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## **ANATOMICAL CHANGES INDUCED BY SALINITY STRESS IN ROOT AND STEM OF TWO ACACIA SPECIES (*A. KARROO* AND *A. SALIGNA*)**

### **SUMMARY**

Soil salinity is one of the main abiotic constraints limiting plant growth. This paper focuses on the concept of internal adaptation in relation to salt tolerance during the vegetative phase. Under saline conditions, we evaluated some anatomical changes in stems (area, perimeter, cortex thickness, stele area, stele perimeter, pith area) and roots (thickness, cortex thickness and stele thickness) of two acacia species (*A. karroo* and *A. saligna*). Plants of 90 days old were cultured at various concentrations of NaCl (0, 200, 400 and 600 mM) for 21 days. The experiment was laid out in completely randomized design with four replications. For microscopic analysis, the stem tissues were cross-sectioned and the root were profile viewing. Results showed that salt caused remarkable changes in some anatomical-related parameters. Microscopic studies showed that every acacia species had made its own anatomical changes in stem and root by increasing/decreasing organ area, such as cortex thickness, stele thickness and pith area compared to control. In conclusion, under saline regimes, both species adapted specific characteristics of the roots and stems for better survival under saline environments.

**Keywords:** Acacia, forestry, salt tolerance, NaCl, osmoprotection.

### **INTRODUCTION**

The response of plants to salinity can be described in two main phases: Shoot ion-independent reaction occurs first (within minutes to days) and is thought to be related to Na<sup>+</sup> detection and signaling (Roy *et al.*, 2014). In this first phase, the effects of salinity can be significant on water relations, causing stomatal closure and inhibition of leaf expansion (Miller *et al.*, 2010). The second phase, the salt-dependent response to salinity, develops over a longer period (days to weeks) and leads to the accumulation of toxic ions in the stem, particularly in old leaves, leading to premature leaves senescence (Munns and Tester, 2008). Salinity can affect the growth and yield of most plants, inducing a reduction in cell division in roots and leaves, auxesis, cell differentiation, as well as genetic, anatomical, biochemical, physiological processes, morphological, ecological, with their complex interactions followed by significant tissue

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damage, resulting in plant senescence under prolonged exposure to salinity (Reynolds *et al.*, 2005 ; Negrão *et al.*, 2017, Kheloufi *et al.*, 2018a).

In Algeria, it has been reported that *A. karroo* could germinate under 400 mM NaCl with 66% of final germination (Kheloufi *et al.*, 2017a). However, the seeds of *A. saligna* could only germinate at 150 mM with only 18% of final germination (Kheloufi *et al.*, 2016). Indeed, in previous studies (Kheloufi *et al.*, 2016 ; Kheloufi *et al.* 2017a ; Kheloufi *et al.*, 2018b), we showed that *A. karroo* was the most salt-tolerant species compared to *A. saligna* whose seeds were sensitive to the germination stage. *A. saligna* seems very sensitive compared to *A. karroo* but can be considered as a salt-tolerant glycophyte. However, the status of 'Sensitive' is only given as a comparison between *A. saligna* and *A. karroo*. Effectively, *A. saligna* is a salt-tolerant glycophyte that has been proven to contribute to the prevention of soil erosion and revegetation with moderate salinity (Sekkour, 2008 ; Mansouri, 2011). Thus, the introduction of *A. karroo* and/or *A. saligna*, as salt-tolerant species, could be an important strategy for the conservation of ecology and wood production in the Algerian salt-affected regions. In addition, no studies have been undertaken to characterize and understand the internal anatomical mechanisms associated with the adaptation of *A. karroo* and *A. saligna* under salt stress. Therefore, in this study, we sought to examine the effects of various levels of salinity on some anatomical changes in both *A. karroo* and *A. saligna* plants under greenhouse.

