

Morphological and physiological studies in three alfalfa varieties (Medicago sativa L.) under salt stress

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Abstract: Responses to salt stress were investigated in three alfalfa varieties (Gabès, Hunterfield, and Hyb.555). Seedlings were submitted to salinity induced by adding NaCl into water at 4 concentrations (0 - 2.5 - 5 and 10g.l⁻¹). Growth parameters (DM, plant height, LA, LAR, SLA, leaves thickness, succulence), and hydraulic parameters (RWC, WC, WUE) were investigated in leaves, stems and roots after three months of salt stress. Results showed that NaCl decreased DMY of the three varieties. However, Gabès variety, compared to Hunterfield and Hyb.555 has a DMY significantly higher, in the absence and presence of NaCl. The severe decrease in the DMY of the introduced varieties was related to the premature senescence accelerated by the salt intensity and the duration. On the other hand, Gabès variety has a well-equilibrated distribution of DM between organs. The LAR and the SLA decreased significantly with the salt increasing. The salt increased the leaves thickness and succulence. WC increased in concomitance with the rise of leaf succulence. WUE under salt stress decreased in the three lucernes. However, Gabès variety has WUE significantly higher in all salt treatments. This study allowed highlighting the interest of the Gabès variety in any selection program for salt tolerance.

Key words: Alfalfa, Growth parameters, hydraulic parameters, Salinity.

I. Introduction

Among abiotic stress, the salinity is one of the most constraints environmental which provokes grave threats for the agriculture and the environment (Lugan, 2008; Shahid et al., 2011). Salted soils and waters constitute an unfavorable environment for the increase of the agronomic yields (Hammad and al., on 2010). Indeed, the salinisation of the soil is a major obstacle to the productivity of the main cultivated species. Tunisia possessed vast salty zones (Chott and Sebkha) and an important water resource with a salinity varying between 1.5 to 3g.l⁻¹ (Al Atiri, 2004; Ben Nouna et al., 2004). According to Hachicha (2007), soils affected by salts in Tunisia covered approximately 1.5 million hectares, which represented about 10% of the country surface.

The salty soils are generally situated in the dry and the Saharan area, and then they are subjected to an intense evaporation which favored the rise of salts on-surface (Lapeyronie, 1982; Marlet et al., 2008) and provoked the mortality of plants, resulting to the decrease of the soil osmotic potential (Da Silva et al., 2008). The strong concentrations of salts in the soil decreased the water extraction by roots (Munns and Tester, 2008). Confronted to salinity, plants developed several adaptation strategies such as: compartmentation (Li et al., 2008; Smethurst et al., 2009; Sun et al., 2009; Hanana et al., 2011) and/or Na⁺ and Cl⁻ dilution by dry matter production (Hamrouni et al., 2011; Ibn Maaouia-Houimli et al., 2011), leaves succulence (Lacerda et al., 2006; Hameed et al., 2010; Fercha et al., 2011), leaves thickness (Suárez, 2011; Boughalleb et al., 2012), ions exclusion to the stem parenchyma (Munns and James, 2003; Suárez and Medina, 2008; Mezni et al., 2010). In Tunisia, the livestock sector constituted in value 36% of the total agricultural production, with more than 600 thousand jobs and 1 million of direct and indirect workdays a year. The area reserved to the rainfed forage crops produced, according to the level of rain, between 460 and 750 million of fodder units (30% of the average cover rate), compared for needs esteemed to 1400 million UF (Dahi, on 2008). This chronic deficit needed a big effort to improve the productivity of the existing forage crops. This was the Tunisia challenge to maintain the agricultural productivity in these zones affected by the salinity by the use of tolerant alfalfa genotypes (Singh et al., 2010; Javid et al., on 2011).

The objective of the study was to investigate the effect of salt stress on the growth and the water balance in three alfalfa varieties, to integrate them into selection programs, in order to create tolerant genotypes for the salinity, to value and develop the Tunisian salty soils and the brackish waters.

