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Effect of salt stress on the growth of *Lippia gracilis* Schauer and on the quality of its essential oil

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ABSTRACT

This study evaluated the effect of salt stress on the growth of *Lippia gracilis* Schauer, a species native to the *caatinga* (shrublands) of Brazil and rich in essential oils, as well as on the quality of its oil. We exposed individuals of *L. gracilis* to NaCl, in the following concentrations, for a period of 40 days: 25 mM; 50 mM; 75 mM; and 100 mM. An additional group of plants was not exposed to NaCl (controls). Data were collected on days 20 and 40. We evaluated relative growth rate; shoot and root dry weight; relative water content; proline concentration in leaves; and chemical composition of the essential oil. At all concentrations, NaCl reduced the relative growth rate in comparison with that observed for the controls. No significant difference in relative water content was observed among treatments. In all treatments, the proline concentration in leaves was highest on day 40. Salt stress did not affect the yield or the concentrations of the constituents of the essential oil of *L. gracilis*, carvacrol and thymol showing the highest concentrations in all treatments.

Key words: Alecrim-da-chapada, carvacrol, salt stress, thymol

Introduction

The productive potential and yield of some plant species are strongly affected by the environment. The presence of salts in the soil and in irrigation water is an important environmental factor because it can directly influence growth and crop production, particularly under certain weather conditions (Stanev 2010; Baser & Buchbauer 2010). Increasing salinization, which is a growing phenomenon worldwide, especially in arid and semi-arid regions, is caused by climatic conditions and agricultural irrigation (Sairam & Tyagi 2004). The unregulated use of fertilizers results in the contamination of irrigation water, which contributes to increasing salt concentrations in certain areas. Salinization and soil saturation problems have been identified in approximately 50% of the 250 million irrigated hectares worldwide; 10 million hectares are abandoned annually as the result of such problems (Lima-Júnior & Silva 2010).

The immediate effects that salinization has on plants are decreased osmotic potential, nutritional imbalance due to high ionic concentration, and the toxic effects of certain ions, especially chlorine and sodium (Munns 2002; Flowers 2004; Willadino & Camara 2010). Soil salinization results in reduced biomass accumulation, because of the metabolic cost of energy associated with adapting to salt stress conditions. The increase in osmotic pressure of the soil solution and the reduction in the water infiltration rate result in water limitation for plants (Taiz & Zeiger 2009).

Water limitation has a negative effect on the growth and development of most plant species. However, water stress and other abiotic stresses have often been shown to have positive effects in medicinal and aromatic plants by influencing the accumulation of active constituents (Gobbo-Neto & Lopes 2007). This becomes relevant when the cultivation of resistant species and production of metabolites of economic importance in semi-arid regions where plants are subjected to abiotic stress are considered in conjunction. In herbaceous plants and shrubs, for instance, the concentrations of terpene compounds are likely to increase in plants grown under stress conditions, especially water stress—even moderate water stress (Lima *et al*. 2003; Gobbo-Neto & Lopes 2007; Taarit *et al*. 2010).

Essential oils are among the chemical compounds of economic importance produced by some plant species. These volatile compounds, which are produced and stored in trichomes, are involved in plant-animal, plant-microorganism, and plant-plant interactions that aid plant

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maintenance, survival, and adaptation to environmental conditions (Castro *et al.* 2008). According to Morais (2009), essential oils from medicinal plant species occurring in the *caatinga* (shrublands) of Brazil are promising sources of new antimicrobials, because the climate of the *caatinga* promotes biosynthesis.

In northeastern Brazil, species in the genus *Lippia* (Verbenaceae) are widely used in folk medicine. One of the most widely used species is *Lippia gracilis* Schauer, known locally as *Alecrim-da-Chapada*, which is a native to the *caatinga*. The species produces an essential oil containing phytochemicals of proven antimicrobial effectiveness, in varying proportions (Albuquerque *et al.* 2006; Oliveira *et al.* 2008; Motta-Neto *et al.* 2010).

