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INDUCED OSMOTIC ADJUSTMENT IN ALFALFA PLANTS CONFERS TOLERANCE TO WATER STRESS

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Abstract

ABID, M., E. MANSOUR, A. BEN KHALED, Kh. D. BACHAR, L. BEN YAHYA and A. FERCHICHI, 2016. Induced osmotic adjustment in alfalfa plants confers tolerance to water stress. *Bulg. J. Agric. Sci.*, 22: 415–425

South Tunisian oases are characterized by arid environment in which drought is a major limiting factor for growth and crop production. In these conditions, understanding plant adaptation is required in order to preserve such regions from degradation. Four levels of water stress were applied to evaluate the effect of drought in three *Medicago sativa* populations (Chenini, Gannouch and Tebelbou): 25%; 50%; 75% and 100% of field capacity. Under water deficit conditions, leaves and roots exhibited a higher increase in proline, total soluble sugars, amino acids and potassium. A decrease in dry matter production was observed especially in alfalfa leaves. Chenini population showed the highest increase in different solutes, in contrary Tebelbou seems to be the most affected by drought as it showed lowest values. A close relationship was observed between proline and several osmolytes indicating that it is an important indicator of drought stress tolerance in alfalfa plants.

Key words: Compatibles Solutes, Drought, *Medicago sativa*, Populations, Responses
Abbreviations: PROL: proline; TSS*:* total solubles sugars; AA*:* total amino acids; PROT*:* proteins; K*:* potassium; Na*:* sodium; DM*:* dry matter; PCA*:* Principal component analysis

Introduction

Drought becomes the most menacing abiotic stress in arid and semi-arid regions Shao et al. (2009) in the world and in Tunisia. Furthermore, to withstand drought, plants showed a wide range of adaptations at different levels XOCONOSTLE-Cazares et al. (2010). Among varied responses, osmotic adjustment is the important physiological mechanism by which plants synthesize and accumulate solutes that functioned as osmolytes in cells in response to water deficit Seki et al. (2007) and it is said to be a drought avoidance mechanism Yang et al. (2011).This beneficial phenomenon resulted in the maintenance of a higher turgor potential that may contributes to limiting the effect of water deficit in plants photosynthesis and growth Link et al. (2010); Ali and Ashraf (2011). In addition, osmoregulation allows protecting membranes and enzyme systems especially in young organs. The compatible osmolytes generally found in higher plants are low molecular weight sugars, nitrogen containing compounds such as free amino acids, amides, proline and soluble proteins.

The ability to evaluate the performance of crops subjected to water stress is very important in research programs aimed to the rehabilitation and improvement of production in arid and semi-arid lands. It is well known that alfalfa or *Medicago sativa* is an extremely adaptable plant that can be grown under a wide range of climate conditions from the equator to almost arctic polar circle regions Michaud et al. (1988).This plant produced around 2600 kg of protein per hectare (Mauries, 1994) which constitutes the greatest protein yield observed beyond classical cultivated plants such as wheat, corn or soy. Due to their capacity of symbiotic

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*Corresponding author: abid.mabrouka@yahoo.fr nitrogen fixation, leguminous plants, like *Medicago sativa*, are often used to improve soil organic fertility and nitrogen economy Erice et al. (2010). In addition, this plant is able to endure water lose until 70% (Morad, 1996). Contrary to the annual alfalfa, perennial alfalfa (*Medicago sativa* L.) is not new in Tunisia; it's very old in the Tunisian oases especially (Le Houérou, 1969). East coastal oases – located especially in the south of Tunisia (Gabes oases) are the main production centers of perennial alfalfa. They are characterized by arid environment that can affect plants negatively (Sghaier, 2010).

The aim of this study is to evaluate the accumulation of solutes in *Medicago sativa* populations in response to four water stress levels: 25%; 50%; 75% and 100% of field capacity and their contribution to adaption of this perennial specie to these conditions. Exploration of the variability in drought responses leaded not only to identify tolerant varieties, but also to determine useful criteria for genetic improvement of water stress tolerance. The present study is part of this approach.

