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Characterization of *Medicago arborea L.* Response to Water and Salt Stress

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Medicago arborea L. is a Mediterranean leguminous fodder shrub, regarded as a promising species in arid and semi-arid lands where it can play an important role in the elaboration of a durable pastoral system.

The aim of this paper is to investigate and characterize the response of *M. arborea* plants to water and salt stress at the early growth stage. Seedlings of the species derived from seeds collected in the Djelfa province, Algeria, were grown in pots under greenhouse conditions and separately submitted to water stress, restoring 20%,40%,60%,80% and 100% of substrate field capacity, and salt stress, supplying irrigation water with 0, 50, 100, 150 and 200 $meql^{-1}$ of $NaCl+CaCl_2$.

Stress effects were determined on fresh and dry-matter biomass, relative water content, leaf pigment content (chlorophyll and carotenoids), proline and total soluble sugars amount.

Results showed that both water and salt stress affected seedlings growth. In particular, the lowest water regime (20% of field capacity) significantly reduced fresh and dry-biomass and relative water content, whereas seedlings under salinity maintained a good water content (>70%).Chlorophyll *a* and *b*, and carotenoids content did not show significant differences among treatments, while proline and total soluble sugars amounts, major osmolytes involved in osmotic adjustment, significantly increased according to salt and water stress intensity.

The findings highlight that *M. arborea* has a remarkable potential of tolerance to water deficit and salinity, involving a range of physiological strategies to cope with stress by regulating metabolism activity and maintaining cell turgor.

Key words: growth, Medicago arborea, salinity, tolerance, water deficit

Drought and salinity are among the main abiotic stresses characterizing the arid and semi-arid lands, they adversely affect plants growth and productivity (Tuteja, 2010; Kyani and Niknam, 2015). In Algeria, these lands cover over 216.000 km^2 of the surface (Le Houérou, 1995). Moreover, the availability of water in the soil is the main limiting factor for Mediterranean pasture production (Medrano *et al.,* 1998).

Several reviews have covered various aspects of the similarities and contrasts in the plant response to drought and salt stress. Salinity reduces the ability of plants to take up water, and this quickly causes reductions in growth rate, along with a suite of metabolic changes similar to those caused by water stress (Munns, 2002; Bartels and Sunkar, 2005).

Photosynthesis and plant growth are among the primary processes affected by drought (Chaves, 1991) and salinity (Munns *et al*., 2006). Under salt stress and low soil water potential, plants accumulate a range of osmolytes including proline and soluble sugars, in order to accomplish osmotic adjustment (Zhu, 2002; 2007). Depending on the species involved and severity and duration of the stress event, proline concentration can reach a level of several hundred folds higher than its background value (KaviKishor and Sreenivasulu, 2014).

Forage shrubs are interesting forages for arid lands because of their adaptation and productivity in dry climates and poor soil (Ventura *et al*., 1999). Among the Mediterranean flora, *Medicago arborea* L. is one of the most important native species in arid and semiarid Mediterranean regions (Hickman, 1993). It is a leguminous fodder shrub, adapted to periodic drought and all kinds of soils, capable to protect fragile landscapes from wind and water erosion, and therefore used in many valorization and restoration programs of damaged steppic areas (Lapeyronie, 1982; De Koning and Duncun, 2000).

M. arborea could provide highly nutritious fodder and may act as a strategic forage species supporting conventional resources in forage systems for sheep farming in semiarid environments (Papanastasis *et al*., 1998; Amato *et al*., 2004).

Notwithstanding its crucial role, the establishment of plants is a critical development phase which depends on the availability of water and nutrients (Lefi *et al*., 2004). Plants tolerance to drought and salinity involves mechanisms at the whole-plant morphological, physiological, biochemical and molecular levels (Farooq *et al*., 2009). In this context, considering the lack of current data on *M. arborea* tolerance to salinity and drought in Algeria, a study on the physiological and biochemical response was carried out under a range of salinity and water stress conditions within controlled greenhouse conditions, in order to understand the mechanisms of tolerance of the species.

