

In vitro Evaluation of *Eucalyptus Urophylla* Behavior Under Salt Stress

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Abstract: *Eucalyptus urophylla* is a tree used in intensive farming and presents an ecological, economic and industrial interests. In this research, morphological, physiological and mineral studies were conducted at the seedling stage under salt stress condition. The results show that, at the seedling stage, salinity using a concentration above 4g/l of NaCl have a significant effect on the plants as it damaged their development, caused a fresh matter, an increasing concentration of NaCl. This decrease is rather greater beyond 4g/l of sodium chloride. For better identification of differences in sensitivity and specific behavior, the salt sensitivity index was determined and it shows a significant decrease in the studied organs related to the concentration of NaCl. A mineral study was conducted for Na⁺, Cl⁻, K⁺, Ca²⁺ contents for both shoots and roots. Results showed high accumulation levels of Na⁺ et Cl⁻ levels starting from the low dose of 34.2mM of NaCl with more significant ion accumulation capacity of the leaves. K⁺ and Ca²⁺ ions levels was reduced starting from 34.2mM NaCl with roots more effected by the elevated salt concentrations. These results also indicated that leaves can withstand up to 144.3mM of NaCl as for roots they can tolerate up to 171.1mM of NaCl. This salt toleration is done through ions compartmentation which is why ions were allocated in the different parts of the plant. This confirms the inclusiveness of *Eucalyptus urophylla* under a salt stress.

Keywords: *In vitro*, Salt stress, Morphological parameter, Physiological, Mineral parameters.

1. INTRODUCTION

The plants are very sensitive to abiotic stresses such as drought, low temperature and especially salinity, which is the most dominant constraint. It is an ever-increasing problem in arid and semi-arid regions (Shanon, 1986). In fact, salinity is one of the limiting factors of agricultural productivity (Petronia C. *et al*, 2011) Worldwide, more than 45 million hectares of irrigated land have been damaged by salt, and 1.5 million hectares are taken out of production each year as a result of high salinity levels in the soil (R Munns & Tester, 2008) Also, about 70% of the earth's surface is covered by saline water and oceans have concentrations of Na⁺ around 500mM, contrasting with the low K⁺ concentrations of 9mM (Flowers, 2004), the remaining 30% of the earth's surface is severely affected by an increasing salinization phenomenon.

Globally salinity remains one of the most serious environmental problems. Zhang and Shi 2014 mismanagement of the irrigation methods combined with inadequate drainage system contributed to soil salinization. Therefore, a double problem arises: first salt absorption threatens the tissue and the good physiological functioning of cells, secondly, the

presence of salt lowers the soil water potential and threatens the water supply.

Salinity is also an impediment for the sustainability of species with ecological and socio-economic interests as well as the natural balance (Errabii, 2007). Same way facing salt stress, some species seem to have their growth reduced as they are unable to withstand salt excess in their cells. Depending on the plants growth ability in saline environments, they are classified as either glycophytes or halophytes, and their response to salt stress differs in terms of toxic ion uptake, ion compartmentation and/or exclusion, osmotic regulation, CO₂ assimilation, photosynthetic electron transport, chlorophyll content and fluorescence, reactive oxygen species (ROS) generation, and antioxidant defenses (Tang *et al*, 2015). Most salinity adaptive mechanisms in plants are accompanied by certain morphological and anatomical changes. (Larcher, 2003) Glycophytes, which includes most crop plants, cannot grow in the presence of high salt levels; their growth is inhibited or even completely prevented by NaCl concentrations of 100–200 mM, resulting in plant death. Such growth inhibition can even occur in the short term (Hernández and Almansa, 2002). In contrast, halophytes can survive in the presence of high NaCl concentrations (300–500 mM) because they have developed better salt resistance mechanisms (Flowers and Colmer, 2015).

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Among tolerant species *Eucalyptus* has significant adaptation capacities that enable them to ensure high industrial wood production and energy even in highly stressed environments. Among them, *E.urophylla* salt stress response was also evaluated. In this study we will try to reassess *E.urophylla* behavior under salt stress through morphological and physiological parameters.