Materials and Methods

Growth conditions and water stress application

Experiment was conducted in spring period in the Institute of Arid Lands in Medenine in the South of Tunisia. Seeds of three populations of *Medicago sativa* (Chenini, Gannouch and Tebelbou) were collected from Gabes Coastal Oases in the South-east of Tunisia (Latitude 33°35′N, Longitude 10º48′3″E, Altitude 105 m). The study was done under controlled conditions of a glasshouse with a thermo-period of 26^{o}C (day) and 18^{o}C (night). Daily maximum relative humidity (RH) ranged from 65 to 70%. Average daily photo-synthetically active radiation (PAR) inside the greenhouse was 500 µmol m^{-2} s^{-1}. Planting were done in pots (10 L plastic pots), each pot contains a mixture of 2/3 sand and 1/3 of soil. All the samplings were well watered until complete leaf sprouting occurred. The plants were then submitted to four levels of water stress: 25, 50, 75 and 100% (control) of field capacity with three replicates for each treatment. Harvesting material collected in the last cut during spring season were used to determine PROL, TSS, AA, PROT, K^+ and Na^+ contents in leaves and roots.

Dry matter determination

The dry matter of the collected material was measured after drying at 85°C for 48 h. The completely dry samples were weighted and the dry weight is taken after stabilization.

Proline determination

PROL content was measured according to Bates et al. (1973) with 3% of sulphosalicyclic acid and toluene. The absorbance was read at 520 nm. PROL content was expressed in µmolg^{-1} of DM.

Total sugars determination

Total soluble sugars (TSS) were measured by (Dubois, 1956). According to this method, fresh material was mixed with 3 ml of ethanol 80% for extraction of sugars. The absorbance at 640 nm was measured by a spectrophotometer according to a calibration curve. Results are expressed in mgg^{-1} of DM.

Total proteins determination

To evaluate PROT content (Bradford, 1977) method was used. The absorbance was measured at wavelength of 595 nm.The standard curve was prepared previously in the same conditions using a solution of bovine serum albumin (BSA). Proteins content are expressed in mgg^{-1} of DM.

Amino acids determination

Ninhydrin method (Aoun, 2009) was used to determine AA content. Absorbance was measured after cooling at 570nm with a spectrophotometer Secomam. Standard curve was established with alanine solution. Results are expressed in mgg^{-1} of DM.

Mineral contents

For the determination of K^+ and Na^+ content, Sherwood 410 flame photometer regulated on the filter of sodium or potassium was used. The contents of sodium (%Na^+) or of potassium (%K^+) in the dry matter plant were calculated as: % Na^+ or % K^+ = (C * DF) / (100 * m) (1), where C is the concentration of sodium or potassium (mgL^{-1}), DF is the factor of dilution and m is the mass of the extract (g).

Statistical analysis

Data were subjected to General Linear Model (GLM) with water Stress treatments and their interaction. When the effect was significant, differences between means were evaluated for significance by using the *LSD* test ($P = 0.05$). Statistical analysis was performed using SPSS software18.0 for Windows statistical software package (SPSS, Chicago, IL, USA). Differences between populations were established by Duncan test. Relationships between parameters were determined using Pearson correlation test at 0.05 Levels and Principal component analysis (PCA).

Results

Effect of drought on osmolytes accumulation

Variance analysis revealed a highly significant treatment effect in all studied parameters in alfalfa leaves and roots (Table 1). Data indicated that there is significant difference between lucerne parts in response to drought for all parameters. Population effect was highly significant (1‰) in TSS, PROT, DM, Na$^+$, K$^+$ contents, significant in proline content (5%) and K/(Na+K) ratio (1%) and not significant for AA content (Table 1). Population X treatment, population X organ and Population X treatment X organ interactions were also detected for all parameters indicating variable performance of populations in different drought levels and alfalfa parts.