## MATERIAL AND METHODS

### Plant material, growth condition and salt treatment

The seeds of *A. karroo* Hayne were collected from Ain El Baïda salt farm area (Oran, Algeria) (latitude: 35°39'34.96" N; longitude: 0°40'4.68" W; elevation: 136 m) and those of *A. saligna* from the region of Terga (Ain Temouchent, Algeria) (latitude : 35°26'32.26"N ; longitude : 1°13'42.80"W ; elevation : 2 m). Pods were collected from 10 trees and the seeds were then mixed. The thousand-seed-weight of *A. karroo* and *A. saligna* were 39 g and 15 g, respectively. 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 for *A. karroo* (Kheloufi, 2017) and 90 minutes for *A. saligna* (Kheloufi *et al.*, 2017b), followed by washing in distilled water. *A. karroo* and *A. saligna* 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) (Figure 1) containing 1 kg of mixed substrate (two volumes of sand mixed with one volume of compost) (EC = 49 mS.m<sup>-1</sup>; pH = 6.2; N = 89 g.m<sup>-3</sup>; P<sub>2</sub>O<sub>5</sub> = 42 g.m<sup>-3</sup>; K<sub>2</sub>O = 27 g.m<sup>-3</sup>) 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).



**Figure 1.** Experimental design and different stages of plant development of two acacia species: (A) *A. karroo* and (B) *A. saligna*.

Three months (90 days) old healthy seedlings of uniform size were selected as initial material and further grown in KNOP's nutrient medium. Plants were subjected to salt treatment by supplementing the nutrient medium with varied sodium chloride (NaCl) concentrations (200, 400 and 600 mM). 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, stem and root samples were harvested from control and NaCl-treated plants for estimation of various parameters. It should be noted that for each measurement or assay, a number of 4 replications were used. In addition, and to ensure the study of the effect of salinity on all parameters with the same conditions of growth and development, a considerable number of plants were used (4 plants  $\times$  4 treatments  $\times$  2 species) (Kheloufi *et al.*, 2019).

### Measurement of anatomical parameters

Osmotic adjustments induced by salinity were deduced by cutting the stems and roots with a razor blade (Gillette). Sections of both control and NaCl-treated plants were observed immediately after cutting the tissues (about 1.5 cm below the root apex and 1.5 cm from the shoot apex).

All sections were viewed under light microscope at 40X magnification and without staining. This microscope (Zeiss Microscope camera) has an integrated camera connected to a computer. The best observations were then captured as images (JPG). These images were subjects of several dimensional analyzes with Motic Image Plus 2.0 software (Motic Instruments Inc., Canada).

#### *For cross sections of a stem:*

SA : Stem area (mm<sup>2</sup>)

SP : Stem perimeter (mm)

SCT : Stem cortex thickness (mm)

SSA : Stem stele area (mm<sup>2</sup>)

SSP : Stem stele perimeter (mm)

SPA : Stem pith area (mm<sup>2</sup>)

***For a root profile observation (without crushing):***

RT : Root thickness (mm)

RCT : Root cortex thickness (mm)

RST : Root stele thickness (mm)

**Statistical analyzes**

All experiments were conducted with four replicates (n = 4) and the results were expressed in average ( $\pm$  standard deviation). All data were subjected to one-way analysis of variance (Treatment), two factors (Treatment and Species) (ANOVA) and Duncan's multiple comparison test ( $p < 0,05$ ) using SAS Version 9.0 (Statistical Analysis System) (2002). Charts were made with Excel 2016.

