of superoxide dismutase (SOD) and catalase (CAT) increased in plants subjected to salinity. In addition, membrane damage (DM) increased with increase in NaCl concentration. The yield of the essential oils decreased but the number of compounds increased in all the NaCl treatments. The compounds neryl-acetate and geranyl-acetate were detected at 100, 150 and 200mM NaCl, suggesting that under salt stress, lemon balm plants activate the metabolic pathways for the production of terpenoids, consequently producing monoterpenes. Salinity negatively affected most of the parameters evaluated in lemon balm plants. Our results show that lemon balm plants are tolerant to low concentrations of salinity (up to 50mM) as proved by their distinct metabolic responses.

Keywords: abiotic stress, essential oils, secondary metabolites, α -citral, neral, citronellal.

Abbreviations: ROS_reactive oxygen species, GPX_glutathione peroxidase, GR_glutathione reductase, CAT_catalase, APX_ascorbate peroxidase, PPO_polyphenol oxidase, EO_essential oil, SH_shoot heights, SFM_shoot fresh masses, RWC_relative water content, FM_fresh mass weight, DM_dry mass weight, TM_turgid mass weight, aw_water activity, REL_relative electrolyte leakage, L1_initial electric conductivity of the extract, L2_final electric conductivity of the extract, DM_damage in the membrane, NBT_nitro-blue tetrazolium, GC/MS_gas chromatograph/mass spectrometer, RI_retention indices, ANOVA_analysis of variance, CA_cluster analysis, PCA_principal component analysis, UPGMA_Unweighted Pair Group Method with Arithmetic Mean.

Introduction

Lemon balm (*Melissa officinalis* L.) is a medicinal plant belonging to the family Lamiaceae. It was originally found in Mediterranean countries and is commonly cultivated worldwide due to its economic importance (Younesi and Moradi, 2015; Szabó et al., 2016). Also known as the balm, common balm, or balm mint, its leaves are used as tranquilizer and sedative (Morgan, 1994). The essential oil from the balm tree is used for the manufacture of various products, such as condiments, flavoring agents, natural insecticides and insect repellents, thus serving the pharmaceutical, food, beverage, cosmetic and chemical industries (Bagdat and Cosge, 2006; Abbaszadeh et al., 2009; Verma et al., 2015). The leaves of balm contain high quantities of secondary metabolites. These include citronellal, citronellol, linalool, neral, geranial and geraniol (both isomers of citral), rosmarinic acid, phenolic acid,

flavoglycoside acid, caffeic acid, isoquercetin, apigenin (apigenin-7-O-glucoside), beta-carophyllene, tannins, flavonoids, luteolin, hesperidin and others (Moradkhani et al., 2010; Argyropoulos and Müller, 2014). The quality and continuous production of essential oils is very important to supply market demands and the industry requirements, even under adverse environmental conditions such as drought, salinity, and extreme temperatures (Silva, 2002; Çoban and Baydar, 2016). These adverse conditions strongly affect the biosynthesis and production of secondary metabolites in medicinal and aromatic plants, causing increase or decrease in the essential oils' yield and changes in their composition (Lopes and Gobbo-Neto, 2007; Kim et al., 2010; Tonelli et al., 2014). Abiotic stresses such as drought and salinity cause negative effects on growth and development of medicinal plants (Khorasaninejad et al., 2010). In response to these

stresses, plants activate regulatory complexes to neutralize the harmful effects and restore cell homeostasis (Suzuki et al., 2012). Salinity reduces plant growth by decreasing the plant's water potential, breaking various cell metabolisms, altering enzymatic activities thereby causing nutritional imbalances, increasing accumulation of solutes, or combining all these events (Munns and Tester, 2008; Tounekti et al., 2012).

Plants can produce and accumulate reactive forms of oxygen (ROS) as a defense mechanism against injuries caused by stress (Jaspers and Kangasjarvi, 2010). The commonly observed ROS are the oxygen molecules with superoxide anion radical ($O_2^{\cdot-}$), hydroxyl radical (OH^{\cdot}), singlet oxygen (O_2) and hydrogen peroxide (H_2O_2). The antioxidant enzymes such as glutathione peroxidase (GPX), glutathione reductase (GR), catalase (CAT), ascorbate peroxidase (APX), and polyphenol oxidase (PPO) are activated to protect cells from oxidative damage (Hu et al., 2009; Gill and Tuteja, 2010; Tripathy and Oelmüller, 2012). Studies on different types of stress and on growth and development, metabolism, and composition of the essential oils of medicinal plants have gained high momentum. It has been reported that each type of stress differently influences plant metabolism and essential oil composition (Alvarenga et al., 2011; Meira et al., 2012; Kasrati et al., 2014). However, few studies have demonstrated the effect of salt stress on development, metabolism, and composition of essential oils of *M. officinalis*, in addition to physiological and biochemical responses (Ozturk et al., 2004; Khalid and Cail, 2011). Therefore, this study aimed at evaluating the effects of salt stress levels in *M. officinalis* plants on morphological aspects, antioxidant responses, and essential oil yield and composition.