Table 1
Mean squares values from analysis of variance of the effects of population (P); stress level (SL); organs (O); and their interactions in osmotic parameters K/Na and K(Na+K) ratios for *Medicago sativa* plants subjected to water stress

Source	df	Prol	TSS	AA	Prot	DM
P	2	16323.341*	1589.666***	0.772^{NS}	5853.993***	5.412***
SL	3	69052.002***	6120.607***	37.511***	28719.011***	2.229***
O	1	7725921.890***	4055.102***	297.810***	718912.411***	62.589***
P * SL	6	2843.977^{NS}	39.901^{NS}	0.624^{NS}	309.392**	0.549***
SL * O	3	40808.215***	624.103***	36.431***	28894.124***	13.397***
P * O	2	15647.174*	893.661***	0.749^{NS}	5844.661***	1.189***
P * SL * O	6	1785.451^{NS}	47.776^{NS}	0.613^{NS}	310.958**	0.316***
Error	48	4321.027	20.007	0.357	64.251	0.011
Source	df	K$^+$	Na$^+$	k/Na	k/(Na+K)	DM
P	2	0.101***	0.000***	444.211***	0.002**	5.412
SL	3	0.043***	0.010***	356.898***	0.003***	2.229
O	1	4.590***	0.290***	17006.109***	13.896***	62.589
P* SL	6	0.001^{NS}	3.188^{NS}	18.521^{NS}	9.963^{NS}	0.549
SL* O	3	0.050***	0.009***	355.359***	0.003***	13.397
P* O	2	0.095***	0.000***	442.565***	7.311^{NS}	1.189
P * SL * O	6	0.001^{NS}	0.000**	18.707^{NS}	0.000^{NS}	0.316
Error	48	0.002	2.743	14.520	0.000	0.011

Prol: proline; TSS: total solubles sucres; AA: amino acids; Prot: proteins; DM: dry matter; K$^+$: potassium; Na$^+$: sodium

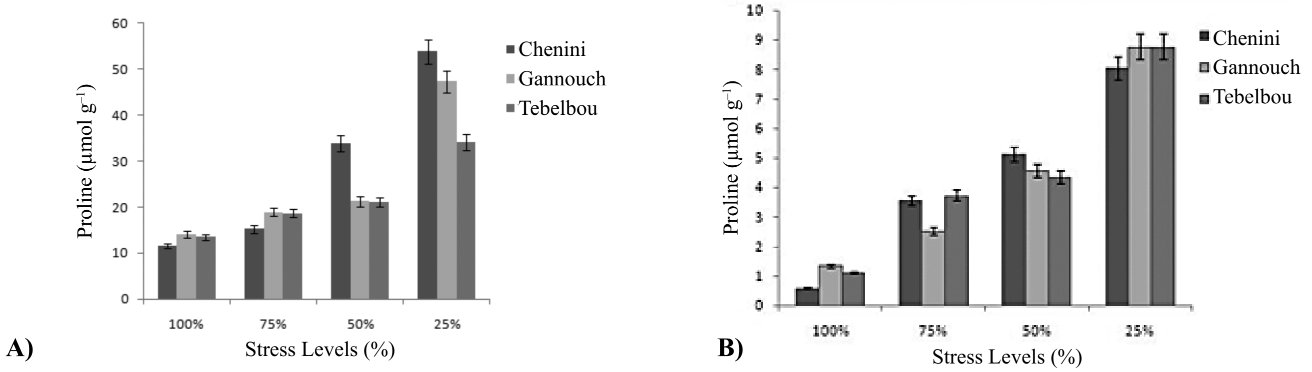

Fig.1. Proline content (μmol g^{-1}) under different water stresslevels (25, 50, 75 and 100% of field capacity) in *Medicago sativa* leaves (A) and roots (B) for the three studied populations(Chenini, Gannouch and Tebelbou)

As a consequence of water stress, PROL accumulation increased significantly ($p < 0.05$) in leaves (Figure 1A) and roots (Figure 1B) in the three studied populations. Of all populations, Chenini presented higher values while Tebelbou showed lowest ones. Among organs, leaves accumulated highest rates of PROL. Values in roots vary between 1umol $g^{-1}$to 8.04 µmol g^{-1}while in leaves; values reached 50 µmolg^{-1}.