II. Materials And Methods

Plant material and growth conditions

Plants were cultivated in greenhouse (natural light; maximal temperatures of 30 to 42°C and minimal of 15 to 28°C; maximal relative humidity of 80 to 95% and minimal of 25 to 45%). The seedling was carried out in plastic pots (volume = 10litres) containing an equilibrated soil with a basic pH (8.2), rich in active chalk (13%).

It has a humidity fluctuating between: 12.1% at wilting point and 22.6% at field capacity. Pots were daily irrigated and continuously drained and drainage water was collected in small bottles. The quantities of water necessary for each irrigation treatment were determined using mini-lysimeters. The soil was maintained at the field capacity throughout the experiment.

Each pot contained ten plants of the same variety. Before achieving this density, an initial sowing of twenty seeds per pot was carried out, followed by a thinning at the four leaves trifoliolate stage to leave only the ten best plants, while respecting an uniform spatial distribution between individuals. Seedlings were first irrigated with tap water during establishment (20 days). Two water samples were taken monthly to monitor their chemical composition. The average concentration was: 0.2meq.l⁻¹ for K⁺, 12 to 17meq.l⁻¹ for Ca²⁺, 2 to 5meq.l⁻¹ for Na⁺, and 2 to 6meq.l⁻¹ for Cl⁻.

At the four leaves trifoliolate stage, salinity stress was induced by adding NaCl into water irrigation at 4 concentrations (0 - 2.5 - 5 and 10g.l⁻¹). The experimental design included 12 treatments (3 varieties x 4 levels of salinity), with randomized 4 replications, resulting in a total of 48pots and 480 plants.

To avoid osmotic shocks, the salt was supplied with irrigation water using increasing gradual fractions until the desired level of each treatment is reached. It took 12 days to fully establish the saline treatments. Thereafter, weekly analyze were done either on soil or on excess drainage water to insure that NaCl concentration in medium was constant. This procedure allowed us to monitor NaCl quantities taken up by alfalfa plants or adsorbed by the exchangeable complex in the soil.

Growth parameters

The growth parameters were carried out on plants harvested at the late bloom-early pod stage, corresponding to a salt stress period of three months. The leaf area (LA) was calculated by the method of weighting, on leaves completely developed. Leaves are quickly photocopied and their imprints carefully cut and weighed. The LA was then deducted, with the weight of a known surface area. The LA per plant was obtained by multiplying the LA average by the corresponding leaves number (Mezni et al., 1999).

The leaf area ratio (LAR) was calculated as the ratio of total leaf area on the whole plant dry matter (DM) and it was expressed in cm² of leaves by mg of DM (cm².mg⁻¹ DM). The specific leaf area (SLA) designed the ratio of leaf area to leaf dry mass and expressed in cm².mg⁻¹ leaves DM (Garnier et al., 2001). Leaves thickness was calculated as water content of leaves (Volume) by specific leaf area.

Water parameters

Water parameters were measured in the morning from 10 hours, on completely developed leaves. *Relative water content* (RWC) was measured in eight fully expanded leaves without petiole per treatment. Leaves were immediately weighed after the harvest, to determine their fresh weight (FW). Leaves were immersed on distilled water in Petri dishes for 4 hours (time required to regain turgidity after many essays). Then, leaves are carefully wiped with the filter paper to remove the water droplets on their surface and weighed to determine turgid weight (TW). The samples were dried at 80°C for 24 h to determine the dry weight (DW). RWC was defined as follows:

$$RWC (\%) = \frac{FW - DW}{TW - DW} \times 100$$

Leaf water content (WC) was determined by weighing leaves immediately on harvest (FW) and after complete dried at 80°C for 24 h (DW) using an analytical balance. WC was given by the following formula:

$$WC (gH_2O.g^{-1}DW) = \frac{FW - DW}{DW}$$

The leaves succulence by treatment was calculated on a sample of eight grown-up leaves by the following relation:

$$Succulence (mgH_2O.cm^2) = \frac{FW - DW}{LA}$$

The water use efficiency (WUE) was determined by successive weighing of the culture pots, constantly maintained in the field capacity. To estimate the losses of water by evaporation, one pot per treatment without culture was installed. For each treatment, the quantity of water consumed was then determined by the difference between the weight of the pot containing plants and without plant.