MATERIALS AND METHODS

Site and experimental treatments

Seeds of *M. arborea* were collected in June 2012, provided by the station of INRF (National Institute of Forestry Research) of the Djelfa province (region of high plateau of the center of Algeria).

The seeds were selected then sterilized for 10 min in 5% of sodium hypochlorite and rinsed with distilled water. Then they were germinated in an incubator maintained at a continuous optimal temperature of 20°C (Aisset and Mehdadi, 2016).

Seedlings were planted in pots (90 x 120 mm) containing 350 g of sandy-loam soil with a pH of 7.2. They were grown at the research greenhouse of the Djillali Liabes university, under a photoperiod of 16h-8h, a day/night temperature of 25-15°C (± 3°C) and a day/night humidity of 70-80%.The pots were irrigated by restoring the water content at the field capacity (FC) with distilled water and, once a week, by nutritional Hoagland solution (Hoagland and Arnon, 1938).

After 120 d (days), the seedlings were submitted for 30 d to water and salt stress separately managed. For water stress, plants were supplied with Hoagland solution by restoring 20% (WS20), 40% (WS40), 60% (WS60), 80% (WS80) and 100% (WS100, control treatment) of water content at field capacity. For salt stress, the following treatments were compared: S0, control treatment, supplied only with Hoagland solution; S50, S100, S150 and S200, supplied with Hoagland solution to which NaCl + $CaCl_2$ were added at the

concentration of 50, 100, 150 and 200 meql^{-1} respectively. Plants were irrigated every 3 d.

For both water and salt stress experiments, the treatments were arranged in a completely randomized design with10 replicates.

Plant sampling and analysis

After 30 d of water and salt stress treatments, plants were harvested, separating shoots and roots biomass; roots were washed from the soil and thoroughly dried.

In order to estimate the plants response, the following parameters were determined.

Biomass: fresh shoot (FSB) and root biomass (FRB) were recorded, while the respective dry shoot (DSB) and root biomass (DRB) were measured after oven drying the samples at 80°C for 48h; the dry root biomass/dry shoot biomass ratio (RATIO) was determined. Fresh and dry biomasses were expressed as g.plant^{-1}.

Relative water content (RWC): RWC was measured with Barrs and Weatherley (1962) method. Fresh leaves were immediately weighed (fresh weight, FW), then placed in distilled water and incubated in cold (2°C) for 24 h, they were after taken out of the water, slightly dried and immediately weighed to obtain fully turgid weight (TW).

Dry weight (DW) was obtained after oven drying at 80°C for 48 h. Relative water content, expressed as percentage, was calculated as:

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

Leaf pigment content: the chlorophyll and carotenoids were extracted using Shabala *et al*., (1998) technique. The absorbance spectra (A) of the extracts were read using UV spectrophotometer at 645, 663 and 470nm.The amounts of chlorophyll a (Chl-a), chlorophyll b (Chl-b) and carotenoids (Cd) were calculated according to Lichtenthaler (1987) and expressed asµgmg^{-1}of FW.

$$Chl-a = 9.78 * A(663) - 0.99 * A(645)$$

$$Chl-b = 21.42 * A(645) - 4.65 * A(663)$$

$$Cd = \frac{[1000 * A(470) - 1.90 * Chla - 63.14 * Chlb]}{214}$$

Proline content: determined using the method described by Troll and Lindsley (1955) and improved by Dreier and Göring (1974). The amount of proline was determined from a standard curve of different concentrations of commercial proline in a range of 0-100 µgml^{-1} and expressed as µgmg^{-1}of DLB (Dry Leaf Biomass).

Total soluble sugars (TSS) content: quantified using the method described by Shields and Burnet (1960). Their content was determined on a standard curve of different concentrations of pure glucose (0-100 µgml^{-1}) and expressed as µgmg^{-1}of DLB.