Results and discussion

Effects of salt stress on development of lemon balm plants

The development of lemon balm plants decreased with the addition of different concentrations of NaCl. The salt stress levels caused by 50, 100, 150 and 200 mM NaCl affected plant height and shoot fresh mass accumulation (Figs. 1A and 1B.). A decreased 17.97% SH and 48.36% SFM was observed at 200 mM NaCl, in comparison with control (Table 1). RWC and WA decreased with the increase in NaCl concentrations (Figs. 1C and 1D.). Further reduction in RWC (%) (Fig. 1C.) and WA (Fig. 1D.) were observed at concentrations of 150 and 200 mM NaCl.

Plant development and water accumulation are related factors. The reduced development of lemon balm plants subjected to salinity is probably as a result of the NaCl action on plants that causes the disturbance in their water relations, especially with respect to turgor, due to changes in osmolarity (Yasar, 2006; Hussain et al., 2011). It means that there is a reduction in water absorption, and consequently, a decrease in water content in the plant tissue and in the turgor, as observed in our work (Fig. 1.). Therefore, cell elongation in plants is inhibited by the reduced pressure of turgescence, under stress conditions. On the same way, the salt stress probably decreases the accumulation of photo assimilates due to the closure of stomatal cells, restricting carbon dioxide absorption and consequently, reducing the availability of metabolites to perform cell division (Baghalian et al., 2011). As consequence, some mechanisms as mitosis, elongation, and expansion of cells are hampered, resulting in reduced plant height and impaired growth (Farooq et al., 2009; Negrão et al., 2017).

Studies on different NaCl concentrations applied to medicinal plants demonstrate similar results. Tounekti et al. (2012) verified great reductions in the growth of *Salvia officinalis* at 100 mM NaCl. Khorasaninejad et al. (2010) observed the same response in the shoot fresh mass of *Mentha piperita* L. at 100 mM NaCl. Kasrati et al. (2014) also observed a low production of shoot fresh mass in a menthe species subjected to 100 mM NaCl and 150 mM NaCl.

Fig. 2 demonstrates that salinity caused physiological disturbances in lemon balm plants. The severity of leaf lesions increased with the increase in NaCl concentrations. The first visual symptoms of salt stress were chlorosis and subsequently leaf drying. Some lemon balm plants lost their vitality from 150 mM NaCl. Such changes occurred due to the release of Na^+ and Cl^- in the plant cell tissues caused by the increased concentrations of NaCl, resulting in structural damages that led to dehydration and cell death (Munns, 2002).

Total chlorophyll content diminished at 200 mM NaCl in comparison with control (Fig. 3A.). As a consequence, yellowing was observed in the leaves (Fig. 2.). It occurred because salt stress normally hampers the entry of water into cell metabolisms. Since water participates in the production of chlorophyll, the decrease in the plant's photosynthesis may result in the plant death (Kumar et al., 2014).

With an increase in NaCl, the number of electrolytes released was increased. The highest DM% was observed in plants subjected to 200 mM NaCl (Fig. 3B.). Kere et al. (2016) observed that NaCl caused damage to plasma membranes of different genotypes of *Cucumis sativus* L., either resistant (11411S and 11432S) or sensitive (HH1-8-57 and 11439S) to salinity. However, the authors reported higher electrolyte leakage in the sensitive genotypes.

Response of antioxidant enzymes to salt stress

Many studies have demonstrated alterations in the activity of antioxidant enzymes as a response to abiotic stresses, suggesting that the increase in such activities is closely linked to stress tolerance (Chen et al., 2014; Choundhury et al., 2016). Understanding plant responses to salt stress will lead to decipher a cascade of biochemical events and complex gene expressions (Bray et al., 2000). In this study, the activity of antioxidant enzymes was differentially modulated by NaCl in the *M. officinalis* leaves. An increased SOD activity was observed at 200 mM NaCl in comparison with control (Fig. 4A.). The maximum activity of the CAT enzyme was verified at 150 mM NaCl. The increased activity of SOD and CAT is related to the maintenance of levels of lipid peroxidation under salt stress conditions (Alscher et al., 2002).

In this study, the CAT activity increased up to the concentration of 150 mM NaCl; therefore, this enzyme was effective to protect cells against oxidation. Among the H_2O_2 degrading enzymes, catalase does not consume equivalent cell reducers, removing H_2O_2 produced in cells under stress conditions through efficient mechanisms (Scandalios, 2005). The CAT enzyme is found in peroxisome and cytosol and is indispensable for cellular detoxification, catalyzing the transformation of H_2O_2 into molecular oxygen and water (Gill and Tuteja, 2010). Lledias et al. (1998) suggested that the CAT enzyme is so efficient that it cannot be saturated with H_2O_2 at any concentration of peroxide. A combined action of CAT and SOD converts the toxic $O_2^{\cdot-}$ and H_2O_2 into the water and molecular oxygen, avoiding cell damages under adverse environmental conditions (Reddy et al., 2004). The increase in the activity of these enzymes is related to the tolerance of plants to oxidative stress.