Statistical analysis

Confidence intervals were calculated to the threshold of 95% probability. General Linear Models of SAS was used to explain the degree of significance of each factor and of the interactions between different factors. The Duncan test was used to compare treatment means for all studied parameters.

III. Results

Effect of NaCl on the dry matter (DM)

The figure 1 represented total DM of whole plant (W.P.), shoots (S) and roots (R). The salt stress affected negatively the alfalfa DM. At the most stressful treatment and compared to the control, the *Gabès* DM decrease was lower and the reduction did not exceeded 37%, whereas the decline was 45% and 59% for *Hyb.555* and *Hunterfield* respectively, at the same treatment. Indeed, the shoots of the introduced varieties was more marked by the salt intensity than the roots biomass, the reduction was 68% and 59% respectively.

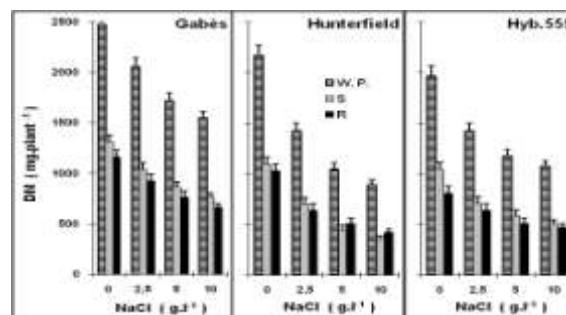


Figure 1. DM of whole plant (W.P.), Shots (S) and Roots (R) of three alfalfa varieties, in presence of increasing NaCl (0 - 2.5 - 5 - 10g.l⁻¹), after three months of salt treatments. Confidence intervals were calculated at $\alpha = 95\%$ probability level.

Distribution of the DM between the various organs

The distribution of the DM between roots (R), stems (S), senescent leaves (Sen. L.) and photosynthetically active leaves (L) is represented by the figure 2. The distribution of the DM showed that roots DM, for the three varieties, never coming down below 42% of the total biomass of the whole plant. The roots DM of *Hunterfield* variety, under the stressful treatment (10g.l⁻¹), represented 55% of the total biomass. While for *Gabès* and *Hyb.555* varieties, the DM fraction of stems and leaves photosynthetically active was appreciably stable and was little modified by the increase of the salt in the culture medium. For these two varieties, the mass of every organ constituted about 25% of the whole plant DM. On the contrary, DM of stems and especially leaves photosynthetically active of *Hunterfield* variety was significantly reduced by the increased doses of NaCl in the culture pots. Indeed, the *Hunterfield* foliar mass, compared to whole plant DM passed for 25% under the control treatment (without salt) to 14% in the most stressful treatment (10g.l⁻¹).

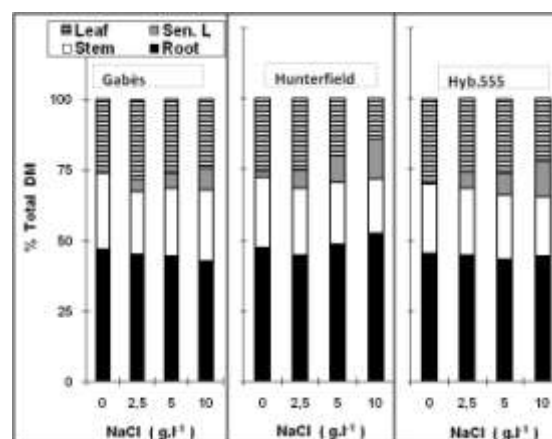


Figure 2. DM distribution between Leaves (L), Senescent Leaves (Sen. L), Stems (S) and Roots (R) of three alfalfa varieties, in presence of NaCl (0 - 2.5 - 5 - 10g.l⁻¹), after three months of salt treatments. Confidence intervals were calculated at $\alpha = 95\%$ probability level.