Data analysis

All the data were subjected to a one way analysis of variance considering a completely randomized design. The means of the parameters were compared using Student Newman Keuls (SNK) post hoc test at P=0.05. Data analysis was performed using the SAS (Statistical Analysis Software) software.

RESULTS

Water stress effect

Fresh and dry biomass showed significant differences among treatments. In particular, FSB and FRB were the lowest in the most stressed treatment (WS20), with decreases of 53 and 71% respectively, compared with the average yield obtained in the other treatments (Table 1). Dry root biomass (DRB) was significantly higher with the optimal supply of irrigation (restoring of 100% of water content at field capacity, WS100), while no differences were observed among the more stressed treatments (WS20, WS40, WS60).

Although not significant, the root/shoot ratio was higher in the most stressed treatment (WS20), with an increase of 31%compared with the average value of the other treatments (Table 1).

Relative water content (RWC) was significantly affected by treatments investigated, in fact the most stressed one (WS20) showed a significant decrease of 47% compared with the average value of the other treatments (Table2).

Chlorophyll *a* and *b* content and carotenoids did not show significant differences among the treatments (Table 2). In any case, the chlorophyll a did not change

with restoring 100, 80 and 60% of water content at field capacity whereas showed a decrease at higher water stress levels. The content of chlorophyll b, instead, gradually reduced with increasing water stress after WS80. Carotenoids showed an increase to the increase of water stress, with the highest average content at greatest water deficit condition (WS20).

The water stress levels strongly influenced leaf proline and TSS content, in fact these parameters significantly increased with the water stress intensity (Figure1). The proline content reached the highest rate (42.33 μgmg^{-1}) at the highest water stress levels (restitution of 20% of water content at field capacity) that resulted about 9 fold higher than the average value of the less stressed treatments (WS80 and WS100; 4.77 μgmg^{-1}) (Figure1, A).

Also TSS accumulation increased as the water stress rise and the highest rate (943.829 μgmg^{-1}**)** was attained at the most stressed treatments with an increase of about 60% compared with WS100 (Figure1, B).

Salinity effect

All parameters investigated were not significantly affected by treatments (Table 3), except for fresh root biomass (FRB) that gradually decreased with increasing salinity, and in the most stressed treatments (S200) it reduced of 37% compared with not saline control (S0).

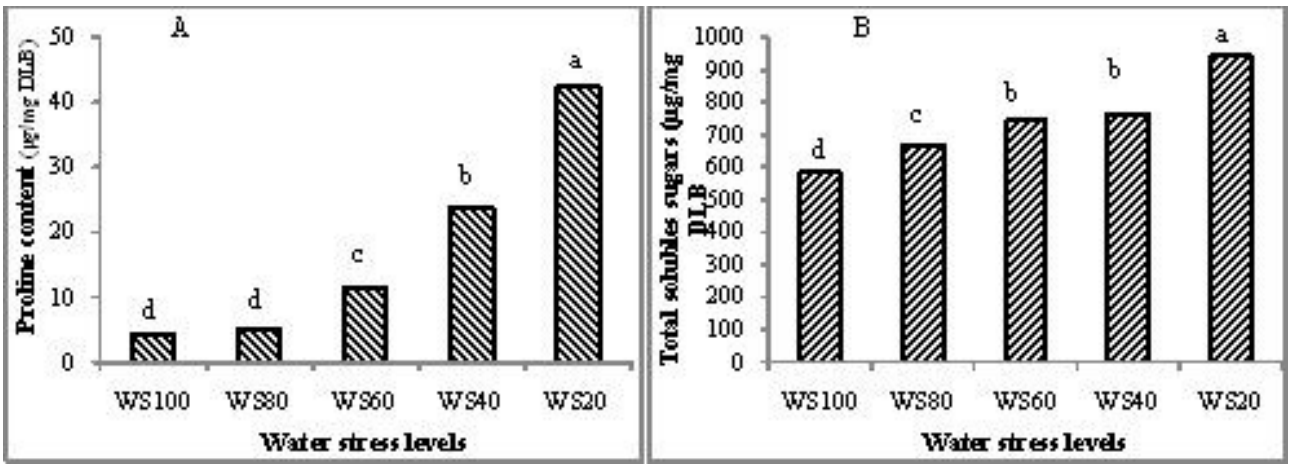

Figure 1: Leaf proline (A) and total soluble sugars (B) content under different water stress levels. Different letters indicate significant differences at P = 0.05 according to SNK test