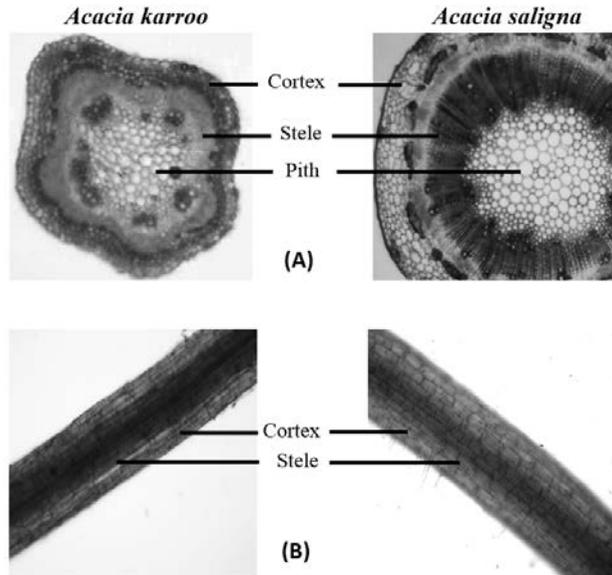
## RESULTS AND DISCUSSION

This anatomical study was performed on the 21<sup>st</sup> day of the application of salt stress by sodium chloride. Our observations suggest that the anatomical characteristics of stems and roots are significantly altered when plants are exposed to salinization of the environment. Indeed, stress due to salt had a very significant effect ( $p < 0,0001$ ) (Table 1) on the internal cell morphology, and the plants subjected to salt stress showed an adjustment of the anatomical characteristics in order to minimize the damage caused by the presence of an excessive amount of NaCl.

### Effect of salinity on stem anatomy

As salt concentration increases, a decrease or increase in the cross-sectional area of the stems was observed in *A. auriculiformis* (Rahman *et al.*, 2017) and *A. ampliceps* (Theerawitaya *et al.*, 2015). Akcin *et al.* (2017) have associated the stem succulence with the adaptation mechanisms to saline conditions in a halophytic species *Salicornia freitagii*. According to Figure 3 and Table 1, the anatomy of the stems has been modified when applying various doses of salt water. For *A. karroo*, the stem area has been gradually reduced compared to the increase in salt concentration up to 400 mM NaCl and then this area tries to recover its initial size at 600 mM NaCl. For *A. saligna*, the SA has also undergone a reduction but stops at 200 mM. Indeed, at 400 and 600 mM NaCl, the SA reached its maximum with an increase of 70% compared to control (Figure 3A).

The stem perimeter of *A. karroo* plants has endured a 35% decrease under the extreme concentrations of NaCl (400 and 600 mM) compared to control and 200 mM NaCl. However, the SP of *A. saligna* plants has undergone a gradual increase that stabilizes at 400 and 600 mM NaCl with a 35% increase over the control (Figure 3B).



**Figure 2.** Anatomical parameters studied of (A) the stem (cross section) and (B) the root (Profile view) at 1.5 cm from the shoot apex and root apex at 21<sup>st</sup> day of the application of different levels of salinity (0, 200, 400 et 600 mM NaCl) on *A. karroo* and *A. saligna* plants.

In addition, it has been found that increasing the thickness of the stems could increase the storage capacity in order to preserve more water and overcome the adverse moisture conditions under the effect of salt stress (Rodriguez *et al.*, 2012). An increase in stem cell thickness under saline conditions has also been reported in *Citrus* (Rewald *et al.*, 2012) and *Tamarix* (Zhang *et al.*, 2016). In general, salinity can cause a reduction in the stem area (Bader *et al.*, 2015). However, in the population of *A. saligna*, the SA showed a considerable increase under salt regime (Figure 3A). This change in the stem dimensions can help to store extra water and can contribute to some resistance to water loss by stem cells to ensure plant survival in an unfavourable environment (Feikema *et al.*, 2010).

The decrease in the SCT is mainly due to the collapse of cortical cells in response to salinity (Al-Tardeh and Iraki, 2013). This can be beneficial for limiting growth under saline conditions by conserving the energy for survival (Naz *et al.*, 2013 ; Quartararo, 2018). According to Figure 3C, the SCT of *A. karroo* was gradually reduced compared to the increase in salt concentration up to 400 mM where this thickness was reduced by 35%. At 600 mM NaCl, the stem cortex regains a thickness equivalent to that recorded in plants subjected to 200 mM NaCl where the difference compared to the control was just 20%. The SCT of the control plants of *A. saligna* was reduced by 30% under 200 mM NaCl. However, at 400 mM NaCl, the cortex regained a thickness equivalent to that of the control and reached its maximum at 600 mM with a value that exceeds the control by 50% (Figure 3C). Decreasing in cortical area of the stems could be

