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MORPHO-PHYSIOLOGICAL CHARACTERIZATION AND PHOTOSYNTHETIC PIGMENT CONTENTS OF ACACIA KARROO HAYNE SEEDLINGS UNDER SALINE CONDITIONS

SUMMARY

Acacia karroo is a leguminous tree listed in most of the Algerian territory. It is a salt-tolerant species and a multipurpose tree in agroforestry. However, the defence mechanisms underlying salt tolerance of this species are still unknown. In this study, the effects of salt stress on various morpho-physiological and biochemical traits of *A. karroo* were investigated. Three-months-old plants were submitted to increasing salt concentrations (0, 200, 400 and 600 mM NaCl), for a period of 21 days. Stem length was not significantly affected by salinity. Increasing salinity reduced the length of root. Number of leaves was maintained constant at 200 and 400 mM NaCl but was reduced slightly at 600 mM NaCl. Also, an increase in crown diameter by 30% under mild and high salt stress was observed. Furthermore, salt tolerance index was not affected at all salinity levels. The leaf mass area was not affected by saline conditions. Salt treatments did not produce a notable change in the relative water content of leaves, indicating a relatively high resistance as well to dehydration, which will certainly contribute to some degree of salt tolerance in *A. karroo*. Relative water loss from excised leaves was significantly higher at 200 mM and similar at high concentration of NaCl as compared to control. The result of variance analysis for the major effect of salinity showed that salt stress significantly decreased the content of photosynthetic pigment in leaves at higher concentrations of NaCl. However, at 200 mM of NaCl, an enhancement of chlorophyll b, total chlorophylls and carotenoids content was observed. At the same level, chlorophyll a presented a constant content compared with control. In conclusion, although plants suffered from salt stress, as shown by the degradation of photosynthetic, they continued their vegetative growth and maintained their internal water potential under salinity conditions. Therefore, *A. karroo* is a potential halophytic species to be cultivated in saline lands and make it favourable for agroforestry practices.

Keywords: *Acacia karroo*, agroforestry, halophyte, NaCl, water potential.

INTRODUCTION

Salinity is a widespread problem, affecting around 831 million hectares of lands that include 397 and 434 million hectares of saline and sodic soils,

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respectively (Teakle and Tyerman, 2010). Salinization of soil and groundwater has been considered as the most critical environmental issue, hindering sustainable agricultural productivity and presenting a challenging task for ecologists and physiologists (Lal, 2009; Mansouri and Kheloufi, 2017). Salinity can affect growth and yield of most plants (Munns, 2002; Nasrin *et al*., 2016), by inducing reduced cell division in roots and leaves (Munns and Tester, 2008), cell elongation, cell differentiation, along with genetic, biochemical, physiological, morphological, and ecological processes, as well as their complex interactions followed by significant tissue damage, leading to the plants' death in case of prolonged exposure to salinity (Ashraf and Harris, 2004).

Halophytes have been regarded as potential new crops for use as forage, vegetable, and oil seed crop (Glenn *et al.*, 2013). However, the potential utilization of halophytic species to grow in salt-affected soil and to facilitate saline soil phytoremediation depends on several factors such as salt accumulation, relative growth rate and biomass conversion, multipurpose utilization, and economic returns to the farmers (Panta *et al*., 2014). *Acacia karroo* Hayne, commonly known as the sweet thorn, is a species of acacia, native to southern Africa from southern Angola to east Mozambique and south Africa (Archibald and Bond, 2003). It belongs to the family of Fabacea (Leguminosae) with the main advantage to make symbiosis with soil microorganisms (rhizobium and mycorrhizae) conferring them the capacity to survive in very poor grounds in nutritional elements (Bashan *et al*., 2012; Boukhatem *et al*., 2016). *A. karroo* varies from a shrub up to 2 m tall to a tree more than 20 m in height, with distinctive white thorns and attractive yellow flowers. The leaves comprise about five pairs of leaflets, each divided into ten or more pairs of smaller leaflets of about 5 mm long (Maroyi, 2017). This species is used for chemical products, forage livestock, domestic uses and environmental management. *A. karroo* is the most widespread acacia in southern Africa and occupies a diverse range of environments from acacia savannahs and woodlands on hills and rocky soils to the banks of dry watercourses in Algeria (Kheloufi *et al*., 2018). *A. karroo* tree can produce seeds prolifically from an early age and is resistant to fire (Midgley and Bond, 2001). It has a lot of potential as a possible source of pharmaceutical products for the treatment of a wide range of both human and animal diseases and ailments. Indeed, *A. karroo* has been used as herbal medicine by the indigenous people of southern Africa for several centuries and several diseases (Maroyi, 2017).