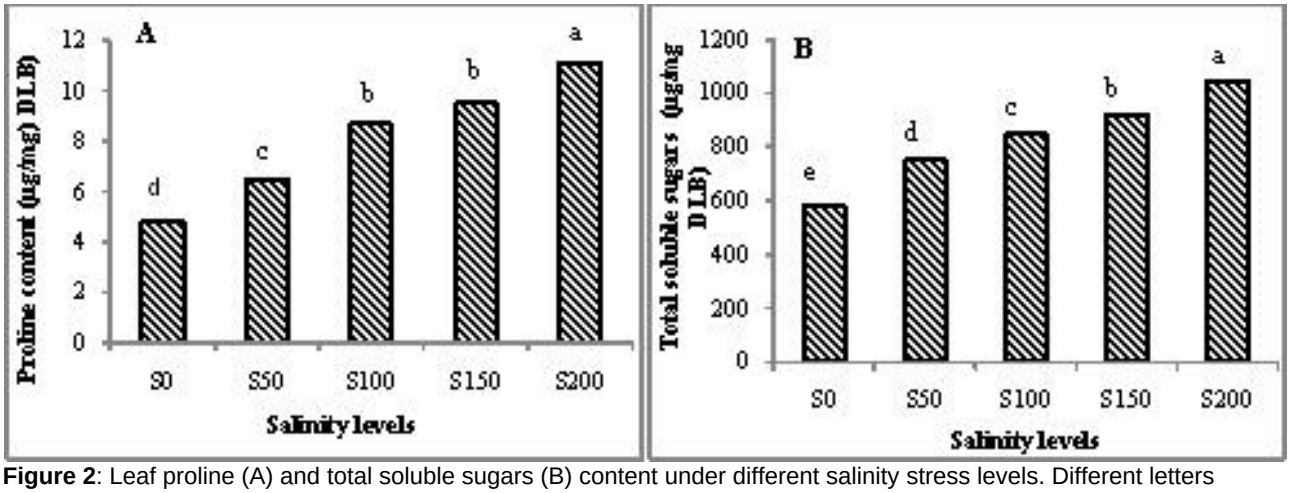

Figure 2: Leaf proline (A) and total soluble sugars (B) content under different salinity stress levels. Different letters indicate significant differences at P = 0.05 according to SNK test

Table 1: Plant growth response parameters as affected by water stress (WS)

Source of variation	FSB (g plant^{-1})	DSB (g plant^{-1})	FRB (g plant^{-1})	DRB (g plant^{-1})	Ratio
P value	*0.0079***	*0.2293^{NS}*	*0.0001***	*0.0040***	*0.7898^{NS}*
WS20	0.238 b	0.147	0.109 b	0.064 b	0.717
WS40	0.442 a	0.147	0.325 a	0.064 b	0.549
WS60	0.504 a	0.167	0.354 a	0.080 b	0.499
WS80	0.505 a	0.200	0.388 a	0.100 ab	0.556
WS100	0.556 a	0.205	0.417 a	0.117 a	0.586

*, ** indicate respectively differences at P≤0.05 and P≤ 0.01; NS indicates not significant differences
Means followed by the same letter in each column are not significantly different according to the SNK test (P=0.05)

Table 2: Plant physiological response parameters as affected by water stress (WS)