Imposition of water deficit conditions had a significant increase effect on total soluble sugars in leaves and roots (Figure 2A, B) as well as drought level increase. TSS rates exceeded 50% especially in leaves. TSS content was comparatively higher in Chenini population plants; moreover, Tebelbou accumulated lowest contents.

AA content increased significantly during drought stress in all populations, while Chenini population showed the ability to accumulate highest content (0.152 mgg^{-1}). Considerable accumulation was reestablished in roots (Figure 3 B) as compared to leaves (Figure 3A). Means reached 0.176 $mgg^{-1}$for leaves and 0.87 $mgg^{-1}$for roots.

Drought stress treatment induced a significant decrease ($p < 0.05$) in total soluble proteins contents in both leaves and roots. Values decreased from 300 $mgg^{-1}$to 119 mgg^{-1}in leaves (Figure 4A) and from 912 to 588 mgg^{-1}in roots (Fig4 (B)). We note that roots accumulated highest rates. Among populations, Gannouch and Tebelbou populations exhibited lowest content than Chenini in both leaves and roots.

The increasing water stress enhanced significantly the potassium uptake by alfalfa leaves and roots ($p < 0.05$). Highest accumulation was observed in leaves (Figure 5A,B). Significant difference was observed between populations. For leaves, Chenini showed the highest rates, however, for roots, Tebelbou exhibited highest values.

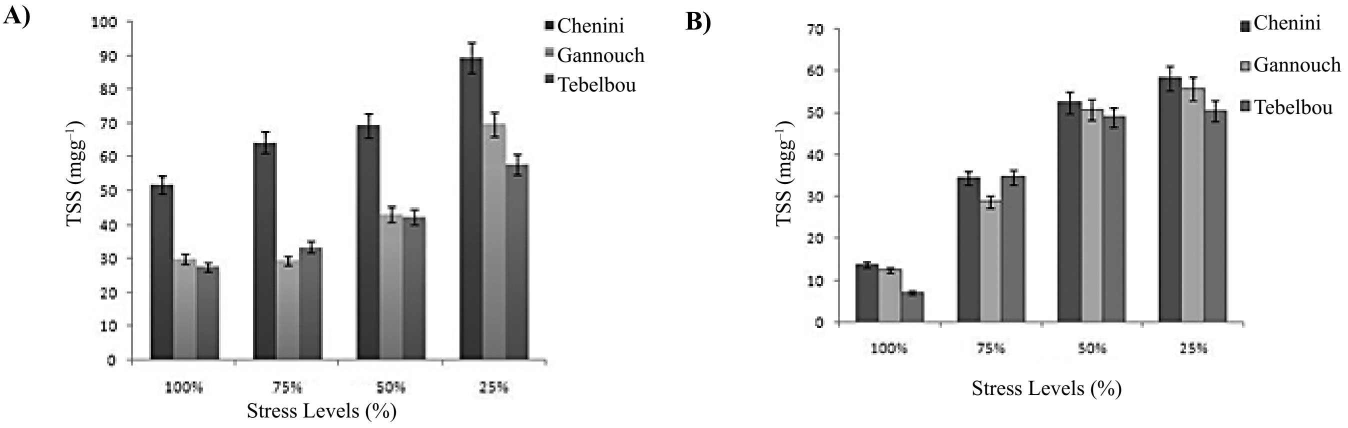

Fig. 2. Total soluble sugars (TSS) content(mgg^{-1}) under different water stress levels (25, 50, 75 and 100% of field capacity) in *Medicago sativa* leaves (A) and roots (B) for the three studied populations (Chenini, Gannouch and Tebelbou)