In Algeria, it has been reported that *A. karroo* can germinate under 400 mM of NaCl with 66% of final germination (Kheloufi *et al*., 2017). Thus, introduction of *A. karroo*, as a salt-tolerant species, could be an important strategy in conserving ecology and wood production in the salt-affected regions of Algeria. Moreover, no study has been conducted at morpho-physiological and biochemical levels to understand the mechanisms associated with the adaptability of *A. karroo* under salt stress. Therefore, in the present study, we aimed to examine the effects of various levels of salinity on some morpho-physiological

parameters and photosynthetic pigment contents (chlorophylls and carotenoids) of *A. karroo* seedlings.

MATERIALS AND METHODS

Plant material, growth condition and salt treatment

The seeds of *A. karroo* Hayne were collected from Aïn El Baïda salt farm area (Oran, Algeria) (latitude: 35°39'34.96" N; longitude: 0°40'4.68" W; elevation: 136 m). The pods were collected from 10 trees and the seeds were then mixed. The thousand-seed-weight was 39 g. Sieving and flotation were used to sort out seeds. The clean seeds were then spread on filter paper to dry. Once dried, the seeds undergo a chemical treatment which consisted of immersion in 96% sulphuric acid for 30 minutes followed by washing in distilled water. *A. karroo* seeds need this pre-treatment to break down the seed coat and induce a high germination rate in a short time (Kheloufi, 2017). Seeds were germinated in plastic pot (Top diameter: 10 cm; Bottom diameter: 7 cm; Height: 14 cm) containing 1 kg of mixed substrate (two volumes of sand mixed with one volume of compost) (EC = 49 $mS.m^{-1}$; pH = 6.2; N = 89 $g.m^{-3}$; P_2O_5 = 42 $g.m^{-3}$; K_2O = 27 $g.m^{3}$) and arranged according to the method of complete randomized blocks with four replicates under greenhouse conditions. Sand was sieved at 2 mm to eliminate wastes and coarser material then washed repeatedly with tap water to eliminate all carbonates and chlorides. The experiment was conducted in the green house of Ecology and Environment Department, University of Batna 2, Algeria (latitude: 35°38'10.32"N; longitude: 6°16'31.52"E; elevation: 926 m).

Table 1. Preparation of saline solution and corresponding hydric potential.

NaCl (mM)	NaCl (g/L)	Ψos Level (MPa) (Braccini *et al.*, 1996)
0	0	0
200	11.68	-0.83
400	23.37	-1.67
600	35.06	-2.50

Three months (90 days) old healthy seedlings of uniform size were selected as initial material and further grown in KNOP's nutrient medium. The plants were subjected to salt treatment by supplementing the nutrient medium with varied sodium chloride (NaCl) concentrations (200, 400 and 600 mM) (Table 1). The control plants were grown in the nutrient medium devoid of NaCl. The nutrient solutions were replaced with freshly prepared solutions at every 7 days intervals. After 21 days of salt treatment, leaf, stem, and root samples were harvested from control and NaCl-treated plants for estimation of various parameters. Leaves occupying the same position were sampled from control and NaCl-treated plants for estimation of photosynthetic pigment contents.

Measurement of morphological parameters

Total stem length (SL), total root length (RL), leaves number per plant (LP) and crown diameter (CD) of four plants (n=4) from each treatment were

recorded after 21 days of treatment. For measurement of fresh and dry weights, leaves were excised from control and NaCl-treated plants and the fresh weight was noted immediately. Later, they were wrapped in pre-weighed aluminium foils and kept in an incubator at 80°C for 48h before the dry weight was recorded. Total green leaf area per plant was measured in both control and NaCl-treated plants, using Image analysis system Digimizer software (version 4.6.1, MedCalc Software, Belgium).