Source of variation	RWC (%)	Chl-a (µgmg^{-1} FW)	Chl-b (µgmg^{-1}FW)	Carotenoids (µgmg^{-1} FW)
P value	*<0.0001***	*0.6515^{NS}*	*0.6726^{NS}*	*0.0909^{NS}*
WS20	43.11 b	1.02	0.81	1.87
WS40	72.18 a	1.11	0.89	0.96
WS60	78.83 a	1.79	1.13	0.84
WS80	86.31 a	1.79	1.44	0.73
WS100	89.83 a	1.86	1.16	0.41

*, ** indicate respectively differences at P≤0.05 and P≤ 0.01; NS indicates not significant differences
Means followed by the same letter in each column are not significantly different according to the SNK test (P=0.05)

Table 3: Plant growth parameters as affected by salinity (S)

Source of variation	FSB (gplant^{-1})	DSB (gplant^{-1})	FRB (gplant^{-1})	DRB (gplant^{-1})	Ratio
P value	*0.2066^{NS}*	*0.6962^{NS}*	*0.0137**	*0.4724^{NS}*	*0.1989^{NS}*
S0	0.943	0.272	0.481 a	0.083	0.306
S50	0.868	0.268	0.418 ab	0.076	0.294
S100	0.851	0.249	0.387 ab	0.081	0.368
S150	0.720	0.258	0.358 ab	0.081	0.358
S200	0.705	0.223	0.303 b	0.066	0.302

*, ** indicate respectively differences at P≤0.05 and P≤0.01; NS indicates not significant difference
Means followed by the same letter in each column are not significantly different according to the SNK test (P=0.05)

Table 4: Plant physiological response parameters as affected by salinity (S)

Source of variation	RWC (%)	Chl-a (µgmg^{-1}FW)	Chl-b (µgmg^{-1}FW)	Carotenoids (µgmg^{-1}FW)
P value	*0.1042^{NS}*	*0.2108^{NS}*	*0.1544^{NS}*	*0.9609^{NS}*
S0	84.79	1.98	1.41	0.85
S50	78.44	2.51	1.85	1.07
S100	75.78	2.25	1.65	0.94
S150	74.41	1.96	1.31	0.93
S200	72.93	1.17	0.78	0.93

*, ** indicate respectively differences at P≤0.05 and P≤ 0. 01; NS indicates not significant difference
Means followed by the same letter in each column are not significantly different according to the SNK test (P=0.05)

Increasing salt concentrations slightly reduced relative water content compared with control; however plants maintained a good water content higher than 70% (values ranged from 78.44 to 72.93% at the 50 to 200 meql^{-1}).

Chlorophyll a and band carotenoids content did not show significant differences among treatments (Table 4). On average chlorophyll a and b increased in the lowest salt concentration (50 meql^{-1}) compared with S0, then gradually decreased with the lowest values in the

most stressed treatment. Differently from chlorophyll, carotenoids content did not change among treatments (average content of about 0.95 μgmg^{-1} of FW).

The increasing water salinity caused significant increases of leaf proline and total soluble sugar content (Figure 2, A-B). In particular, proline content ranged from 4.843 μgmg^{-1} in control to 11.137 μgmg^{-1} in the most stressed treatment (S200).Total soluble sugars content gradually rose with increasing salinity, passing from a content of 584.33μgmg^{-1}in the control to 1046.88 μgmg^{-1} in S200 treatment.

DISCUSSION

The inhibition of plant growth and yield, due to soil salinity and water deficit, is the result of osmotic, ionic, oxidative and hormonal imbalances effects. To cope with salt and drought stress and to achieve success in the adaptation and survival to limiting growth conditions, plants have developed several stress-responsive signaling pathways and sophisticated defense mechanisms (Huang *et al*., 2012). The stress response depends on species, genotypes and developmental stages as well as on soil salt and water level and timing of exposure. The response mechanisms may exert positive and negative effects over plants growth (Forni *et al*., 2017).