Plant height

The result recorded on the figure 3 represented the evolution of the plant height, under increasing salt intensities. The height growth for the three varieties decreased with the increase of NaCl in the medium. So, the *Hyb.555* height was more affected by the salt, compared to the other varieties, with a height level significantly lower at the stressful treatment.

The analysis of variance of the plant height showed a highly significant effect ($p < 0.0001$) for variety, salinity and their interaction. The Duncan test ranked the varieties in the following order for the height growth: *Gabès* \geq *Hunterfield* $>$ *Hyb.555*.

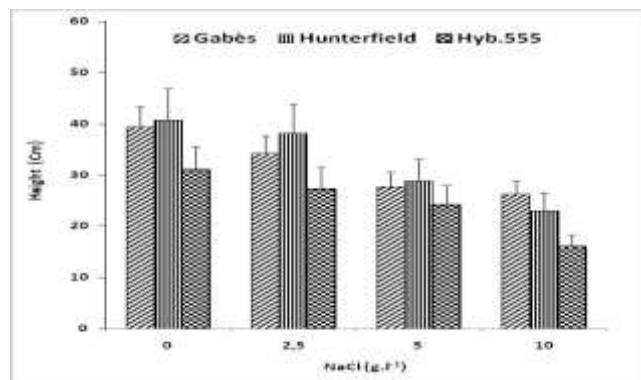


Figure 3. Height growth of the three alfalfa varieties, in presence of increasing NaCl (0 - 2.5 - 5 - 10g.l⁻¹), after three months of salt treatments. Confidence intervals were calculated at $\alpha = 95\%$ probability level.

Leaf area (LA)

The results showed that the salt stress decreased the LA growth (figure 4). However, the LA of *Gabès* variety was significantly more important at 2.5 and 5g.l⁻¹ treatments, compared to the two introduced varieties. For the most stressful treatment (10g.l⁻¹), the three alfalfa varieties have appreciably the same LA with a reduction of the number of leaves resulting of their premature senescent.

The analysis of variance of the LA showed a highly significant effect ($p < 0.0001$) for variety, salinity and their interaction. The Duncan test ranked the varieties in the following order for the LA: *Gabès* $>$ *Hyb.555* $>$ *Hunterfield*.

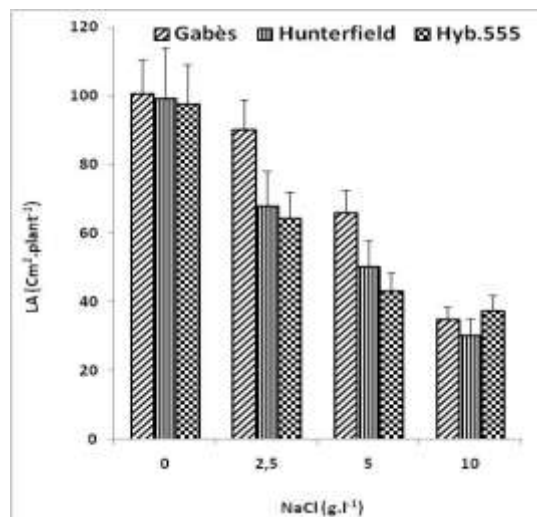


Figure 4. LA of the three alfalfa varieties, in presence of increasing NaCl (0- 2.5 - 5 - 10g.l⁻¹), after three months of salt treatments. Confidence intervals were calculated at $\alpha = 95\%$ probability level.

Leaf area ratio (LAR)

The figure 5 described the LAR evolution of the three alfalfa varieties in absence and presence of increasing concentrations of NaCl. The results showed that the dose of 2.5g.l⁻¹ increased the LAR in the three alfalfa varieties. Beyond this concentration, the LAR decreased for the three varieties with however a severe reduction for *Hunterfield* in the most stressful treatment (10g.l⁻¹).