Table 1. Inhibition (%) of the shoot height (SH) and the shoot fresh mass (SFM) of plants subjected to different concentrations of NaCl.

Treatments with NaCl (mM)	% Inhibition	
	SH	SFM
50	6.98 b*	0.58 c
100	8.46 b	27.34 b
150	10.50 b	46.48 a
200	17.97 a	48.36 a

*Means followed by the same letter did not differ significantly at $p < 0.05$, according to Tukey's test.

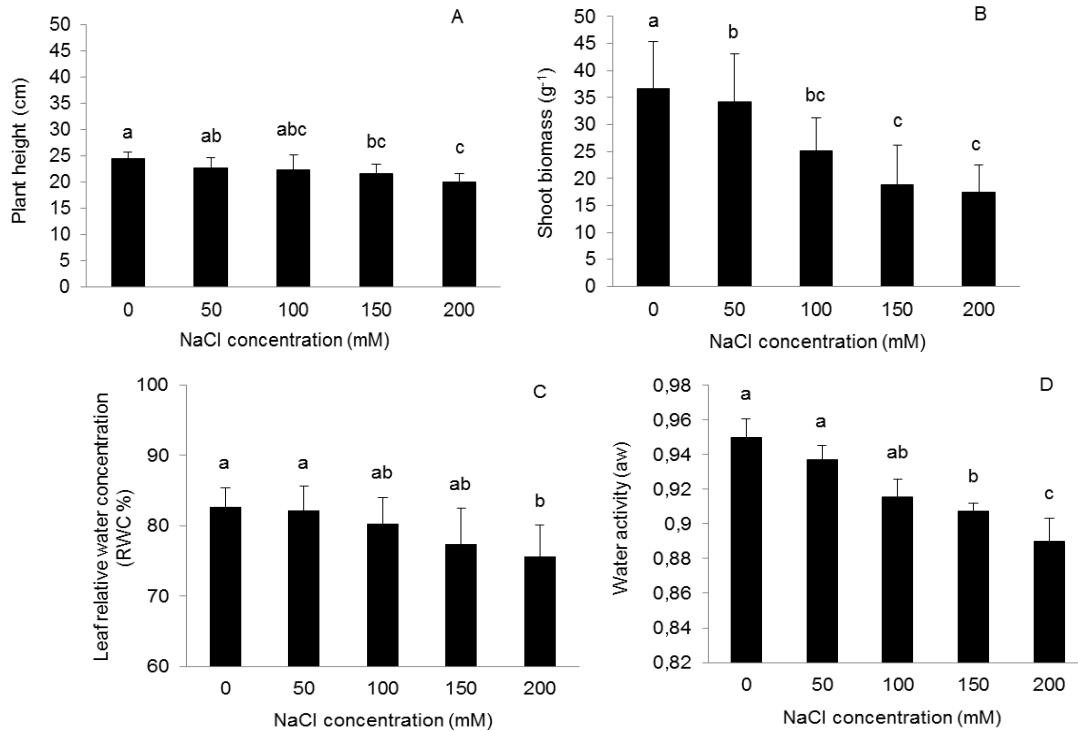


Fig 1. Effects of salt stress on plant height (A), shoot biomass (B), relative water content in leaves (C), and water activity (D). Means with the same letter did not differ significantly at $p < 0.05$, according to Tukey's test.

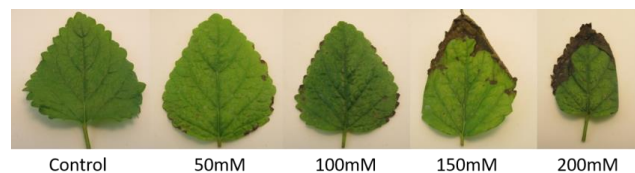


Fig 2. Leaf toxicity symptoms on lemon balm leaves (*Melissa officinalis* L.) subjected to different NaCl concentrations for sixty days.

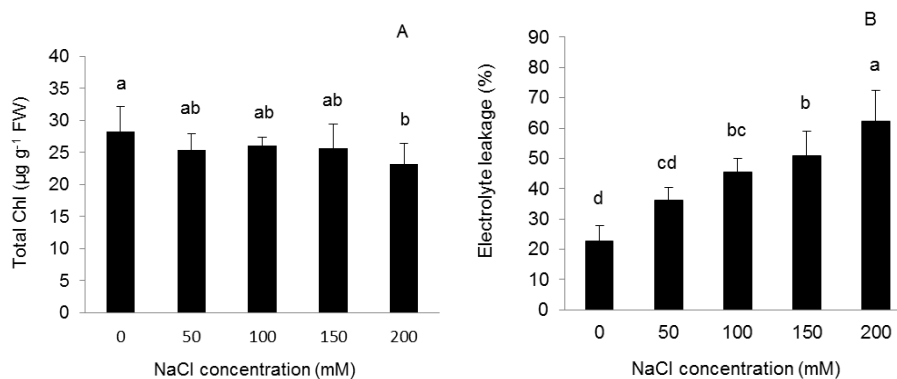


Fig 3. Effects of saline stress on total Chl (A) and electrolyte leakage (B) in *Melissa officinalis* L plants. Means followed by the same letter did not differ significantly at $p < 0.05$, according to Tukey's test.