2. MATERIAL AND METHODS

We selected approximately 150 seeds of *Eucalyptus urophylla* from Brazil and stored at a temperature of 4°C for 8 months. In order to conserve and protect them from fungi infection that reduces embryo viability.

These seeds were disinfected using consecutive wash of soapy water with tween 20 for 20 minutes then in antifungal solution of Benlate (2g/l) for 30 minutes, passed quickly through alcohol 70%, bleach at 30% with tween 20 for 35 minutes. Of course, after each wash seeds were rinsed with distilled water to finally left to drain under a sterile hood. Once dried seeds were cultured on Murachige and Skoog (1962) medium with macro-elements, micro-elements, vitamins, iron, sugar, and agar with a pH regulated between 5.6-5.8 with NaOH 0.1N then autoclaved and poured into flasks and petri dishes.

The petri dishes with the medium and seeds are put in a chamber under controlled conditions:

- Photoperiod: 16h light (1500µmol/h/phot) and 8h dark
- Relative humidity: 90% day and night
- Temperature: 25°C day and night

The plantlets obtained, after 45 days, are transplanted into 5 flasks (5 explants in each) containing MS medium with different NaCl concentrations added (2, 4, 6, 8, 10, 12 g/l). Every 2 months transplanting is performed until the third subculture.

Once the third subculture is reached the plant material is collected and subjected to a number of measurements

2.1. Determination of the Morphological Parameters of the Plant

After 6 months of transplanting and exposure to salt stress, the plants shoot elongation, leaf expansion,

aspect and number were measured and closely observed for any signs of toxicity due to the increasing amount of salt.

The plants treated with different doses of NaCl had their growth attributes checked; their shoot and roots elongation, leaf number and fresh and dry weight, also their RGR was determined using Hunt, 1990 formula:

$$RGR = \frac{(\ln DW_f - \ln DW_i)}{(t_i - t_f)}$$

In addition, water content was determined as well as sensitivity index using Slama, 1982 formula:

$$SI = \frac{\Delta DW_{NaCl} - \Delta DW_{control\ sample}}{\Delta DW_{control\ sample}} \times 100$$

2.2. Determination of the Physiological Parameters of the Plant

In order to analyze the transport and storage behavior of ions we determined the concentrations of mineral ions (sodium, chlorine, Potassium, calcium and magnesium) in the various organs (root, stem and leaves) of the plants cultivated in mediums with different NaCl concentrations.

The determinations of Na⁺, Mg²⁺, Ca²⁺ and K⁺ elements are measured directly by means of atomic absorption (Perkin Elmer Atomic absorption spectrometer 3110). Chlorine (Cl⁻) is assayed using a chloridometer (Haake Büchler type)

The ionic selectivity was tested through the Na⁺/K⁺ ratio using Abdelly, 1997 formula:

$$SK+/Na+ = S1 / S2 \text{ with } S1 = \Delta QK / (\Delta QK + \Delta QNa) \text{ and } S2 = [K+] / ([K+] + [Na+])$$

2.3. Statistical Analysis

The program SAS (statistical analysis system, 1988) was used to determine Variance, averaging and correlations.

3. RESULTS

3.1. Morphological Parameters

3.1.1. Morphological Aspect

After 8 to 9 weeks of treating *Eucalyptus urophylla*'s vitro plants with different doses of NaCl from 34.2mM to 205.3mM, we noticed an impact on plants development for doses higher than 68.4mM. the shoot

elongation, leaf expansion as well as their aspect and number were affected by the higher doses. In fact, *in vitro*-plants showed symptoms of foliar toxicity with yellow-colored spots, and necrosis on the leaves and at the end of the shoot due to excess salt accumulation. (Figure 1).

3.1.2. Growth Attributes

After measuring the effect of the elevated doses on the plants growth attributes as well as their relative growth rate and comparing them to a control sample, one non-exposed to salt stress, we could conclude that a dose of 34.2mM of NaCl have insignificant effect on the shoot and roots elongation, leaf number, their fresh or dry weight and therefore on their relative growth rate (RGR) (Figure 2). However, using doses above 34.2mM (starting from 68.4mM of NaCl) significantly reduced shoot and roots elongation and the effect was more severe at higher doses of 171.1mM and 205.3mM. the dry and fresh weight of both shoots and roots were also decreased abruptly at higher salt levels. (Figure 2) As for the RGR we recorded an unbalance distribution of the biomass wich lead to a sharp decline of this attribute for the roots after applying the smallest dose of 34.2mM of NaCl whereas for the aerial parts this dose had hardly any effect on the RGR, the 68.4-171.1mM NaCl had little effect and it

remained stable it is only when using 205.3mM of NaCl that we noticed a rather significant effect on the RGR that reached 0.12g comparing to the control sample of 0.36g. These results revealed a more significant effect of salinity on roots rather than shoots.