The ability to grow native medicinal plants adapted to conditions of high concentrations of salts in the soil while maintaining production of their active metabolites would provide small local producers the opportunity to grow low-cost crops. Few studies have evaluated the effect of salt stress on plants with medicinal potential, especially with respect to the production of secondary metabolites. This study evaluated the effect of salt stress on the growth, yield, and quality of essential oils of *Lippia gracilis*.

Material and methods

The study was conducted in a greenhouse at the State University of Rio Grande do Norte, in the city of Mossoró (06°12'43"S; 37°20'39"W). The city is located in northeastern Brazil, in the semi-arid region of the country, and has an annual average temperature of 27.5°C, an annual average relative humidity of 68.9%, an annual average cloud cover of 4.4 tenths, and an annual average rainfall of 673.9 mm. According to the Köppen climate classification system, the climate of the region is type BSh (hot, dry steppe), summer being the rainy season (Carmo-Filho *et al.* 1987).

Lippia gracilis plants were collected in the municipality of Felipe Guerra, also in the state of Rio Grande do Norte, and planted in 8-L pots containing a substrate composed of washed sand, clay, and a commercial compound (Polifértil Home®; Polifértil Nutrição, Importação e Exportação Ltda., Uberaba, Brazil), at 1:1:1. The water retention capacity of the substrate was evaluated before the cuttings were planted. Plants were cultivated for approximately 60 days to obtain individuals of comparable sizes. Size-selected plants were exposed to no salt stress (controls) or to one of four levels of salt stress resulting from the addition of 25, 50, 75, or 100 mM of NaCl (corresponding to 0.49, 2.97, 5.73, 7.79, and 8.49 mS cm^{-1} of electrical conductivity, respectively) to the irrigation water. The experiment lasted 40 days, during which time we evaluated shoot and root dry weight; relative growth rate (RGR); relative water content (RWC); proline concentration; and the chemical composition and yield of the essential oil.

We evaluated growth by determining the dry weight of shoots and roots after 20 and 40 days of exposure to salt stress. The plant material was dried in a forced-air kiln at 70°C. The RGR (expressed in $g.g^{-1}.day^{-1}$) reflects the temporal increase in dry weight and was calculated by the following equation:

$$RGR = \frac{\log W_2 - \log W_1}{d_2 - d_1}$$

where W_2 and W_1 are the dry weights at 40 days of exposure (d_2) and 20 days of exposure (d_1), respectively.

The RWC was evaluated in three leaf discs of known diameter, taken with a hole punch from the fourth leaf below the shoot apex. The discs were immediately weighed to determine the fresh weight, after which they were placed in Petri dishes over water-soaked filter paper in a biological oxygen demand incubator (at 25°C and 80% relative humidity) for 10 h in the dark. Subsequently, the discs were weighed to determine the turgid weight and dried in a forced-air kiln at 70°C. The RWC (expressed as a percentage) was quantified by the following formula:

$$RWC = \frac{Wf - Wd}{Wt - Wd} \cdot 100$$

where *Wf*, *Wd*, and *Wt* are the fresh, dry, and turgid weights, respectively. The RWC was analyzed at 20 and 40 days of exposure.

Proline concentration was determined in expanded leaves harvested between 5:00 a.m. and 6:00 a.m. Fresh leaves (750 mg) were crushed and placed in a test tube containing 15 ml of 5-sulfosalicylic acid and centrifuged at 2000 rpm for 3 min. A mixture of 3 ml of supernatant, 3 ml of acetic acid, and 3 ml of ninhydrin was heated in a water bath at 100°C for 1 h. After color development, samples were cooled in an ice bath and 6 ml of toluene was added for phase separation. The colorless fraction was discarded, and the color fraction was read at 520 nm. Absorbance values were logged in the proline standard curve equation (Silva *et al.* 2010); results were expressed in mM of proline/g of fresh weight. This variable was assessed at 20 and 40 days of exposure.