Measurement of physiological parameters

Salt Tolerance Index

Salt tolerance index (STI) was calculated by using the following formula developed by Seydi (2003):

$$\mathrm{STI} = \frac{\mathrm{TDW\ at\ Sx}}{\mathrm{TDW\ at\ SI}} \times 100$$

TDW: Total dry weight (oven at 80°C for 48h)
SI: Control treatment
Sx: Salt treatment

Leaf mass area

The leaf mass area (LMA) was calculated using (Hernández and Kubota, 2016) formula:

$$\mathrm{LMA\ (mg/cm2)} = \frac{\mathrm{LDW}}{\mathrm{LA}}$$

LDW: Leaf dry weight (mg)
LA: Leaf area (cm^2)

Leaf relative water content

Leaf fresh weight (LFW) was immediately noted after sampling and subsequently immersed into distilled water for 8 h at room temperature. Leaves were then blotted dry and leaf turgid weight (LTW) was taken prior to incubating at 80°C for 48h. After incubation period, leaf dry weight (LDW) was also noted. The leaf RWC was calculated using following formula (Barrs and Weatherley, 1962):

$$\mathrm{RWC\ (\%)} = \left[\frac{(\mathrm{LFW} - \mathrm{LDW})}{(\mathrm{LTW} - \mathrm{LDW})}\right] \times 100$$

Rate water loss

The rate water loss (RWL) was calculated using (Clarke *et al*., 1989) formula:

$$\mathrm{RWL\ (mg/cm2.min)} = \frac{(\mathrm{FW} - \mathrm{FW2h})/\mathrm{DW}}{\mathrm{LA} \times 120}$$

FW: Leaf fresh weight determined immediately after leaf harvesting
FW2h: Leaf fresh weight measured after 120 minutes under laboratory conditions
DW: Leaves dry weight measured after drying in an oven at 50°C for 2 hours.
LA: Leaf area (cm^2).

Chlorophylls and Carotenoids

Chlorophylls (Chl a, Chl b and Total Chl) and carotenoids (mg.g^{-1} LFW) were extracted by 100% acetone from fresh leaves samples (LFW). After centrifugation (10 000 rpm for 5 minutes), supernatants were used for the analysis of pigments. Absorbances were determined at 645, 652, 662, and 470 nm, respectively, using UV/visible light spectrometer (4-16K, Sigma) and the following equations were used for calculations (Lichtenthaler and Wellburm, 1983):

$$\text{Total Chl} = \frac{\text{A652} \times 27.8 \times 20}{\text{mg LFW}}$$

$$\text{Chl a} = \frac{[(11.75 \times \text{A662}) - (2.35 \times \text{A645})] \times 20}{\text{mg LFW}}$$

$$\text{Chl b} = \frac{[(18.61 \times \text{A645}) - (3.96 \times \text{A662})] \times 20}{\text{mg LFW}}$$

$$\text{Car} = \frac{[((1000 \times \text{A470}) - (2.27 \times \text{Chl a}) - (81.4 \times \text{Chl b}))/227] \times 20}{\text{mg LFW}}$$

Statistical analysis

All the experiments were conducted with four replicates (n=4) and the results were expressed as mean ± standard deviation (SD). All the data were subjected to one-way analysis of variance (ANOVA) and Duncan's multiple-range test ($P<0.05$) using SAS Version 9.0 (Statistical Analysis System) (2002) software.

RESULTS AND DISCUSSION

Morphological traits

The effect of sodium chloride was significant for root length ($p = 0.0199$), leaves number per plant ($p = 0.0278$) and crown diameter ($p = 0.0010$), except stem length, which was not significantly affected by salinity ($p = 0.2178$) (Figure 1, Figure 2, Table 2).

Figure 1. *Acacia karroo* seedlings of 111 days-old cultivated under different salinity levels (0, 200, 400 and 600 mM NaCl) after 21 days of treatment.

Table 2. Mean comparison and analysis of variance effects of salinity on stem length, root length, leaves per plant, crown diameter, crown diameter, leaf mass area, rate water loss, rate water loss, relative water content, salinity tolerance index, total chlorophylls content, chlorophyll a content, chlorophyll b content and carotenoids content.