Table 2. Chromatography (GC/MS) of the essential oils of lemon balm plants (*M. officinalis* L) subjected to different concentrations of NaCl (T50=50 mM; T100=100 mM; T150=150 mM and T200=200 mM) and control (TC=0 mM).

Peak ^a	Compounds	RI ^b	Area (%)					IM ^c	Peak ^a	Compounds	RI ^b	Area (%)					IM ^c
			TC	T50	T100	T150	T200					TC	T50	T100	T150	T200	
	Monoterpenes Hydrocarbons									Sesquiterpenes Hydrocarbons							
1	α -pinene	936	T	0.33	0.26	t	0.33	a,b,c	29	Geraniol	1247	-	2.93	2.37	3.04	3.65	a,b,c
2	Sabinene	976	T	0.09	t	t	0.11	a,b,c	30	n,i	-	-	-	-	0.65	a,c	
3	β -pinene	979	T	0.32	0.23	t	0.31	a,c	31	α -citral	1276	45.05	42.22	41.38	41.73	40.45	a,b,c
4	Myrcene	991	5.05	5.34	5.59	4.79	6.03	a,b,c	32	Neryl acetate	1385	-	-	-	4,56	-	a,b,c
5	α -phellandrene	1005	T	0.35	0.29	t	0.38	a,b,c	33	Geranyl acetate	1390	-	-	2,06	-	3,72	a,b,c
6	δ -3-carene	1011	T	t	t	-	t	a,b,c	34	Trans-caryophyllene	1413	2.69	2.93	3.17	3.25	2.85	a,b,c
7	p-cymene	1027	T	-	t	t	0.19	a,b,c	35	Trans- α -bergamotene	1443	-	-	t	-	-	a,b,c
8	o-cymene	1027	-	0.18	0.4	-	-	a,b,c	36	Aromadendrene	1452	-	-	0.47	-	-	a,b,c
9	Limonene	1031	T	-	t	-	-	a,b,c	37	α -humulene	1479	-	t	t	t	t	a,b,c
10	β -phellandrene	1031	T	0.38	-	t	0.45	a,b,c	38	α -curcumene	1484	-	-	t	-	-	a,b,c
11	n,i	-	-	0.36	0.43	-	0.48	a,c	39	α -amorphene	1494	-	0.69	-	-	-	a,b,c
12	γ -terpinene	1033	0.45	-	-	0.41	-	a,b,c	40	Germacrene-D	1524	-	-	t	-	-	a,b,c
13	terpinolene	1100	T	-	1.4	1.94	-	a,b,c	41	α -zingiberene	1581	-	-	t	-	-	a,b,c
	Oxygenated Monoterpenes								42	δ -cadinene	1672	-	-	1.34	-	-	a,b,c
14	Linalool	1104	1.04	0.35	0.47	0.65	1.16	a,b,c		Oxygenated sesquiterpenes							
15	n,i	1105	-	-	-	-	-	a,b,c	43	Tumerol	1705	-	-	t	-	-	a,b,c
16	Trans-thujone	1147	1.09	-	-	-	0.55	a,b,c	44	Caryophyllene-oxide	2108	-	-	t	-	-	a,b,c
17	Cis-verbenol	1147	-	-	-	-	1.18	a,b,c	45	Tumerone-dihydro	2109	-	-	t	-	-	a,b,c
18	Camphor	1148	0.65	0.72	0.71	0.50	-	a,c	Total identified (%)								
19	Isopulegol	1155	T	1.36	1.2	1.09	-	a,b,c				99.09	97.11	94.98	100.00	98.97	
20	Citronellal	1167	9.89	8.93	5.87	5.51	9.13	a,b,c	Groups of compounds (%)								
21	Lavandulol	1169	-	0.79	-	-	-	a,b,c	Monoterpenes Hydrocarbons								
22	Methyl-chavicol	1180	-	-	0.61	-	-	a,b,c	Oxygenated Monoterpenes								
23	Estragol	1184	-	-	-	-	0.62	a,b,c	Sesquiterpenes Hydrocarbons								
24	Citronellol	1198	2.11	2.63	1.74	1.42	2.43	a,b,c	Oxygenated Sesquiterpenes								
25	Nerol	1198	T	-	0.62	0.60	-	a,b,c	EO yield (%)								
26	Pulegone	1198	1.40	1.76	1.70	1.57	1.58	a,b,c				0.091 \pm	0.069 \pm	0.065 \pm	0.079 \pm	0.076 \pm	
27	Neral	1230	28.25	24.45	22.67	27.90	22.72	a,b,c				0.034	0.011	0.016	0.004	0.028	
28	Chavicol	1243	1.42	-	-	1.04	-	a,b,c									

^aCompounds listed in order of elution by capillary HP5ms GC/MS columns, ^bRI = Retention indices calculated with n-alkanes (C7-C25) on HP-5MS column, ^cIM = Identification method based on comparison with the mass spectra of the Wiley 275 library, t = traces, n,i, = not identified.