Essential oil constituents were analyzed in fresh leaves. Data were collected in April 2012, between 5:00 a.m. and 6:00 a.m., on a 27°C day without rainfall. Essential oils were extracted through steam distillation using a modified Clevenger apparatus. For each treatment, we collected leaves from three plants (1 kg per plant). The leaves were weighed and placed in a 2-L round-bottom flask containing 1.5 L of distilled water. The extraction process was performed over a 2-h period at ≈100°C. The yield (mass of the essential oil extracted) is expressed as a percentage of the wet mass of plant material (w/w).

The analysis and quantification of the most prevalent essential oil constituents (thymol, carvacrol, and *p*-cymene) followed the methods reported by Hajimehdipoor *et al.* (2010), with modifications. We used high-performance liquid

chromatography (HPLC) in a Shimadzu system (Class-VP; Shimadzu, Kyoto, Japan). The system consists of three pumps (LC-10ATvp; Shimadzu), a photodiode array detector of ultraviolet spectra (SPD-M10Avp; Shimadzu), a column oven (CTO-10ASvp; Shimadzu), an autosampler (SIL-10AF; Shimadzu), an automatic collector (FRC-10A; Shimadzu), and an in-line degasser (CTO-10AS; Shimadzu). The essential oil constituents were separated on a 3-µ, reversed-phase, 4.6 × 150 mm analytical chromatography column (HyperClone ODS [C18]; Phenomenex, Torrance, CA), at a 1-µl injection volume, with an acetonitrile:water gradient (from 50:50 to 90:10 in 20 min), and at a flow rate of 0.8 ml/min.

Samples of each oil at known concentrations were analyzed in triplicate using HPLC to verify the reproducibility of the method. The concentrations of thymol, carvacrol, and *p*-cymene were quantified with an external standard method; calibration curves were built as described by Ciola (1998), from the arithmetic mean of the corresponding peak areas of metabolites and their concentrations under the conditions described above. The solvents used for the chromatographic analysis were HPLC grade; absorbance reads were obtained at 202 nm (for thymol and carvacrol) or 211 nm (for *p*-cymene).

The experimental delineation was a randomized block design, with four blocks and five treatments. The experimental plot consisted of nine vessels, each containing an established plant. Six plants were randomly selected to assess growth (3 plants on day 20 and 3 plants on day 40). The three remaining plants were used in evaluating the other variables studied. Data were compared by Tukey's test at a 5% probability.

Results and discussion

Among the *Lippia gracilis* plants evaluated, the RGR values decreased markedly in parallel with increasing salinity. A growth rate reduction of approximately 30% was observed in plants exposed to the highest concentration of NaCl (100 mM), the RGR was approximately 30% lower than that observed for the control plants. The RGR was not affected in plants exposed to the lowest concentration of NaCl (25 mM), although decreases in the RGR were observed at the higher concentrations (Tab. 1). Similar results were obtained by Hendawy & Khalid (2005), who reported a 34-48% reduction in the dry weight of *Salvia officinalis* exposed to 50 mM of NaCl. In that same species, Taarit et al. (2010) observed a more dramatic (61%) decrease in plants exposed to 100 mM of NaCl. According to Sudério *et al.* (2011), salinity affects plant growth by impeding cell elongation, a process mediated by alpha- and beta-galactosidases. Those authors demonstrated that high salinity levels reduce the activity of these enzymes, resulting in delayed stem growth in *Vigna unguiculata* seedlings.

Salt stress also inhibits plant growth by reducing the osmotic potential in the soil solution. A reduction in osmotic potential restricts water availability or causes excessive ion accumulation in plant tissues, resulting in ion toxicity, nutritional imbalance, or both (Tester & Davenport 2003). Consequently, photosynthetic efficiency is compromised, because salt stress induces stomatal closure to prevent "sweating", thereby limiting the absorption of CO_2 (Taarit *et al.* 2010).