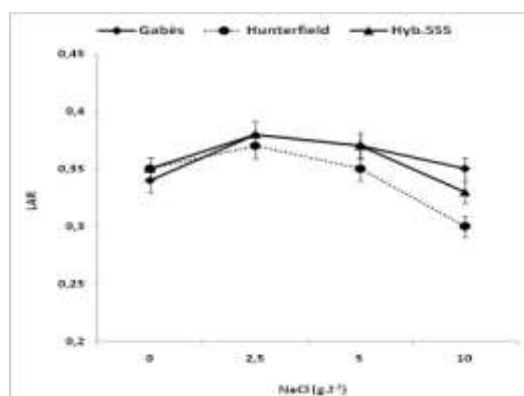


Figure 5 - The LAR of the three alfalfa varieties ($\text{cm}^2 \cdot \text{mg}^{-1}$ DM of whole plant), in presence of increasing NaCl (0 - 2.5 - 5 - 10g.l⁻¹), after three months of salt treatments. Confidence intervals were calculated at $\alpha = 95\%$ probability level.

Specific leaf area (SLA)

The SLA decreased with the NaCl increasing in the culture medium, for the three alfalfa varieties (figure 6). In the control treatment, the three varieties had the same SLA. The SLA of the introduced *Hunterfield* and *Hyb.555* varieties was affected by the salt with the same intensity. The SLA decrease was more important for those varieties (52%) compared to *Gabès* decrease (34%). In 10 g.l⁻¹ of NaCl treatment, the local variety *Gabès* has a SLA significantly higher; this was confirmed by the variance analysis which revealed a very highly significant effect ($p < 0.0001$) of "varieties", "salinity" and of their interaction.

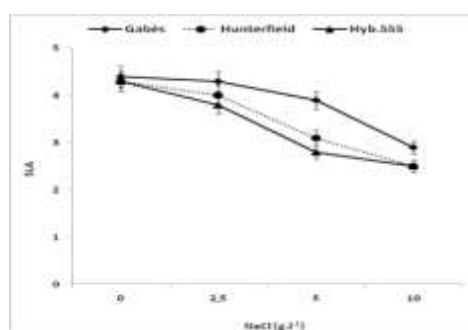


Figure 6 - Specific leaf area ($\text{cm}^2 \cdot \text{mg}^{-1}$ Leaves DM) of the three alfalfa varieties, in presence of increasing NaCl (0 - 2.5 - 5 - 10g.l⁻¹), after three months of salt treatments. Confidence intervals were calculated at $\alpha = 95\%$ probability level.

Leaves thickness

The leaves thickness was calculated by dividing the leaves water content by the corresponding foliar specific surface. The figure 7 represented the evolution of the leaves thickness of three alfalfa varieties after three months of salt stress. Results showed that the three varieties had practically the same leaves thickness in the control treatment. The thickness increased with the rise of NaCl. However, the leaves thickness of *Gabès* variety, compared to the introduced varieties, augmented linearly with NaCl increasing and reached a level significantly higher at the most stressful treatment (10g.l⁻¹).

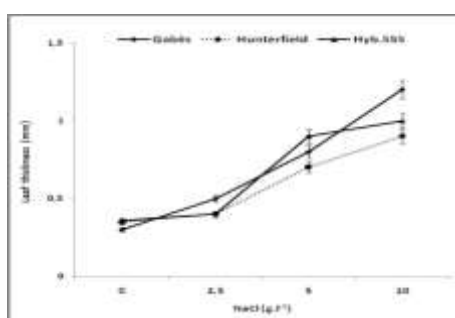


Figure 7 - Leaves thickness of the three alfalfa varieties, in presence of increasing NaCl (0 - 2.5 - 5 - 10g.l⁻¹), after three months of salt treatments. Confidence intervals were calculated at $\alpha = 95\%$ probability level.

Leaves succulence

Results showed that the three alfalfa varieties developed a succulence highly correlated with the increase of the salt in the medium culture ($r = 0.98$). Under 2.5 and 5g.l⁻¹ NaCl treatments, the leaves succulence of *Gabès* was significantly lower, compared to the introduced varieties (figure 8). However, after 5g.l⁻¹ NaCl treatment, the succulence of *Gabès* quickly increased to reach at 10g.l⁻¹, a level significantly higher. The variance analysis of the leaves succulence showed a very highly significant effect ($p < 0.0001$) of "salinity" and the interaction " salinity x varieties " but no significant effect of varieties (0.2254).