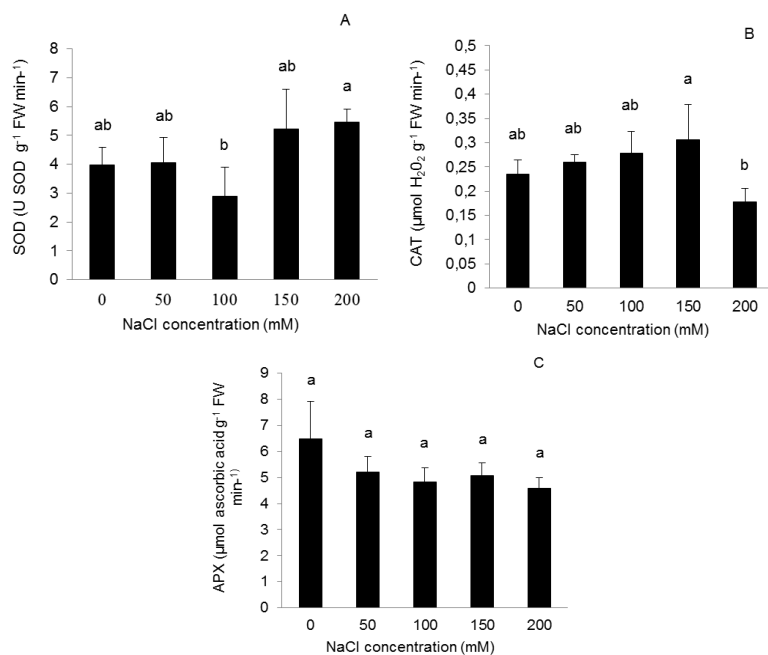


Fig 4. Antioxidant responses of the enzymes SOD (A), CAT (B), and APX (C) due to the effects of salinity stress. Means followed by the same letter did not differ significantly at $p < 0.05$, according to Tukey's test.

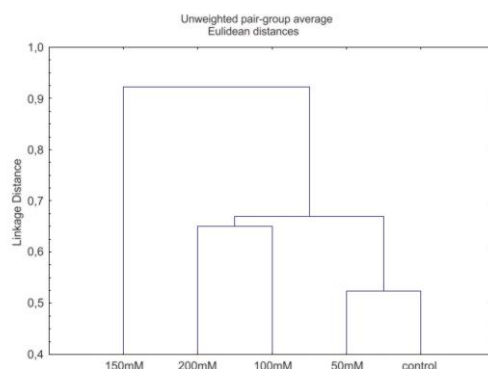


Fig 5. Hierarchical clustering dendrogram obtained in the cluster analysis of the essential oils of *Melissa officinalis* L. leaves treated with different NaCl concentrations. Data extracted from Table 2.

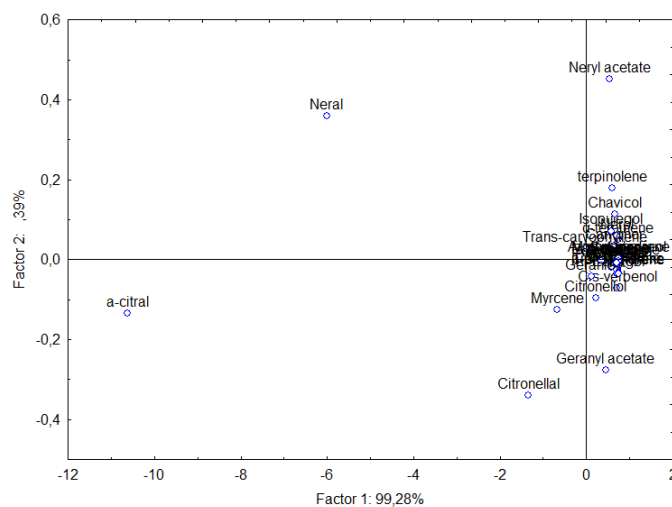


Fig 6. A biplot representation of the essential oil (OE) of *Melissa officinalis* L. plants subject to NaCl stress according to the principal component analysis (PCA).

The APX is located mainly in chloroplasts and partially in cytosols. This is another enzyme of defense that uses H₂O₂ as substrate (Çoban et al., 2016). The results observed in the current study suggest that the CAT and SOD differed significantly, the APX activity did not present significant differences among treatments; however, decreased activity was observed in lemon balm plants subjected to salt stresses (Fig. 4C.). Sabra et al. (2012) compared some enzymatic activities at three NaCl concentrations (50, 75 and 100 mM) in three Echinacea species (*E. purpurea*, *E. pallida*, and *E. angustifolia*) and observed that SOD and APX were effective only in *E. purpurea*. Reduction of the CAT activity was observed at the concentrations of 75 mM and 100 mM NaCl in the three Echinacea species evaluated (Sabra et al. 2012). According to these authors, it probably occurred due to the enzymatic interactions that eliminated superoxide radicals (O₂^{•-}) and H₂O₂ molecules present in cell metabolisms of plants submitted to NaCl stress. With respect to the enzymatic antioxidant mechanisms, our results indicate that the evaluation of resistance to salt stress should be conducted with moderation, taking into account the plant growth stages.