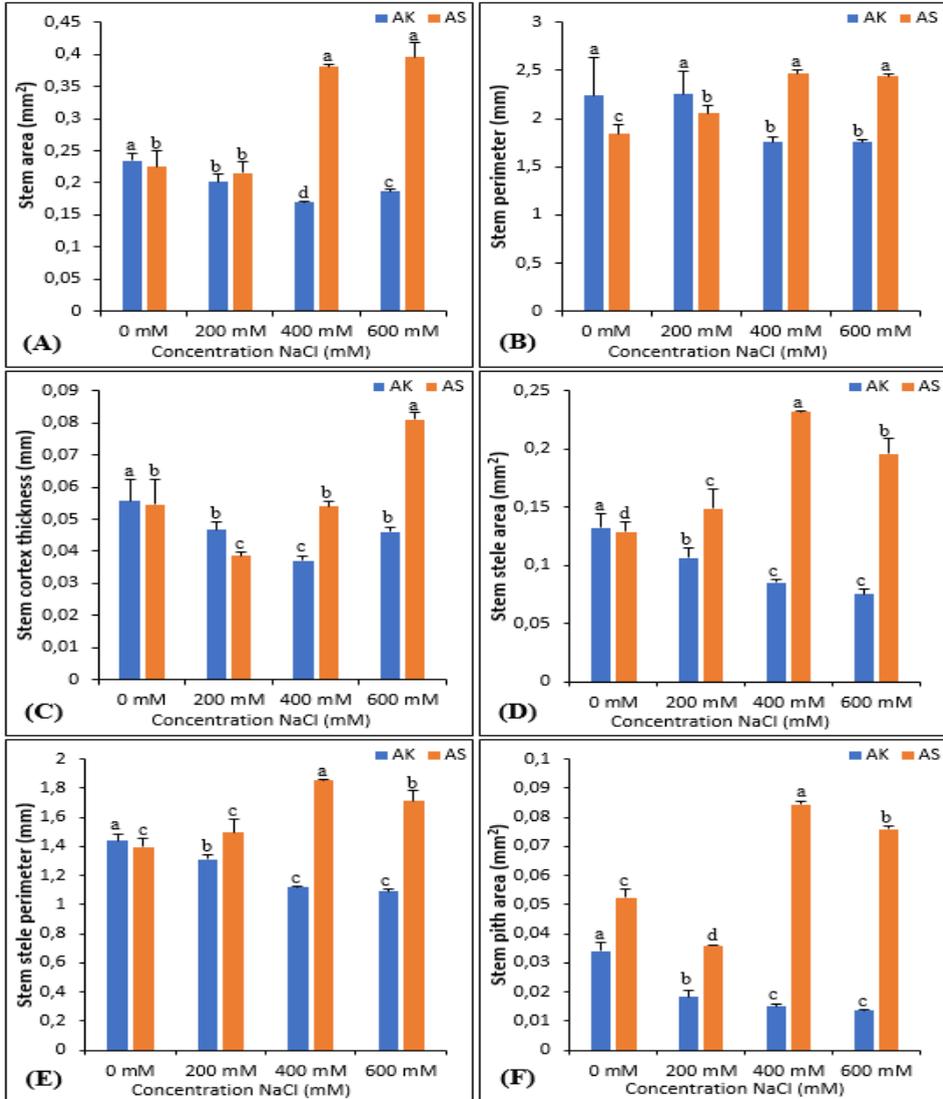
a defensive strategy for plants to limit growth under the salt regime by conserving the essential energy for plant survival in a difficult environment (Chen and Polle, 2010). In contrast, increasing stem cortex thickness in the presence of high salinity may be considered as a defensive strategy to reduce Na<sup>+</sup> toxicity (Mudgal *et al.*, 2010).

**Table 1.** Analysis of the variance of the effects of salinity on anatomical parameters of the stems (SA : Stem area, SP : Stem perimeter, SCT : Stem cortex thickness, SSA : Stem stele area, SSP : Stem stele perimeter, SPA : Stem pith area) and the roots (RT : Root thickness, RCT : Root cortex thickness, RST : Root stele thickness) in two acacia species (*A. karroo* and *A. saligna*) (SP) under saline treatment (TRT).