Parameters	Sources of variation	Df	F	P
Stem length (SL)	NaCl treatment	3	1.73	0.2178
Root length (RL)	NaCl treatment	3	4.82	0.0199
Leaves per plant (LP)	NaCl treatment	3	4.32	0.0278
Crown diameter (CD)	NaCl treatment	3	10.81	0.0010
Salinity tolerance index (STI)	NaCl treatment	3	1.96	0.1738
Leaf Mass Area (LMA)	NaCl treatment	3	7.36	0.0047
Relative water content (RWC)	NaCl treatment	3	1163.37	<0.0001
Rate water loss (RWL)	NaCl treatment	3	8.04	0.0033
Chlorophyll a content (Chl a)	NaCl treatment	3	27.41	<0.0001
Chlorophyll b content (Chl b)	NaCl treatment	3	24.65	<0.0001
Total chlorophyll content (TChl)	NaCl treatment	3	25.17	<0.0001
Carotenoid content (Car)	NaCl treatment	3	20.81	<0.0001

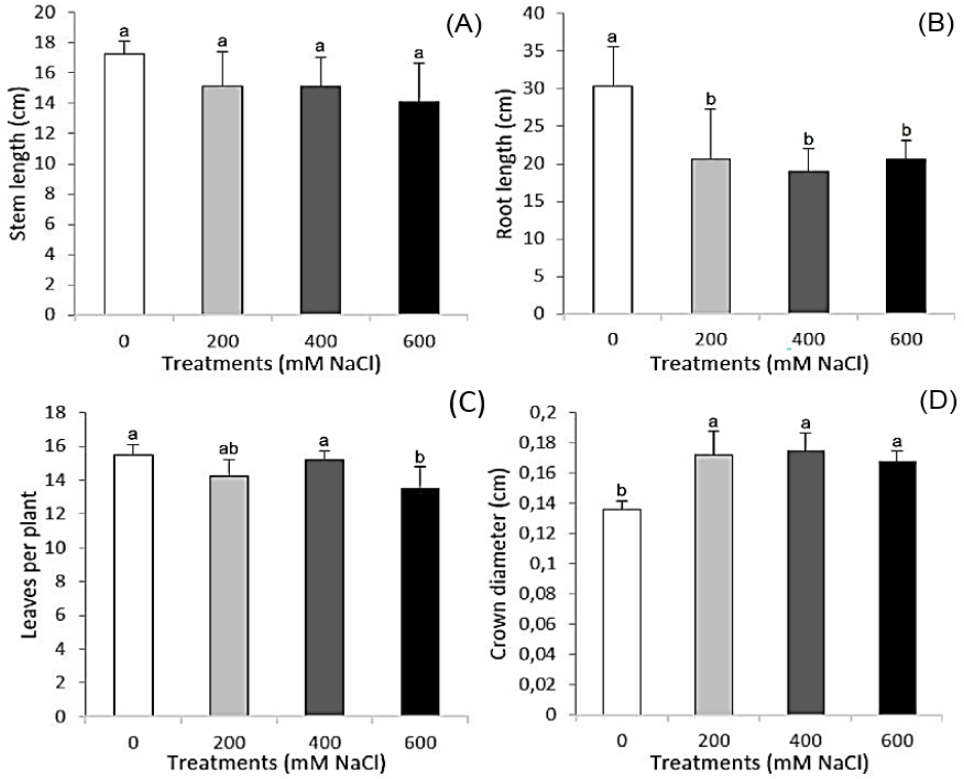

Figure 2. Effects of salt stress on (A) Stem length, (B) Root length, (C) Leaves per plant and (D) Crown diameter of *Acacia karroo* seedlings after 21 days of various levels of saline treatments. Means, in each box, with similar letters are not significantly different at the 5% probability level using Duncan's test.

Increasing salinity reduced the length of root by 10 cm at lower and higher concentrations compared with control. Reduction in plant height and other growth parameters are the most distinct and obvious effects of salt stress, since inhibition of growth is probably the most general response of plants to stress (Munns and Tester, 2008).

In this study, all results indicated that different growing characteristics were significantly affected by salinity stress, except stem length. Depressed growth due to high salinity is attributed to several factors such as osmotic stress, specific ion toxicity and ion imbalance, and induced nutritional deficiency (Giri *et al*., 2003; Morant-Manceau *et al*., 2004; Meloni *et al*., 2008).