3.1.3. Water Content Evaluation

The gathered results related to water content of aerial and root parts, show that *E.urophylla* leaves, under the influence of different concentrations of NaCl, have reacted to this salt stress by a decrease in their water content percentage (Figure 2). This reduction is about 42.5% compared to the control sample for the treatment of 8g/l of NaCl for the aerial part and it is about 53% for the roots part. Therefore we can conclude that the salinity had a more significant effect over roots than leaves and an obvious reduction when using a concentration higher than 8 g/l of NaCl any less dose hold almost no effect on *E.urophylla*'s water content.

3.1.4. Sensitivity Index

The sensitivity index showed a significant decrease for all organs. For the shoots the numbers extend from -35.88 to -78.00 for the treatment of 68.4mM to 205.3mM of NaCl. As for the roots results exhibited sensivity index of -37.53 for 68.4mM NaCl to -79.63 for

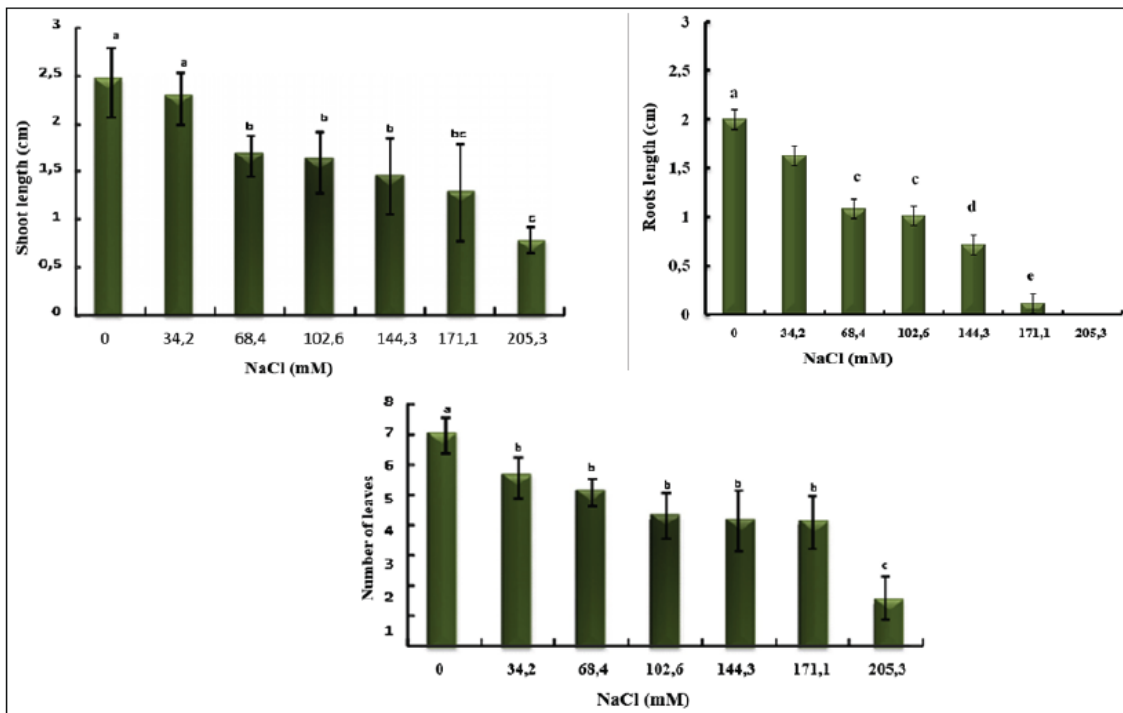


Figure 1: Effect of the elevated NaCl doses on the morphological development of *E. urophylla* plants (The numbers followed by the same letter are not significant to the 5% threshold according to Student-Newman-Kleuls test).