Table 1. Relative growth rate in *Lippia gracilis* between 20 and 40 days of exposure to salt stress.

NaCl concentration	Mean RGR ($g.g^{-1}$ day^{-1})
0 mM	1.82 a
25 mM	1.67 ab
50 mM	1.63 b
75 mM	1.49 bc
100 mM	1.32 c

RGR – relative growth rate.
Means followed by different letters differ significantly ($p \leq 0.05$), according to Tukey's test.

The energy required for osmoregulation is another factor that accounts for reduced growth in plants exposed to high levels of salinity (Gheyi *et al.* 2005). In the present study, increased concentrations of proline, which plays a role in osmoregulation, were observed in plants under salt stress. At 20 days of exposure, the mean proline concentration was significantly higher than that observed in the control plants only in the plants exposed to ≥ 75 mM of NaCl. However, at 40 days of exposure, the plants exposed to ≥ 25 mM of NaCl showed proline concentrations that were significantly higher than that observed in control plants. This indicates that exposure time influenced osmoregulation by increasing the production of proline (Tab. 2). In plants, the osmoregulation process involves the synthesis and distribution (in various organs and within the cells) of organic solutes, for the protection of macromolecules, maintenance of membrane integrity, and regulation of ion transport (Willadino & Camara 2004; Willadino *et al.* 2011).

Evaluating the dry weight of shoots and roots separately, we found that higher NaCl concentrations and longer exposure to salt stress translated to lower dry weight values in both (Fig. 1). Linear growth and biomass accumulation in vegetative organs depend on the intensity of cell division and differentiation, both of which are affected by salinity (González 2001). Our results showed that exposure to salt stress for 20 and 40 days affected the growth of roots more than that of the shoots (Fig. 1). Although limited space for root development might have contributed to this result, other authors have reported that root systems are less affected by salinity than are shoots, despite the fact that the roots receive greater exposure to the saline environment (Cavalcanti *et al.* 2005; Silva *et al.* 2011).

The RWC did not differ statistically among the treatments evaluated here. The RWC varied from 60% to 68% and from 66% to 73% at 20 and 40 days of exposure, respectively, indicating that water absorption capacity was not

Table 2. Proline concentration in leaves of *Lippia gracilis* after 20 and 40 days of exposure to salt stress.

NaCl concentration	Proline concentration (mM/g fresh weight)	
	Day 20	Day 40
0 mM	1.03 a	1.68 a
25 mM	0.90 a	5.55 b
50 mM	1.97 a	5.95 b
75 mM	4.26 b	6.54 b
100 mM	4.17 b	7.56 b

Means followed by different letters differ significantly ($p \leq 0.05$), according to Tukey's test.

affected by the intensity of the salt stress. It is quite likely that the stability of the RWC reflects the accumulation of proline in leaves (Tab. 2), which allowed osmoregulation and the maintenance of hydration. Távora *et al.* (2001) obtained contrasting results, reporting a decrease in RWC in guava plants exposed to increasing concentrations of NaCl. The authors observed no significant difference in the RWC values in relation to the length of exposure, which was probably attributable to the adaptation of the studied species to water scarcity. Fumis & Pedras (2002) studied the effects of water stress on wheat cultivars and found that, between two wheat cultivars that maintained constant RWC values, proline concentrations were higher in the cultivar that was more tolerant to water stress. The maintenance of high RWC values might indicate better adaptation to water deficit through greater osmoregulation (Fumis & Pedras 2002; Maia *et al.* 2007).

In the present study, we found that salt stress reduced RGR, from 1.23% in the control plants to 1.07% in the plants exposed to 100 mM of NaCl. However, the yields of essential oil were not altered by exposure to any concentration of NaCl. Nevertheless, other authors have reported that water stress and salt stress, as well as other stressors, promote increased yields of essential oils (Nerfatti & Marzouk 2008; Gobbo-Neto & Lopes 2007). Conversely, Ansari *et al.* (1998) found that salt stress reduced the yields of essential oils in three species of *Cymbopogon*.