Effects of salt stress on yield and chemical composition of lemon balm's essential oil

Essential oils are secondary metabolites that contain various compounds. Lemon balm plants are rich in essential oils that are widely used in food and healthcare products, medicines, and cosmetics (Mokhtarzadeh et al., 2016). The commercial value of lemon balm plants depends on the yield and chemical composition of their essential oils. In this study, the salt stress reduced the content of essential oils in lemon balm plants. In control plants, the EO yield was 0.091% and the plants subjected to salt stress showed reduced levels of essential oils for the different NaCl concentrations (Table 2). Moderate and high levels of salinity (150 and 200 mM) resulted in EO yields of 0.079 and 0.076%, respectively. Low levels of NaCl (50 and 100 mM) resulted in a decreased EO production of *M. officinalis*. These results suggest that lemon balm plants are sensitive to salinity and high concentrations of NaCl in soil would decrease the EO biosynthesis in these plants. Aziz et al. (2008), suggested that the reduced levels of EO production could be due to the decreased photosynthesis and/or further alterations in metabolism. Any alteration in the normal plant metabolism can affect the EO biosynthesis and therefore decrease the EO yield (Srivastava et al., 1998; Çoban et al., 2016). Some authors reported significant reductions in the total EO content in different medicinal species subjected to salt stress (Aziz et al., 2008; Khorasaninejad et al., 2010; Çoban et al., 2016).

Forty-five compounds were identified in the EO of *M. officinalis* leaves, 97.66% of them belonged to four chemical classes: monoterpene hydrocarbons, oxygenated monoterpenes, sesquiterpene hydrocarbons, and oxygenated sesquiterpenes. The predominant class was oxygenated monoterpenes and the major compounds identified in all NaCl treatments were α -citral (TC=45.05%; T50=42.22%; T100=41.38%; T150=41.78% and T200=40.45%); neral (TC=28.25%; T50=24.45%; T100=22.67%; T150=27.90% and T200=22.72%); citronellal (TC=9.89%; T50=8.93%; T100=5.87%; T150=5.51% and T200=9.13%). In addition to these compounds, myrcene, trans-caryophyllene, citronellol, pulegone, and linalool were also identified in all the treatments, but in lower concentration (Table 2). These compounds are commonly found in the EO of *M. officinalis* leaves; however, their occurrence may vary with local cultivation method, environment conditions and EO

extraction method (Tagashira and Ohtake, 1998; Silva et al., 2005; Khalid and Cail, 2011; Szabó et al., 2016). In addition to alterations in the percentage of major compounds, the different NaCl concentrations induced an increased number of compounds as identified in the EO of all the NaCl treatments (Table 2). The same was observed by Taarit et al. (2011), who reported an increased amount of compounds in *Salvia esclarea* L. Some compounds such as *cis*-verbenol (1.18%), estragole (0.62%) and *p*-cymene (0.19%) were exclusively found at 200 mM NaCl, the highest salt concentration. High production of compounds at high salinity concentrations probably occurred due to the increased enzymatic activity for the EO biosynthesis (Taarit et al., 2010; Khalid and Cail, 2011).

The hierarchical analysis was applied to understand the chemical variability of the EO of *M. officinalis* plants subjected to salt stress. A dendrogram was obtained based on the Unweighted Pair Group Method with Arithmetic Mean (UPGMA), which grouped data into three main clusters classified by salinity levels. The first cluster included the control and the low salinity level represented by 50 mM NaCl treatment. The second cluster was formed by the higher levels of salinity, represented by 100 and 200 mM NaCl treatments. The third cluster included only the 150 mM NaCl treatment (Fig. 6.). The clustering showed three groups distinguishing by salinity level, in which above 100 mM NaCl, the EO biosynthesis was modified to alter the quantity and quality of compounds of the lemon balm's EO (Table 2, Fig. 5.).

Principal Component Analysis (PCA) allows conjointly evaluating all variables in the analysis. In the present study, a PCA was performed in order to investigate the behavior of the chemical components of the EO of *M. officinalis* plants subjected to different NaCl concentrations. The PCA analysis showed a total variance of 99.67%, in which the principal components PC1 and PC2 explained 99.28% and 0.39% of the total variance, respectively (Fig. 6.). It was observed that some EO compounds moved away from each other (Fig. 5.). The results of the PCA corresponded to those obtained in the cluster analysis (Figs. 5 and 6). Citronellal, α -citral, and neral were positioned far from the others, forming three distinct groups. Although predominant and present in all treatments, α -citral and neral decreased to 10.21% and 19.58% respectively, at 200 mM NaCl, in comparison with control. The same was observed for citronellal, which shows decline by 40.64% and 44.28% in the concentrations of 100 and 150 mM NaCl, respectively. Neryl-acetate and geranyl-acetate were exclusively found in plants subjected to salt stress, neryl-acetate was detected at 150 mM NaCl and geranyl-acetate at 100 and 200 mM NaCl. These two compounds were split into two different clusters by the PCA analysis. Probably this monoterpene production occurred due to the activation of a metabolic pathway that produces terpenoids and consequently monoterpenes, which have an important role in protecting plants against abiotic stresses (Loreto and Velikova, 2001; Loreto et al., 2004; Holopainen and Gershenzon, 2010; Savoi et al., 2016).