Organs	Parameter	Sources of variables	Degree of freedom	F of Fisher	P
STEM	SA	TRT	3	55,54	<0,0001
		SP	1	424,06	<0,0001
		TRT×SP	3	137,83	<0,0001
	SP	TRT	3	0,60	0,6202
		SP	1	10,29	0,0038
		TRT×SP	3	22,12	<0,0001
	SCT	TRT	3	45,33	<0,0001
		SP	1	56,84	<0,0001
		TRT×SP	3	46,92	<0,0001
	SSA	TRT	3	17,47	<0,0001
		SP	1	521,00	<0,0001
		TRT×SP	3	106,72	<0,0001
	SSP	TRT	3	5,09	0,0072
		SP	1	455,54	<0,0001
		TRT×SP	3	108,60	<0,0001
	SPA	TRT	3	241,02	<0,0001
		SP	1	4455,50	<0,0001
		TRT×SP	3	487,53	<0,0001
ROOT	RT	TRT	3	17,91	<0,0001
		SP	1	1,25	0,2752
		TRT×SP	3	1,58	0,2204
	RCT	TRT	3	21,99	<0,0001
		SP	1	0,00	0,9471
		TRT×SP	3	11,48	<0,0001
	RST	TRT	3	4,77	0,0095
		SP	1	14,89	0,0008
		TRT×SP	3	21,85	<0,0001

For *A. karroo*, the SSA decreased with increasing stress and stabilizes from 400 mM with a 45% loss in size compared to the control. However, the SST of *A. saligna* increased gradually with increasing salt levels reaching a considerable value compared to the control of a difference of 80% at 400 mM NaCl and 50% at 600 mM NaCl (Figure 3D). Figure 3E showed that the SSP of

*A. karroo* decreased with increasing salt concentration by stabilizing from 400 mM NaCl with a slight loss of 23% compared to the control. On the other hand, the SSP of *A. saligna* increased progressively with the increase in stress level reaching a gain of 32% at 400 mM NaCl and 22% under the extreme concentration of 600 mM NaCl (Figure 3E).



**Figure 3.** Effects of salt stress on the anatomical parameters of the stem: (A) Stem area, (B) Stem perimeter, (C) Stem cortex thickness, (D) Stem stele area, (E) Stem stele perimeter and (F) Stem pith area, in two acacia species (*A. karroo* and *A. saligna*) after 21 days of treatment at different levels. Means, in each box, with similar letters are not significantly different at the 5% probability level using Duncan's test.

Decreased in the stele area under excessive saline conditions was considered an adaptation strategy to reduce water loss in several forest species in arid zones (Rewald *et al.*, 2011a). Reduced stele size may also imply a reduction in saline water absorption by xylem and a reduction in water loss through transpiration (Rewald *et al.*, 2011b) and this is the case of the plants of *A. karroo* in our study. On the other hand, the increase in the stele thickness of *A. saligna* with the increase of the salinity level can also constitute a defensive characteristic in saline conditions as it has already been reported by Srikanth *et al.* (2016).

According to Figure 3F, the SPA of *A. karroo* decreases with the increase in the degree of stress and stabilizes from 400 mM with a loss of 55% compared to the control. Cependant, the SPA of *A. saligna* increased progressively with the progression of salt levels reaching a considerable value compared to the control of a difference of 61% at 400 mM NaCl and 46% at 600 mM NaCl (Figure 3F). It should be noted that in this species, the SPA recorded a 30% loss at 200 mM NaCl compared to the control. These changes in anatomical dimensions can be considered as an adaptive mechanism of *A. saligna* in order to maintain a regular water movement in aerial parts of plants, especially under saline conditions (Polle and Chen, 2015). Unlike *A. saligna*, the plants of *A. karroo* of this study and the case of many plants have undergone a decrease in the stem pith under salt regime: *Leptochloa fusca* (Ola *et al.*, 2012); *Leucaena leucocephala* (El-LAMEY, 2015); *Phaseolus vulgaris* (Bargaz *et al.*, 2016); *Salicornia freitagii* (Akcin *et al.*, 2017). These same authors have pointed out that this reduction due to salinity is indeed another strategy of salinity and water-stress tolerance.