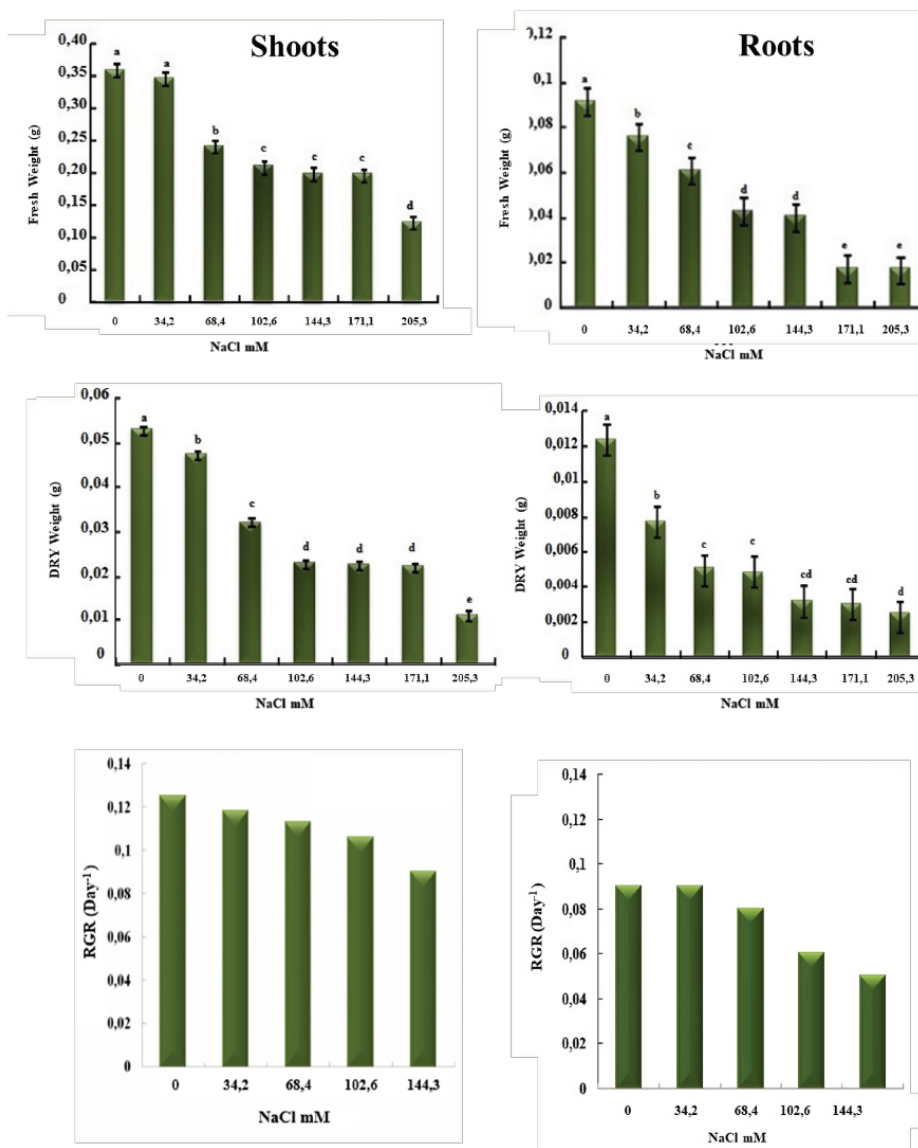


Figure 2: In vitro-plants growth attributes under different levels of salt (The numbers followed by the same letter are not significant to the 5% threshold according to Student-Newman-Kleuls test).