The highest EO production was observed in lemon balm plants treated with NaCl, in which different classes of compounds were detected. It suggests that the production of isoprenoids (monoterpenes and sesquiterpenes) occurs even under stress conditions, when photosynthesis is almost or completely inhibited or when carbon is unavailable (Monson and Fall, 1989; Brili et al., 2007). This suggests that under stress conditions isoprenoids have benefited plants in adverse environments (Siemens et al., 2002). These compounds can activate responses to oxidative stress since they are lipophilic

and can physically stabilize hydrophobic interactions in cell membranes, minimizing lipid peroxidation, reducing oxidative stress and ROS accumulation (Vickers et al., 2009).

Materials and methods

Plant materials

The lemon balm seeds (TOPSEED®) were immersed in distilled water for 48h at 6°C to break seed dormancy. Then, the seeds were sown in polystyrene tray containing commercial substrate (Carolina Padrão®). Five seeds were distributed on each tray cell and subjected to daily irrigation. The experiment was conducted in a greenhouse conditions with an average a 16 h light/8 h dark regimen at 24°C day/14°C night temperature in a random block design. The 30-day-old seedlings were transplanted to 3L plastic pots containing soil, sand, and organic compost in 2:1:1. One seedling per pot and nine pots per treatment were tested for four concentrations of sodium chloride (50, 100, 150 and 200 mM NaCl) (Khorasaninejad et al., 2010; Roodbari et al., 2013). The seedlings treated with water were maintained as control. The concentrations of salt solutions were gradually increased to 50 mM NaCl every week, in order to avoid osmotic shock, until the completion of each treatment. Plants were irrigated with Hoagland solution (Hoagland and Arnon, 1950) weekly, until the end of the experiment. The plant material was collected after 60 days of stress and stored at -80°C in ultra-freezer, in a hermetically sealed container, until analysis.

Morphological evaluation

Shoot height and shoot fresh mass

Plant shoot heights (SH) (cm) were obtained by tape measure, from the lap of the plant to the apex. The shoot fresh masses (SFM) (g) were weighed by an analytical balance.

Biochemical analysis

Chlorophyll content measurement

Total chlorophyll content was measured in five leaves of the middle third with three biological replications of each treatment, using the ClorofiLOG® chlorophyll meter model CFL 1030, according to the manufacturer's instructions (Falker®).

Relative water content

Parameters for relative water content (RWC %) were recorded in five leaves of the middle third with three biological replications of each treatment, according to Rouached et al. (2013). Then, the RWC calculation was performed using the following equation (Schonfeld et al., 1988):

$$RWC = \frac{FM - DM}{TM - DM} \times 100$$

Where:

RWC% = relative water content in plants;
FM = fresh mass weight;
DM = dry mass weight;
TM = turgid mass weight.

Water activity

The water activity (aw) was determined in leaves using the Lab Master-aw apparatus, Novasina-Tecnal®. Ten 1cm-diameter discs were collected in the middle third with three biological replications of each treatment and placed into the apparatus for analysis.

Plasma membrane integrity

Membrane permeability was measured through relative electrolyte leakage (REL) with an ITMCA 150P® micro-processed portable electric conductivity meter, according to the method described by Wang et al. (2005). Ten 1cm-diameter discs were collected in the middle third with three biological replications for each treatment. The damage in the membrane (DM%) was obtained by the following equation:

$$REL = \left(\frac{L1}{L2} \right) \times 100$$

Where:

REL% = relative electrolyte leakage;

L1 = initial electric conductivity of the extract;

L2 = final electric conductivity of the extract.

Antioxidant enzymes

Plant tissues were collected in liquid nitrogen and transferred to the experimental laboratory. Samples of approximately 0.3 g were homogenized in 1.5 mL of 200 mM potassium phosphate buffer (pH 7.8) containing 10 mM EDTA, 200 mM ascorbic acid and 10% of polyvinylpolypyrrolidone (PVPP) using pestle and mortar. The homogenate was centrifuged at 12,000 g for 20 min at 4 ° C and the supernatant was collected and stored in ultra-freezer (-80 ° C) until analysis. The extracts were used for testing the antioxidant enzymes superoxide dismutase (SOD), catalase (CAT) and ascorbate peroxidase (APX). All assays were performed with three biological replications in triplicate.