The first plant part interacts with salt is the roots and it is almost inevitable that the crops are affected by salt concentration. Therefore, the results obtained in present study agree with previous studies on *A. karroo* seedlings and other species of the same genus, reporting the negative effect of salt concentration on plant height: Kheloufi *et al*., 2016a (*A. saligna* and *A. decurrens*); Kheloufi *et al*., 2016b (*A. tortilis*, *A. ehrenbergiana* and *A. dealbata*), Kheloufi *et al*., 2017 (*A. karroo*); Rahman *et al*., 2017 (*A. auriculiformis*) and Theerawitaya *et al*., 2015 (*A. ampliceps*). The delay of the radicle growth under salt stress may be due to the reduction in the turgor of the radicle cells (Bradford, 1995; Saroj and Soumana, 2014). The reason that the root and shoot length are affected negatively by salt stress is due to toxic effect of salts as well as inhibition of cytokinesis and cell expansion (Kurum *et al*., 2013). The increase in osmotic pressure around the roots because of saline environment can also prevent water uptake by root and results with short root (Aroca *et al*., 2011).

Number of leaves was maintained constant at 200 and 400 mM but was reduced by two leaves at 600 mM of NaCl treatment. The crown diameter was the most affected of the morphological parameters and showed an overall increase as salinity increased (Figure 2). Salinity stress had also remarkable effects on other plant growth parameters such as leaf number and crown diameter. Salinity usually results in a biochemical loosening of the cell wall under turgor pressure, which initiates cell expansion followed by water and solute uptake, and an increased succulence (Chen *et al*., 2015). In this investigation, an increase in crown diameter (30%) under mild and high salt stress in this salt tolerant species may be vital under physiological drought for its better water storage, which is an adaptation for ion dilution to minimize the effect of Na^+ and Cl^- in plant tissues (El-Lamey, 2015). Reduction in cell size was also attributed to the plant ability to reduce its size to minimize salt uptake (Zapryanova and Atanassova, 2009). The reduction in biomass increased with the increase in salinity which is obvious because of disturbances in physiological and biochemical activities under saline conditions as shown by Vinocur and Altman (2005) that may be due to the reduction in leaf area and number of leaves.

Physiological traits

STI, a reliable criterion for salt tolerance (Ali *et al*., 2013), was not affected by salt stress at low and high levels ($p = 0.1738$) (Figure 3A, Table 2).

The higher STI at seedling stage indicate that the key mechanisms of salt tolerance in plants may be associated with (i) accumulation of compatible solutes like proline, total sugars, reducing sugars and total free amino acids; (ii) increase amount of K^+, Ca^{2+} and Mg^{2+} in phyllodes than roots; (iii) increase K^+ retention in photosynthetic tissues through hindering Na^+ uptake; (iv) anatomical adjustment by increasing the size of spongy parenchymal tissue of phyllodes, endodermal thickness of stems and roots, and pith area of roots; (v) efficient Na^+ sequestration in vacuoles that would be facilitated by a decrease in stomatal density and (vi) the enhanced Na^+ exclusion **(**Rahman *et al*., 2017).