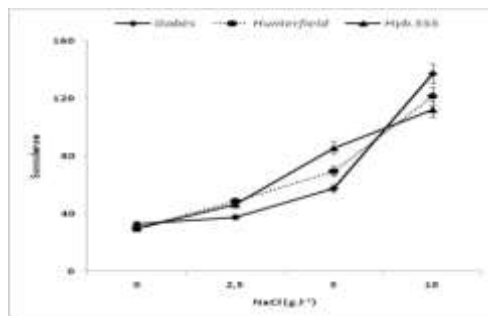


Figure 8 - Leaves succulence (mgH₂O.cm²LA) of the three alfalfa varieties, in presence of increasing NaCl (0 - 2.5 - 5 - 10g.l⁻¹), after three months of salt treatments. Confidence intervals were calculated at $\alpha = 95\%$ probability level.

Relative water content (RWC)

The relative water content of the introduced varieties (*Hunterfield* and *Hyb.555*) decreased appreciably with the same intensity (figure 9). On the other hand, RWC of *Gabès* decreased up to 2.5g.l⁻¹ treatment, and increased to achieve in 10g.l⁻¹ of NaCl, a RWC level identical under 2.5g.l⁻¹ of NaCl treatment.

The variance analysis of the RWC, revealed a very highly significant effect ($p < 0.0001$) of "varieties", "salinity" and of their interaction. The Duncan test ranked varieties in the following order: *Gabès* \geq *Hyb.555* \geq *Hunterfield*.

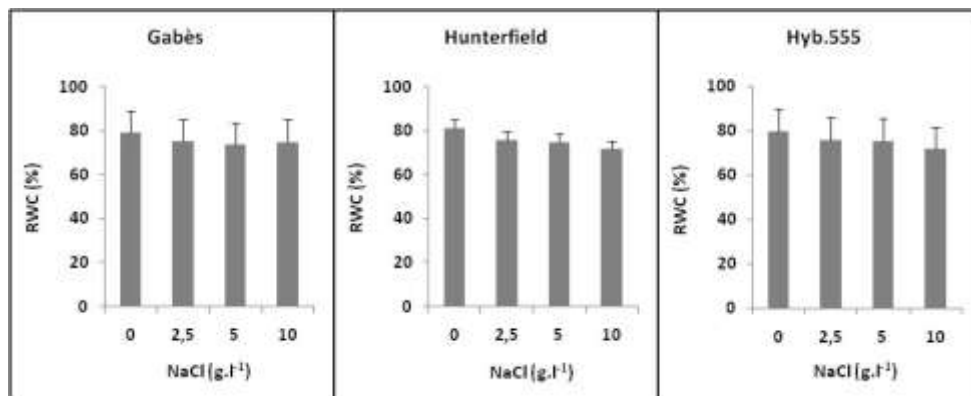


Figure 9 – RWC of the three alfalfa varieties, in presence of increasing NaCl (0 - 2.5 - 5 - 10g.l⁻¹), after three months of salt treatments. Confidence intervals were calculated at $\alpha = 95\%$ probability level.

Water content (WC)

The water content of *Gabès* variety under 2.5g.l⁻¹ of NaCl was practically the same with the control treatment. For *Gabès* and *Hyb.555*, WC increased significantly with the rise of NaCl in the medium environment (figure 10). Compared to the introduced varieties, the WC of *Gabès* variety was significantly higher at the most stressful treatment (10g.l⁻¹). For *Hunterfield*, the WC increased very slowly with the salinity intensity.

The analysis of variance of the water content showed a very highly significant effect ($p < 0.0001$) of "varieties", "salinity" and of their interaction. The Duncan test showed the superiority of *Gabès*, compared to two introduced varieties.