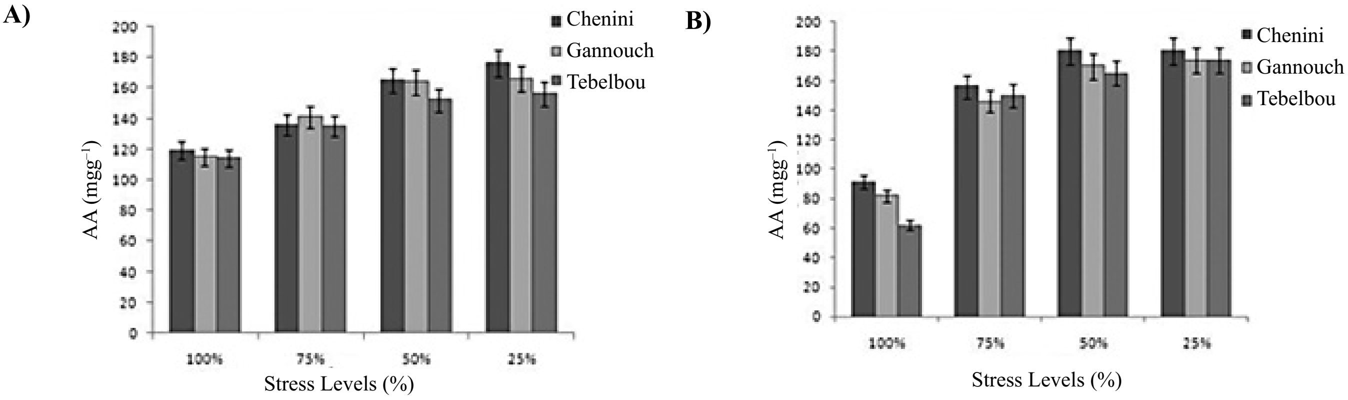

Fig. 3. Amino acids content (AA) content(mgg^{-1}) under different water stress levels (25, 50, 75 and 100% of field capacity) in *Medicago sativa* leaves (A) and roots (B) for the three studied populations (Chenini, Gannouch and Tebelbou)

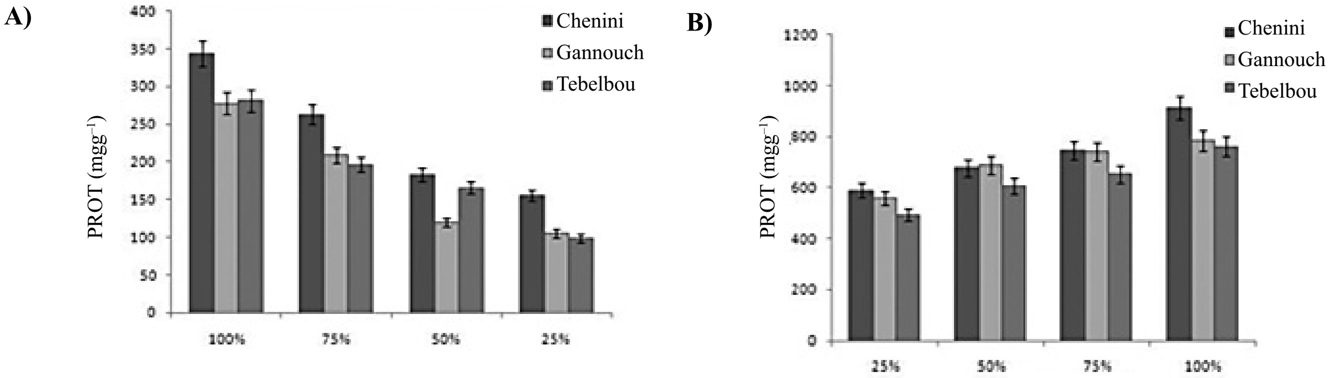

Fig. 4. Total protein content (PROT) content(mgg^{-1}) under different water stress levels (25, 50, 75 and 100% of field capacity) in *Medicago sativa* leaves (A) and roots (B) for the three studied populations (Chenini, Gannouch and Tebelbou)