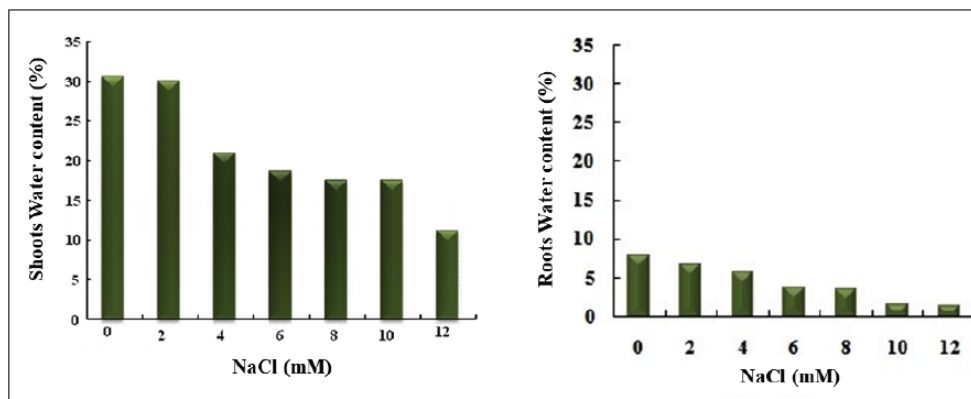


Figure 3: Salt effect on the evolution of the percentage of relative water content of shoots and roots.

205.3mM NaCl. (Table 1). This proves that the roots are more sensitive to the salinity with a significant

effect for doses equal and higher than 68.4mM of NaCl.

Table 1: Variation of Salt Sensitivity Index of Shoots and Roots

Concentration of NaCl (mM)	0	34.2	68.4	102.6	144.3	171.1	205.3
SI Shoots	0	-34	-35.88	-54.59	-55	-56	-78
SI Roots	0	-37.53	-58.45	-60.54	-74.33	-75.68	-79.63

3.2. Physiological Parameters

3.2.1. Ion Accumulation

The mineral analysis showed a markable increase of Na⁺ and Cl⁻ levels in both shoot and roots for the different doses of 34.2mM to 205.3mM of NaCl. This

led to an increase in Na⁺ and Cl⁻ ion accumulation (Figure 4). In addition, the analysis revealed that the shoots accumulate more ions than the roots.

As for K⁺ and Ca²⁺ results proved a decrease in their accumulation levels with the increased salt doses