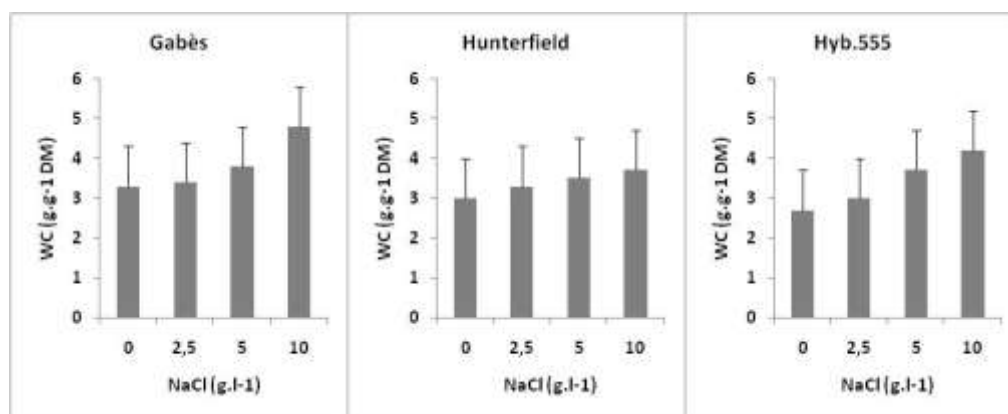


Figure 10. Water content ($\text{H}_2\text{O g.g}^{-1}\text{DM}$) of the three alfalfa varieties, in presence of increasing NaCl (0 - 2.5 - 5 - 10g.l⁻¹), after three months of salt treatments. Confidence intervals were calculated at $\alpha = 95\%$ probability level.

Water use Efficiency (WUE)

The salt stress decreased the WUE for three alfalfa varieties (figure 11). Indeed, water requirements to produce one gram of dry matter for *Gabès* variety passed of 0.6kg (control) to 2kg under the most stressful treatment (10g.l⁻¹). While water requirements for the introduced varieties, varied between 0.7kg and 3kg in absence and in the presence of 10g.l⁻¹ NaCl respectively. The decrease of the WUE was highly correlated with the increasing of doses of NaCl in the medium. The coefficients of determination for the three varieties are very high ($R^2=0.99$).

The *Gabès* variety was distinct from the two introduced varieties, by a better WUE, for all the salt treatments and especially for the most stressful treatments. The *Hunterfield* and *Hyb.555* varieties had appreciably the same WUE. This similarity was translated by a bad valuation of the water absorbed by the plant to produce DM.

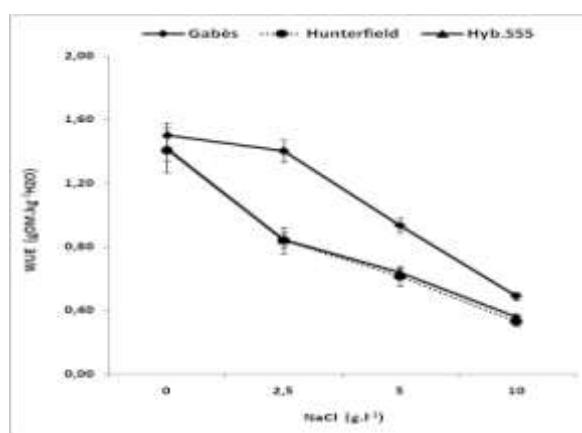


Figure 11. WUE of the three alfalfa varieties, in presence of increasing NaCl (0 - 2.5 - 5 - 10g.l⁻¹), after three months of salt treatments. Confidence intervals were calculated at $\alpha = 95\%$ probability level.