According to Combrinck *et al.* (2007), the distribution and structure of trichomes in a plant contribute to controlling transpiration and regulating the temperature of the organ on which they are located. In addition, it has been postulated that trichome density and phenolic compounds produced in these structures provide protection against ultraviolet-B radiation and other abiotic stresses. Although the number of trichomes was not quantified in the present study, this might explain why the plants exposed to salt stress showed essential oil yields similar to those of the plants under no stress, despite the lower RGR observed for the former.

Our analysis of the composition of the essential oils of *Lippia gracilis* showed that, in the control plants, the principal constituent was carvacrol (at 42.07%), followed by thymol (at 32.35%), and *p*-cymene (at 17.12%). These results are in agreement with those obtained by Albuquerque *et al.* (2012) in plants of the same species.

In the present study, the concentration of NaCl did not influence the concentrations of the major components of the essential oil. Other factors, such as the method of extraction and the environmental conditions under which the plants are cultivated, can influence the concentrations of chemical compounds in plants exposed to salt stress. According to Neffati & Marzouk (2008), the concentrations of chemical compounds in plants can decrease in response to higher levels of salinity. The authors found that the major components of the essential oil of *Coriandrum sativum* increased at low concentrations of NaCl and decreased when the concentration was increased to 75 mM. In contrast, Taarit *et al.* (2010) reported that exposure to 25-75 mM of NaCl increased the yield of sage oil in *Salvia officinalis*. Increased concentrations of essential oils have also been observed in plants exposed to high temperatures (Morais 2009).

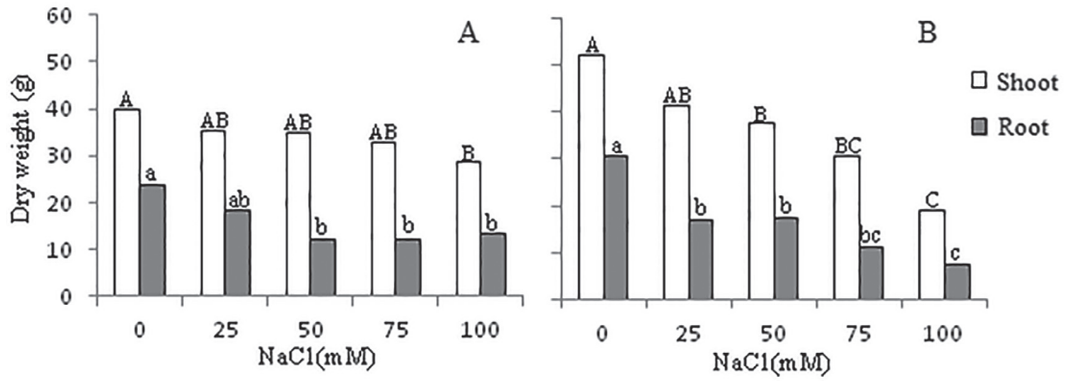

Figure 1. Dry weights of shoots and roots of *Lippia gracilis* after exposure to various concentrations of NaCl, for 20 days (**A**) and 40 days (**B**). Different letters (upper-case over unfilled bars and lower-case over filled bars) indicate significant differences ($p \leq 0.05$), according to Tukey's test.

Our results show that, under the conditions studied, salinity had a negative influence on the growth of *Lippia gracilis*. That finding might be attributable to the osmoregulation that occurred in the plants, proline concentrations increasing in parallel with increases in the concentration of NaCl.

As previously mentioned, we observed no significant difference in RWC among the treatments evaluated. The highest concentrations of proline in leaves were observed at 40 days of exposure, regardless of the NaCl concentration. The exposure to salt stress did not affect the yield or the constituent concentrations in the essential oil, carvacrol and thymol showing the highest concentrations in all treatments.

Our results provide information for growing medicinal native plants under conditions of high salinity. It appears that these plants and their active metabolites can be produced at low cost.

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