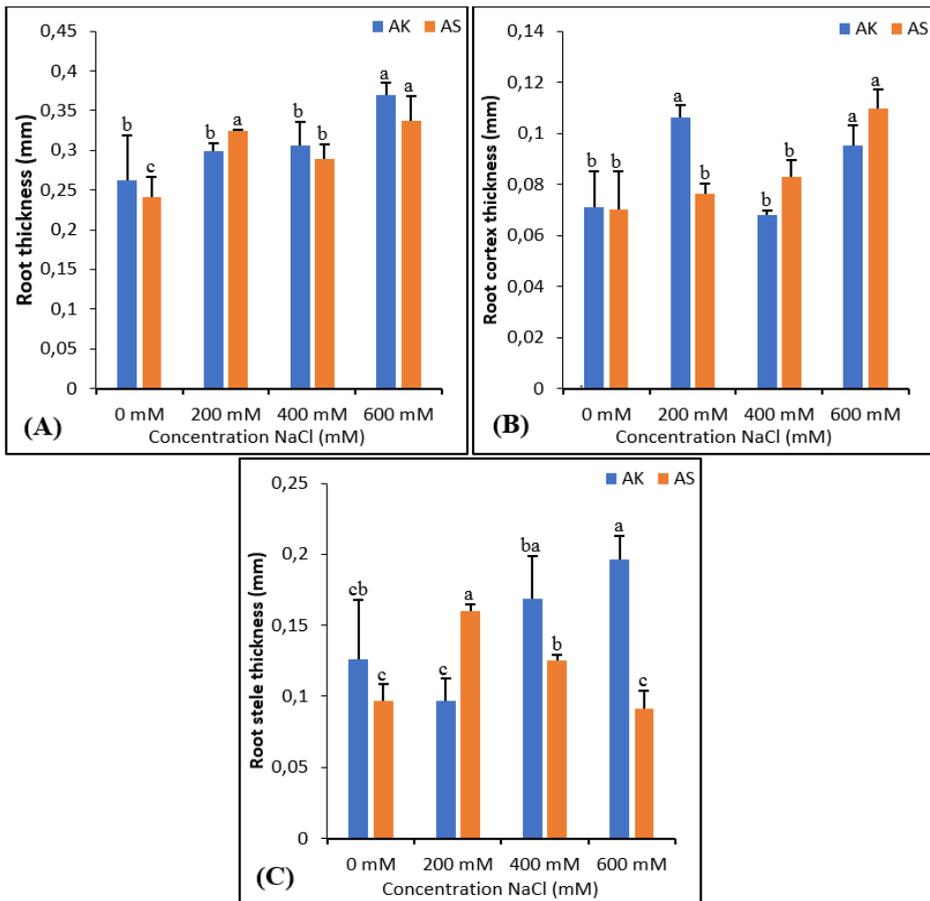
### **Effect of salinity on root anatomy**

The root system is the main organ affected by salinity and is known to rearrange its anatomical characteristics in order to confer adaptation to the species under adverse environmental changes induced by salinity (Aroca *et al.*, 2011; Patakas, 2012). Figure 4A showed that the root thickness is constant at 200 and 400 mM NaCl and increased with the increase in salt stress and this was observed in both acacia species. Indeed, the RT recorded highest values under 600 mM NaCl with a gain of 41% in *A. karroo* and 37% in *A. saligna*. This increase in root thickness under salt stress could be an adaptation of these two species to reduce the transpiration rate and thus to maintain the water content in the internal tissues of the root.

The increase in root and stem thickness not only improves the plant water-use efficiency, but also provides additional compartment for Na<sup>+</sup> sequestration in cells (Parida and Jha, 2010). In contrast to our study, the root thickness was reduced in soybean with different levels of salinity (Dolatabadian *et al.*, 2011), suggesting that there is a differential anatomical-adaptation between halophytes and glycophytes in response to salinity. Based on our results, the main increase in root thickness occurred to prevent the influx of Na<sup>+</sup>.