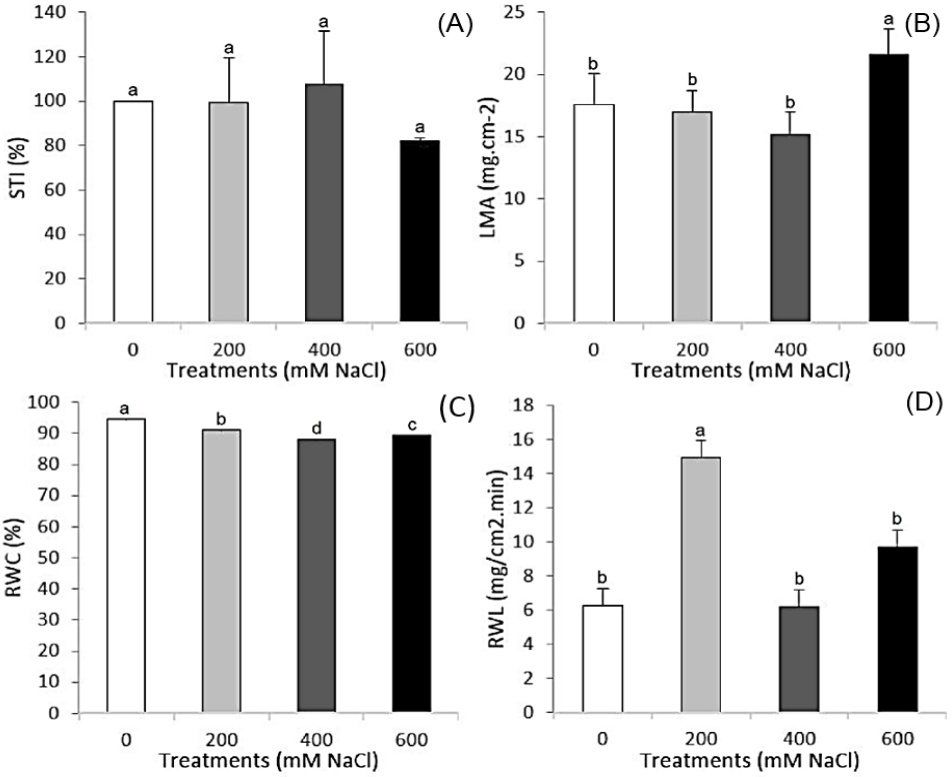

Figure 3. Effects of salt stress on (A) Salinity tolerance index, (B) Leaf mass area, (C) Relative water content (D) Rate water loss of *Acacia karroo* seedlings after 21 days of various levels of saline treatments. Means, in each box, with similar letters are not significantly different at the 5% probability level using Duncan's test.

A meta-analysis on Figure 3 showed that leaf-related parameters (leaf mass area, relative water content and rate water loss) were significantly affected by salinity (Table 2). The LMA was not affected by salinity at 200 and 400 mM compared with control but was improved by 22.2% at 600 mM of NaCl treatment. Our results are inconsistence with Munns and Termaat (1986) and Franco *et al*. (1997) who reported that NaCl highly reduced leaf mass area. Leaf mass per area is a composite structural parameter.

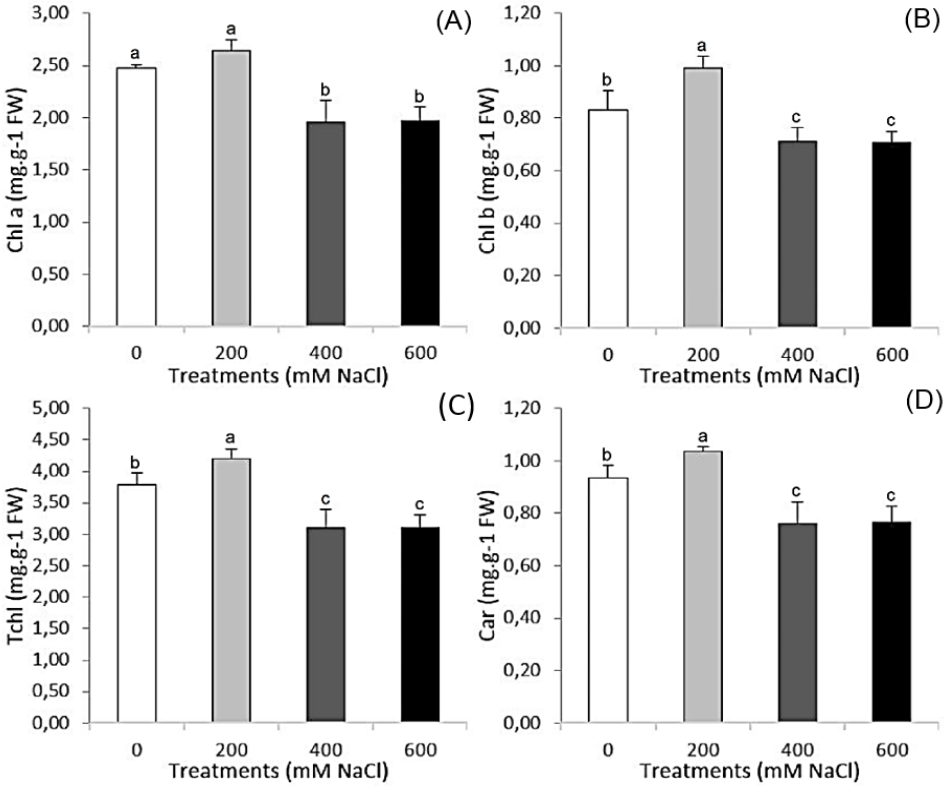

Figure 4. Effects of salt stress on (A) Total chlorophylls content, (B) Chlorophyll a content, (C) Chlorophyll b content and (D) Carotenoids content of *Acacia karroo* leaves after 21 days of various levels of saline treatments. Means, in each box, with similar letters are not significantly different at the 5% probability level using Duncan's test.