IV. Discussion

The objective of this investigation is to study the physiological responses of three alfalfa varieties (*Medicago sativa* L.) to salt stress for salt tolerance screening. The alfalfa tolerance was estimated by the biomass production under salt stress. Indeed, the effects of NaCl on the growth, showed a decrease in dry matter yield (DMY) of the three Lucerne varieties. However, the *Gabès* variety was characterized by a DMY significantly higher than the two other Lucernes, in the absence and presence of NaCl. On the other hand, the *Hunterfield* and *Hyb.555* varieties presented a marked sensibility to the salt. The severe decrease in the DMY of these two introduced varieties was especially related to the elimination of a party of the foliage by premature senescence, what was accelerated by the intensity and the duration of the salt stress, and also the result of the increasing of the difficulty to regenerate a new photosynthetic active leaves. According to Ghanem et al., (2008) and Zhang et al., (2012), leaf senescence is one of the most limiting factors to tomato and cotton productivity respectively, under salinity.

The sensibility to the salt of *Hunterfield* variety resulted from a bad dry matter repartition between roots, stems and leaves. Indeed, at the most stressful treatments, the leaves DM fraction was severely reduced by the salt. Besides, we noted in the leaves of this variety a necroses lesion what was accelerated their senescence. On the other hand, the native *Gabès* variety was characterized by a well-equilibrated distribution of the DM between the various organs.

In the presence of a moderate salt stress (2.5g.l⁻¹), the LAR of the three alfalfa varieties increased. But, for the stressful treatments (5 and 10g.l⁻¹), the LAR decreased significantly with the salt increasing in the culture medium for the three varieties; so *Hunterfield* has the LAR significantly lower. This was the result of the severe leaves sensibility to salt which was accelerated their senescence. On the contrary, the *Gabès* variety presented a low LAR decrease for the most stressful treatments; while *Hyb555* occupied an intermediate position. In the three alfalfa varieties, the SLA was negatively affected by salt increasing. Decrease in alfalfa SLA under salt stress was attributed to the different sensitivity of leaf area expansion and photosynthesis. The sensibility of alfalfa leaves under salt stress resulted in an excessive accumulation of Na⁺ and Cl⁻, entailing their premature senescence for the introduced varieties (data not shown). Our results confirm those of Rahimi et al., (2011) on the strawberry plants subjected to different NaCl concentrations.

The salt modified plant anatomical by increasing the leaves thickness. In our study, NaCl increased significantly the alfalfa leaf thickness at the stressful treatments. Our results confirmed those found by Longstreth and Nobel, (1979) working on three species represent a wide range of salinity tolerance, since bean (salt-sensitive), cotton (moderately tolerant), and *Atriplex* (halophyte), they found that the gradient salinities increased the leaf thickness due to the increase of palisade cells length and the number of spongy cell layers. The thickness of the leaf is related to the extension in the cell wall due to a higher turgor pressure on the cell wall. An increase in the number of spongy parenchyma and palisade parenchyma layers may also result in increased leaf thickness.

In salt stress, the succulence in dicotyledonous plants is an anatomical adaptive way to escape toxic ions Na⁺ and Cl⁻ by increasing in cell size due to the increases in vacuole volume. The three alfalfa varieties increased succulence with the rise of salt concentrations. The succulence permitted the accumulation of large amounts of water and dissolved Na⁺ and Cl⁻ ions in the leaves and preserved the physiological function like photosynthesis. In the three alfalfa varieties, the water content increased in concomitance with the increasing of the leaves succulence.

The water use efficiency (WUE), under salt stress decreased in the three alfalfa varieties. Indeed, the need of water supply to produce one gram of dry matter increased with increasing of salt concentration in the medium. However, compared to the introduced alfalfa varieties, *Gabès* variety has the WUE significantly higher in all of salt treatments. *Hunterfield* and *Hyb.555* varieties had practically the same WUE. The decrease in WUE in the three varieties was attributed to the CO₂ diffusion throw stomata to mesophyl cells. Indeed, Ashraf et al., (2000) showed on wheat differing in salt tolerance, that WUE was more higher at the tolerant variety than on the sensitive one. For Chaves et al., (2009), WUE was function of the CO₂ concentration in internal cells. In conclusion, this study allowed highlighting the interest of the *Gabès* variety in any selection program for salt tolerance.

Acknowledgements

This study was performed within the support of Ministry of Higher Education and Scientific Research through the financing of research work.

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