The same observation was indicated for the cortex thickness of the root where the root preserved its size compared to the control at 200 and 400 mM

NaCl in *A. saligna*, recording a 55% increase over the control under 600 mM NaCl. For *A. karroo* plants, The RCT increased at 200 and 600 mM NaCl recording a gain of 49% and 33%, respectively (Figure 4B). Salinity is known to stimulate suberization and increased the thickness of root cortical cells (Franco *et al.*, 2011; Byrt *et al.*, 2018). These results are in agreement with those of the present study where the root cortex had undergone an increase proportional to the salt levels for the two populations of acacia. This is the characteristic of salt-tolerant species which will certainly avoid the water loss through the roots (Muchate *et al.*, 2016). In fact, halophytes or salt-tolerant species generally have a thick root cortex, which is an effective mechanism against water loss in case of combined stress between salinity and drought (Rewald *et al.*, 2013).



**Figure 4.** Effects of salt stress on the anatomical parameters of the root: (A) Root thickness, (B) Root cortex thickness and (C) Root stele thickness, in two acacia species (*A. karroo* and *A. saligna*) after 21 days of treatment at different levels. Means, in each box, with similar letters are not significantly different at the 5% probability level using Duncan's test.

Figure 4C showed in turn that the increase in salt stress had an effect on the stele thickness of the root by increasing its thickness in *A. karroo* and reaching its maximum under 600 mM NaCl with more than 55% compared to the control. The RST of *A. saligna* behaved differently where it reaches its maximum just at 200 mM NaCl with a 64% increase over the control. However, the RST tend to decrease after this concentration and stabilized at 600 mM recording the same value as the control (Figure 4C). When size and thickness of the root stele increased, the water storage capacity increased to overcome the conditions of water stress conditioned by salt stress (Karimi *et al.*, 2012). It has also been reported that the roots water-potential is much more dependent on the size of the internal structure such as stele and vessels (Munns and Gilliam, 2015). The structure and the stele area are an adaptive mechanism for storing or transmitting water under salt regime (Deinlein *et al.*, 2014). In previous studies, it has been reported that the expansion of the stele plays an important role in the plant adaptation to salinity (Abbas *et al.*, 2013). When transporting water and mineral elements such as Na<sup>+</sup> ions from the rhizoderm to the stele; the lignification of the exoderm, endoderm, metaxylem and protoxylem, prevents the excessive ion movements (Han *et al.*, 2015). Indeed, the endoderm layer is considered to be the most important barrier against the passive flow of ions in root tissues (Singh and Stasolla, 2016).

Overall, Na<sup>+</sup> exclusion, tissue tolerance, osmoprotection and mineral homeostasis play a key role in salt acclimation and tolerance in these two acacia species. *A. karroo* is considered tolerant also on the basis of total dry biomass of stressed plants (Kheloufi *et al.*, 2018b). Under saline conditions: the synthesis of the results of several authors such as Läubli and Epstein (1990) ; Blumwald (2000) ; Yamaguchi and Blumwald (2005) ; Munns and Tester (2008) ; Hauser and Horie (2010) ; Wu *et al.* (2015) ; Mansouri and Kheloufi (2017) and Hamed *et al.* (2018 ) summarize the plant acclimation by three main mechanisms: Ion-exclusion - Net exclusion of toxic ions - Compartmentalization of toxic ions in specific subcellular tissues, cells and organelles - Maintaining growth and water absorption - Anatomical changes in root, stem and leaves.

## CONCLUSION

In conclusion, under saline regime, both acacia species adapted specific characteristics of the roots and stems for better survival under saline environments. The parameters estimated during this study are valid only for the case of young plants in pots and under defined conditions of salinity. It would be necessary to validate our results *in situ* where these species grow naturally on saline soils.

However, this study does not exclude that these acacia species are considered as potential halophytic species to be cultivated in saline lands, thus making them favourable to agroforestry practices, especially since these forests leguminous have the capacity to revegetate nutrient-poor soils.

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