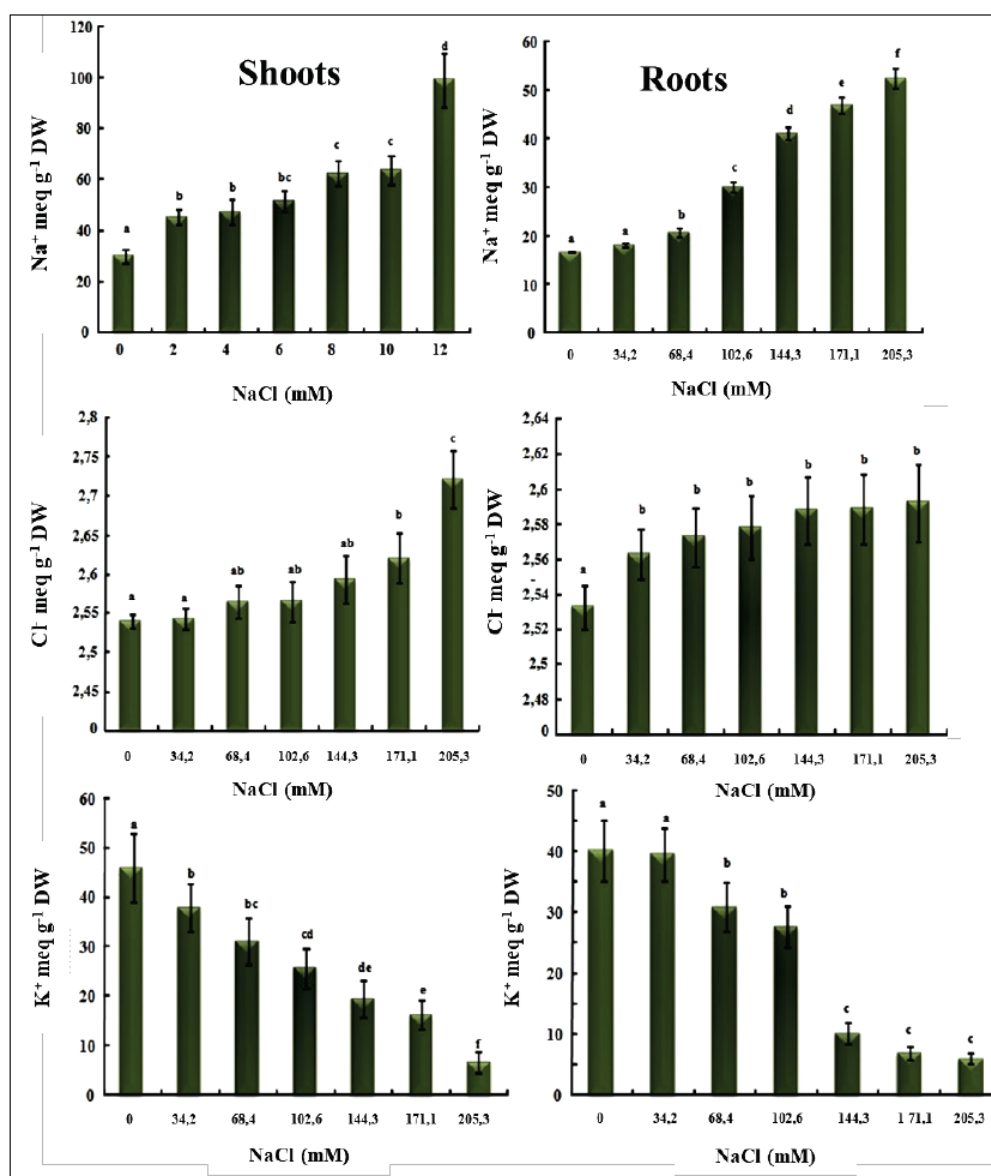


Figure 4: Variation of Na⁺, Cl⁻, K⁺, Ca²⁺ contents in the different parts of the plant depending on NaCl concentration in the medium (Average of 3 repetitions and security interval threshold of 5%. The values followed by different letters are significantly different.)

however the significant decrease was notable for high NaCl doses.

Through this test we can conclude that, shoot and leaves could upstand to 144.3mM of NaCl as for roots they could tolerate up to 171.1mM (Figure 4).

Na^+/K^+ ratio

this ratio showed a significant decrease with the elevated doses of NaCl with a clear effect starting from the smaller dose of 34.2 mM of NaCl specially for the shoots. it was clear that the decrease is important and significant with the increased doses of NaCl the effect is rather clear starting from 2g/l of NaCl and it is more obvious with higher doses. However, the effect is more pronounced in the shoots more than the roots as it decreased almost by half for the shoots for the lowest dose of 2g/l (Figure 5)

4. DISCUSSION

In the light of the present study, *E.urophylla* plantlets were placed under different and increased doses of NaCl. After a period of time morphological and physiological parameters were assessed.

As for the morphological parameters results have shown signs of foliar toxicity due to the rising levels of salt, Maas (1993), explained these symptoms by either an increase of the osmotic potential in the medium by salinity, or they can be the result of a salinity-induced deficiency or even due to the accumulation of toxic ions, or it can be the result of a salinity-induced deficiency or even due to the accumulation of toxic ions. Walker and al, (1981) suggest that the decrease of growth could be linked to a loss of turgidity. The similar researches conducted by Ruiz *et al* (2001) on

Citrus aurantium as well as that of Meloni *et al* (2001) on Tomato justified this decrease could be caused by the osmotic effect of salinity accompanied by a significant reduction in the mass of the shoot, the number of leaves per plant, the root length and leaf area. This biomass reduction was also noted by Hajji (2004) with *Lippia modiflora*, with jojoba by Roussos *et al* (2006) and *Cynodondactylon* by Mansoor and Ashraf (2008). Such a decrease is a response to the salt constraint and a common characteristic within glycophytes (Munns, 1993 and Hajji, 2004). The results also proved that the roots are more sensitive to this salinity constraint than the aerial parts.

To investigate more about *E.urophylla* behavior and its accumulation capacity, a mineral analysis was conducted. The results affirmed that salinity induced an important accumulation of Na^+ and Cl^- within the aerial parts. The consequences of these accumulations were manifested through chlorosis appearing on the leaves after the start of the stress followed by falling leaves. These results are in agreement with those found by Banuls and Primo (1992), which reported that the plant defoliation is tightly correlated with the levels of Na^+ and Cl^- in the leaves. When their levels become way too elevated, it could lead to the abscission of leaves.