SOD enzyme (EC 1.15.1.1)

The SOD activity is based on its ability to inhibit the reduction of nitrobluetetrazolium (NBT) by superoxide, forming blue formazan, as described by Giannopolitis and Ries (1977). The reaction medium (1mL) consisted of 50 mM KPO₄ buffer (pH 7.8), 13 mM methionine, 0.1 μM EDTA, 75 μM NBT, 2 μM riboflavin and 50 μL of the crude sample extract. Absorbance was read at 560 nm and the SOD activity was expressed as U SOD g⁻¹ FW min⁻¹, where a unit of (U) SOD activity was defined as the amount of enzyme required to inhibit 50% of the NBT reduction. Analyses were performed in triplicate.

CAT enzyme (EC 1.11.1.6)

The CAT activity was determined according to methodology described by Haver and McHale (1987). The reaction medium (1 mL) consisted of 200 mM KPO₄ buffer (pH 7.0), 20 mM H₂O₂ and 50 μL of the crude sample extract. Catalase activity was determined by the H₂O₂ consumption monitored by spectrophotometry at 240 nm for 1 minute and quantified using the 36 M⁻¹cm⁻¹ molar extinction coefficient (Anderson et al., 1995). The CAT activity was expressed in μmolH₂O₂ g⁻¹ FW min⁻¹. Analyses were performed in triplicate.

APX enzyme (EC 1.11.1.11)

Ascorbate peroxidase catalyzes the reduction of H₂O₂ to H₂O by oxidizing ascorbate. The APX activity was determined according to methodology described by de Nakano and Asada (1981). The reaction medium (1 mL) consisted of 50 mM KPO₄ buffer (pH 7.0), 10 mM ascorbic acid, 1 mM H₂O₂ and 50 µL of the crude sample extract. APX activity was determined by the H₂O₂ degradation monitored by spectrophotometry at 290 nm for 1 minute and quantified using the 2.8 mM⁻¹cm⁻¹ molar extinction coefficient (Nakano and Asada, 1981). The APX activity was expressed as µmol ascorbic acid g⁻¹ FW min⁻¹. Analyses were performed in triplicate.

Lemon balm EO extraction

The EO extraction of 100 g/L lemon balm fresh leaves was made by hydrodistillation for 3h (Verma et al., 2015). The lemon balm's EO was extracted with n-Hexane, filtered with anhydrous sodium sulfate (Na₂SO₄) and kept under refrigeration (4°C) until total solvent evaporation (Brasil, 2010).

Chemical analysis of EO by GC/MS

The essential oil was subjected to GC/MS analysis using Agilent® (7890B) gas chromatograph coupled to Agilent® (5977A) mass spectrometer. The fused silicacapillary column was HP-5MS (30 m × 0.25 mm × 0.25 µm). The conditions of the equipment were the same as those used by Verma et al. (2015). Helium was used as carrier gas at a flow rate of 1 mL min⁻¹. The temperature of the injector was maintained at 220 °C. The essential oil samples were diluted with dichloromethane (1:10), the injection volume was 2.0 µL and split mode (1:30). The temperatures of the transfer line, ions source, and quadrupole were 285, 230 and 150 °C, respectively. The column temperature was initially programmed at 60 °C, heated at 2 °C min⁻¹ to reach the temperature of 180 °C for 4 minutes, and then heated at 10°C min⁻¹ to reach 260°C and lastly heated at 40°C min⁻¹ to reach the final temperature of 300°C. The detection system was MS with scan mode in the mass/charge range of 40–550 m/z with "Solvent Delay" of 3 min. The retention indices (RI) of compounds of the essential oils were determined based on the alkane series C7-C26. EO chemical compounds were identified by comparison of their mass spectra with the mass spectra in the NIST 11.0 library and by means of their retention indices, compared with those in the literature (Adams, 2007).

Statistical analysis

The completely randomized statistical design was applied for the four concentrations of NaCl (50, 100, 150 and 200 mM) and the control (without NaCl), with nine replications per treatment. Data were submitted to analysis of variance (ANOVA) and the means compared by the Tukey mean test (p<0.05) using the software Sisvar v. 5.6 (Ferreira, 2011). Cluster analysis (CA) and principal component analysis (PCA) were performed in order to discriminate the essential oil composition based on different NaCl levels. Variables were analyzed by the software Statistica v 13.3 (STATSOFT., 2017).

Conclusion

Conclusively, the development of *M. officinalis* was negatively affected by salt stress. Decreases in growth, biomass accumulation and water potential of shoots were some of the apparent responses to salinity. In addition, salinity also caused physiological disturbances such as chlorosis. Necrosis was also observed in plants subjected to high concentrations of NaCl (100, 150 and 200 mM), as a consequence of decreased chlorophyll content and increased membrane damage. Our results demonstrated that lemon balm plants are tolerant to low salinity concentrations (up to 50 mM). Different metabolic responses were identified in this species. Plants subjected to higher NaCl concentrations presented an increased activity of the antioxidant enzymes including SOD and CAT, in addition to a secondary metabolism, maintained by the production of essential oil. The monoterpene synthesis was influenced by salinity, mainly the compounds neryl-acetate and geranyl-acetate, which are responsible for protecting plants against different environmental stresses.

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