In this study a negative relationship was observed between both salinity and water deficit and vegetative growth parameters of *M. arborea* seedlings, even if water stress significantly reduced plant biomass and relative water content, especially under highest stress conditions (20% of FC). Our results are in agreement with those of Elboutahiri *et al*., (2008) in *Medicago sativa* and of Laouar *et al*., (2001) in populations of *Medicago intertexta*. Growth inhibition under severe soil salinity and water deficit conditions can be attributed to a decrease in carbon assimilation due to stomatal limitation and/or metabolic impairment (Hajiboland *et al*., 2014). Moreover, the reduction in plant growth under both stresses conditions can be the result of direct inhibition of cell division and expansion (Munns, 2002). In fact, cell growth is one of the most drought-sensitive physiological processes, due to the reduction in turgor pressure (Taiz and Zeiger, 2006).

Leaf and shoot growth is generally more sensitive to osmotic stress than root growth. Reduced leaf size is generally considered to be beneficial to plants under water deficit conditions because of a concomitant reduced rate of transpiration, even though it may impact on the photosynthetic rate. The roots are often reported to play a key role in the salt and water deficit tolerance of plants as they represent the first organs that control the uptake and translocation of water, nutrients and salts throughout the plant. Despite the direct exposure of these organs to low water availability and saline environment, root growth is less vulnerable than that of the shoots (Munns, 2002). Plants, subjected to water and salt stress, tend to thicken the roots in order to explore a greater soil volume and facilitate water uptake from deeper soil layers (Tuteja, 2010; Forni *et al*., 2017). In our study, the root/shoot ratio was indifferent to saline and water treatments, in any case plants under less water availability (20% of FC) showed the highest root/shoot ratio. Therefore, in extreme conditions of water scarcity shoots biomass of *M. arborea* decreased more than roots, promoting more distribution of biomass toward roots. In several previous studies, the reduced expansion of aboveground plant organs was observed also for the most resistant species (such as Lucerne, Fescue, Sorghum) and that was explained as an adaptive strategy required to surviving plants submitted to abiotic stress (Lefi *et al*., 2004; Durand, 2007).

In our investigation, soil salinity and water deficit did not affect the relative water content that resulted higher than 70% in all treatments, except in WS20, where the RWC value was below 50%. The finding indicated the good water status of plant tissues despite the high salts concentrations and the water deficit applied. Therefore, this plants response can be regarded as a mechanism to avoid water loss, maintaining water absorption at a sufficient level in order to prevent dehydration of plants tissues, dilute salts present in cells and guarantee the continuity of the metabolic process (Bissati *et al*., 2011).

Generally, the decrease of RWC under moderate and severe salt stress leads to limiting photosynthesis, as a consequence of stomata closure (Kicheva *et al*., 1994) and suppression in mesophyll conductance (Flexas *et al*., 2004).

Photosynthesis is among the primary processes to be affected by drought (Chaves, 1991) and salinity (Munns *et al*., 2006). In particular, water and salt stress can affect synthesis or breakdown of the photosynthesis pigments, including chlorophyll a, chlorophyll b (Ashraf, 2002). Drought stress can cause an alteration of the photosynthetic metabolism directly by damaging photochemical system functioning, carboxylation and regeneration of the carbon dioxide acceptor I (Lawlor and Cornic, 2002; Hopkins, 2003). The chlorophyll content commonly decreased at severe water stress (40-20% of FC), which may be regarded a protective adaptive mechanism to prevent excess of photon absorption (Ait Said *et al*., 2013).

The excess of Na^+ and Cl^- ions induces an alteration of the photosynthetic machinery (Munns *et al*., 2006): sodium affects chlorophyll b biosynthesis pathway (Tewari and Singh, 1991) and chlorine inhibits the synthesis of Rubisco (Ashraf and Harris, 2013).