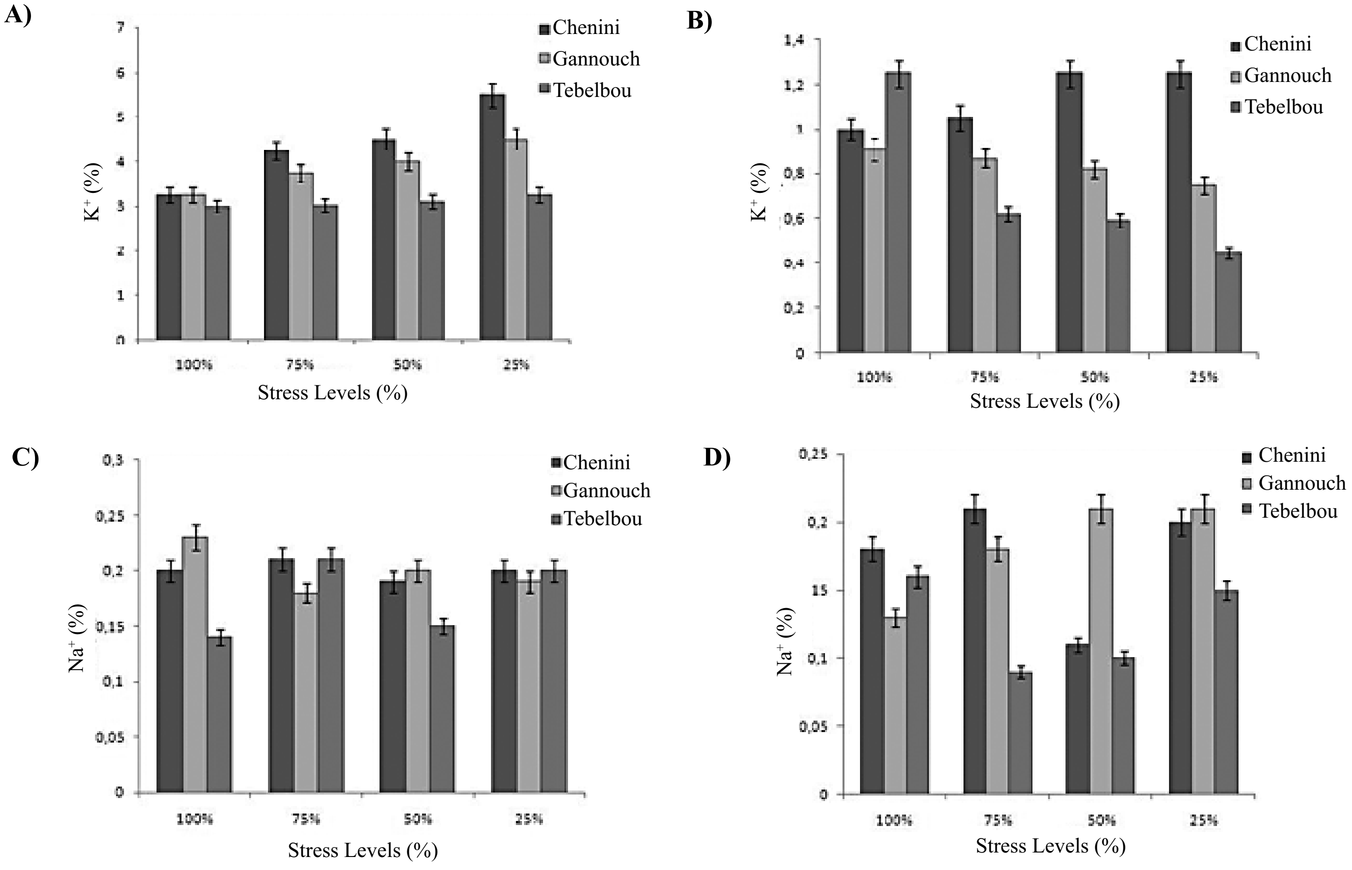

Fig. 5. K$^+$ and Na$^+$ contents (%) under different water stress levels (25, 50, 75 and 100% of field capacity) in *Medicago sativa* leaves and roots for the three studied populations (Chenini, Gannouch and Tebelbou)

Drought stress has no significant effect on Na$^+$ content. These results are the same for roots and leaves and for different populations (Figure 5C,D).

Root and leaf K$^+$/Na$^+$ and K/ (Na$^+$ +K$^+$) ratios were also calculated and increased significantly in alfalfa populations under drought conditions. However, this increase was observed significantly greater in leaves than roots (Figure 6A,B) and (Figure 6C,D). Leaves and roots ratios were higher in Chenini.