This proves that *Eucalyptus urophylla* when placed under salt stress, the sodium and chlorines are accumulated and compartmented in vacuoles contributing this way to the osmotic adjustment to a non-toxic level of salinity.

However, we noticed an important decrease of K^+ level and a less significant reduce in Ca^{2+} levels. of levels and a less significant for is observed. The high accumulation of ions had a direct impact on other ions

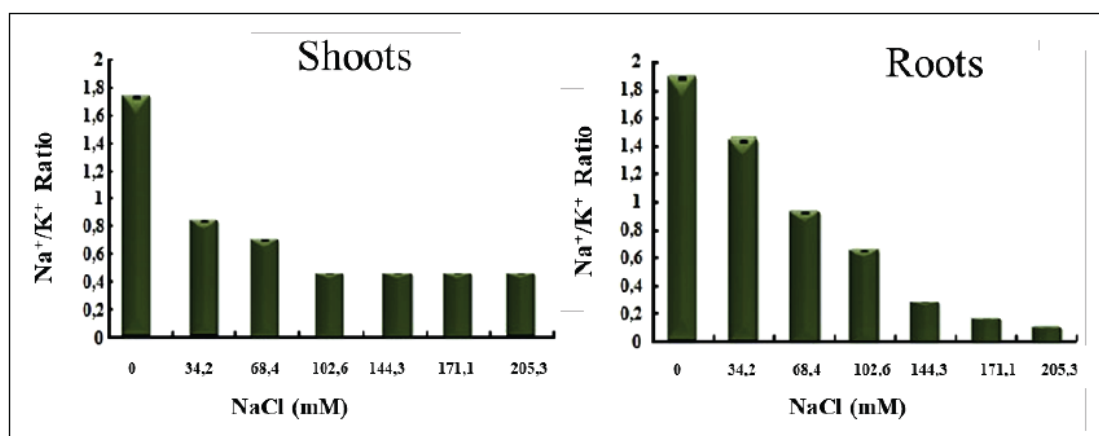


Figure 5: Variation of the K^+/Na^+ accumulation ratio within different plant organs of *Eucalyptus urophylla* (leaves and roots) depending on the NaCl concentration in the medium.

concentration, like it was reported for potassium for other species (Garcia-Sanchez *et al*, 2002 and Roussos *et al*, 2006). In the leaves there is a compartmentation of sodium more than potassium thus potassium content decrease as the salinity increases. Furthermore, potassium content in the roots is less than that recorded in the leaves. These ionic characteristics lead to saying that there is some sort of competition between the absorbance and transport of potassium and sodium ions and that the plant is acting like an includer with leaves loaded with sodium more than the roots.

5. CONCLUSION

Applying salt stress on young *Eucalyptus urophylla* plants showed that their growth is affected by this constraint. Also, salinity led to a depressive effect on the length of shoots and roots as well as the number of leaves. This effect is aggravated when using salt concentration above 34.2mM. Dry and fresh matter were also affected specially the roots for concentration above 68.4mM NaCl. Sensitivity index showed an important decrease with the elevated salt concentrations until it gets above (-50). For *E.urophylla*'s roots they are sensitive starting from the lowest NaCl concentration, as for the shoots they are touched starting from 68.4mM NaCl.

The mineral analysis showed that, *E.urophylla* leaves started absorbing Na⁺ ions from 34.2mM NaCl as for roots, sodium content started rising from 102.6mM NaCl. This build up in salinity lead on one hand to a elevated chlorine content but in the other hand a reduction in potassium and calcium levels compared to the control samples. These results incite that *E.urophylla* behaved as a sensitive glycophyte and confirmed its "includer" character with leaves more loaded in sodium than the roots.

For all plant species, glycophytes as well as halophytes, the degree of inhibition depends on the genre, specie, variety as well as the stage of development and the organ nature. For that the salt resistance is a phylogenetic character that can be controlled by different levels of organization, from a single cell to the entire plant. Yet, the diversity of salt effects on the plants offers an important range of physiological and biochemical criteria that could be the base of fast tests used for grand mass selection.

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