It is not only closely related to many physiological responses of plants, but also can measure the investment of dry mass per unit of light-intercepting leaf area (Poorter *et al*., 2009). LMA is considered an important indicator of plant ecological strategies and has been studied widely in plant ecology, agronomy, forestry, and plant physiology (Liu and Liang, 2016).

The RWC decreased slightly with increase in salinity levels (Figure 3C). Indeed, salt treatments did not produce a notable change in the water content of the plants leaves, indicating a relatively high resistance as well to dehydration, which will certainly contribute to some degree of salt tolerance in *A. karroo*. Salt tolerance is also depending on the plant capacity to accumulate Na^+ and Cl^- in the vacuole, to avoid reaching toxic concentrations in the cytoplasm, a mechanism that is especially efficient in some succulent, highly tolerant dicotyledonous halophytes (Haque *et al*., 2016).

RWL from excised leaves was significantly higher at 200 mM of NaCl by 5 mg/cm^2.min and similar at high level of NaCl compared with control (Figure 3D, Table 2). This improvement could be due to stomatal closure, it will typically

induce the limitation of gas exchange and alter the rate of photosynthesis and metabolism (Wang and Nii, 2000). RWL has been suggested as a screening technique to identify genotypes under drought stress (Gunes *et al*., 2008). Indeed, this trait is a direct measurement of plant water deficit and a good criterion for the selection of drought tolerant plants (Farshadfar *et al*., 2001).

Chlorophylls and carotenoids

Chlorophyll a is the principal photosynthetic pigment while chlorophyll b is an accessory one. The result of ANOVA for the major effect of salinity showed that salt stress significantly decreased ($p < 0.0001$) the content of photosynthetic pigment in leaves (Table 2) at higher concentrations of NaCl (400 mM and 600 mM). Indeed, Chl a, Chl b, Tchl and Car were degraded by 20.5, 14.4, 17.7 and 18.2% of control, respectively, under extreme concentration of NaCl (400 and 600 mM NaCl). However, at 200 mM of NaCl, an enhancement of chlorophyll (b), total chlorophylls and carotenoids content was observed as compared to control (Figure 4). At the same level, chlorophyll (a) presented a constant content compared with control (Figure 4A).

The reduction of photosynthetic pigment content is likely due to chlorophyll degradation induced by toxic levels of NaCl (Hassanein *et al*., 2009). These results are consistent with those reported by Theerawitaya *et al.* (2015), who indicated that chlorophyll content significantly decreased in the leaves of *A. ampliceps* with increasing NaCl concentration. Reduction of chlorophyll levels in salt-treated plants is due to the inhibition of chlorophyll synthesis, together with the activation of its degradation by the enzyme chlorophyllase. Yet, this is not the only reason for the inhibition of photosynthesis in the presence of salt, since NaCl also inhibits key enzymes involved in this process (Parihar *et al*., 2015).

Under salt stress, leaf chlorophyll content could be altered due to impaired biosynthesis and accelerated degradation of the pigments (Mäkelä *et al*., 2000). Therefore, the levels of photosynthetic pigments, such as Chl a and Chl b, are vital for steady photosynthesis in plants during salt stress (Richardson *et al*., 2002). It has been reported that photosynthesis in some halophytes remains unaffected by salinity or even increases at low salinity (Flowers and Colmer, 2015). Increased chlorophyll and carotenoid content under saline stress may be related to a decrease in leaf area, it also can be a defensive response to reduce the harmful effects of drought stress (Farooq *et al*., 2009).

CONCLUSION

In conclusion, although plants suffered from salt stress, as shown by the degradation of photosynthetic, they continued their vegetative growth and maintained their internal water potential under salinity conditions. Therefore, *A. karroo* is a potential halophytic species to be cultivated in saline lands and make it favourable for agroforestry practices. However, this screening is not sufficient for a complete characterization of *A. karroo* Hayne as a halophyte. It will be necessary to go further at the biochemical (e.g., proline, soluble sugar, ion

accumulation) and molecular levels, and to explore other stages of development such as flowering and fruiting in response to salt conditions in situ.

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