In our study, chlorophyll a and b content slightly changed among treatments. In particular, under water deficit these pigments decreased with increasing stress, whereas an unexpected slight increase of chlorophyll content was observed at low salt stress (S50 and S100), which might be explained as an abrupt shock of the photosynthesis metabolism exhibited at mild stress (Kurban *et al*., 1999). In addition, carotenoids content did not change in the *M. arborea* plant under salt stress, whereas increased with increasing water deficit. The carotenoids are considered auxiliary pigments and effective antioxidant which protect and stabilize photochemical processes of photosynthesis under stress conditions (Ashraf and Harris, 2013). Thus, they could be used as reliable selection criteria for salt and water stress tolerance. It increases under stress conditions, where the photosynthetic apparatus appears to be more resistant (Kebbas *et al*., 2015), suggesting that at severe stress leaves start to develop a chlorosis and finish by falling (Agastian *et al*., 2000).

Water and salt stress provided a significant increase of proline and total soluble sugars in *M. arborea* leaves. The synthesis and accumulation of organic compounds such as sugars and amino acids in the cytoplasm occur as metabolic response to stress conditions and play an important role in osmotic adjustment in plants (Ashraf and Foolad, 2007; Per *et al*., 2017). The increase of proline and sugars concentration reduces the water potential inside the cell and water continues to move from high water potential to low water potential sites, in this way intracellular water loss is avoided (Tuteja, 2010) and turgor pressure in plant tissues is maintained also under limited water availability conditions (Farooq *et al*., 2009; Chen *et al*., 2011).

Proline is recognized as a multi-functional amino acid, able to protect cells from damage by acting as both an osmotic agent and a radical scavenger (Verbruggen and Hermans, 2008). It is well known that under stress condition many plant species accumulate proline as an adaptive response to adverse conditions. Thus, proline may act as a possible drought-injury indicator (Irigoyen *et al*., 1992). Under stress condition, proline seems to have diverse roles, such as stabilization of proteins, membranes and cellular structures, and protecting cellular functions by scavenging reactive oxygen species (Szabados and Savourée, 2010). Sanchez *et al*., (1998) suggested that there is a controversy about proline increase whether it is an adaptive response to minimize the damages of the dehydration or a biochemical change due to these damages.

Sugar accumulation might make a greater contribution to osmotic adjustment than proline as also showed on bentgrass by Liu *et al*., (2015). High TSS levels in tissues highlight the aptitude of plants to tolerate abiotic stress conditions, such as drought and salinity. Moreover, sugar accumulation indicates a good metabolic status and promotes growth and carbohydrate storage (Rosa *et al*., 2009; Chen *et al*., 2011). Total soluble sugars increase under water stress could be also a consequence of smaller translocation from the leaf and of slower consumption due to decreased growth or starch hydrolysis (Kameli and Loésel, 1996). Kebbas *et al*., (2015) noted that the sugar accumulation can be attributed to a passive dehydration process rather than an active stimulation of their synthesis.

CONCLUSION

In this study, *M. arborea* seedlings showed morphological and physiological changes under water and salt stress. In particular, the plant growth and the

relative water content significantly reduced in extreme water stress. Moreover, a slight increase of root/shoot ratio was observed in the most stressed water treatment. Leaf pigments were not significantly affected by the treatments investigated, even though the increasing water stress induced a gradual decrease of chlorophyll content combined with an increase in carotenoids. Finally, the exposure of *M. arborea* seedlings to salinity and drought induced a significant accumulation of proline and soluble sugars in leaves. The increase of these organic compounds allowed to maintain a good osmoregulation in plant tissues, since it is well known that proline is a multifunctional amino acid that can also provide stabilization of proteins, membranes, subcellular structures, and protection of cellular functions. Therefore, the seedlings showed to be able to put in action, in a rapid and efficient manner, specific mechanisms to cope with osmotic stress and protect membrane integrity.

Although the selection of aridity and salt-tolerant plants is a very complex process, and deeper studies are required to elucidate biochemical strategies involved in tolerance, our results showed that *M. arborea* seems to be drought and salt tolerant at early stage of growth. In addition, these legume shrubs may provide other key ecosystem services, such as protection from wind erosion and land degradation which may represent, particularly in sensitive areas as the high plateau of the center of Algeria, a barrier against desert expansion.

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