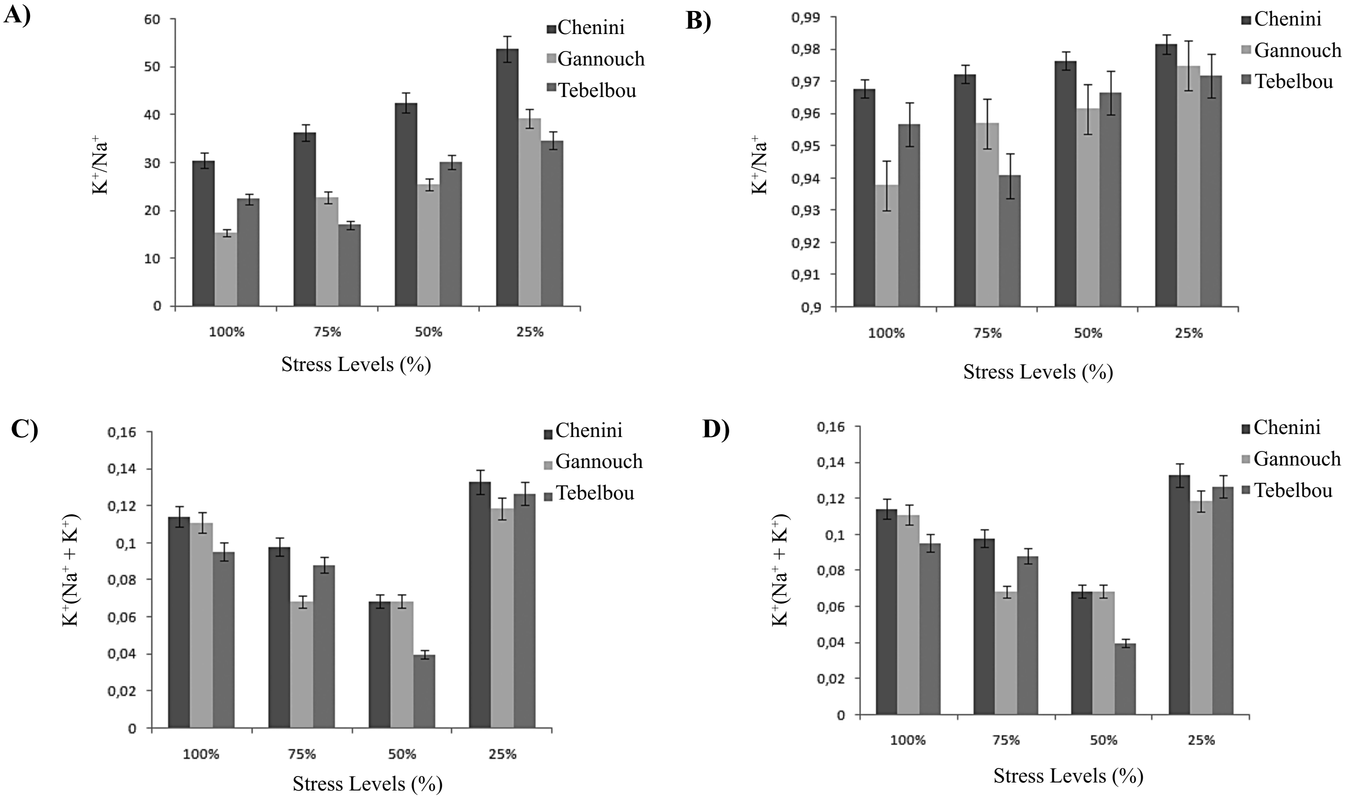

Fig. 6. K^+/Na^+ and $K + (Na^+ + K^+)$ ratios under different water stress levels (25, 50, 75 and 100% of field capacity) in *Medicago sativa* leaves and roots for the three studied populations (Chenini, Gannouch and Tebelbou)

Table 2
Correlations between parameters in *Medicago sativa* leaves showed by Pearson index
Prol: proline; TSS: total solubles sucres; AA: amino acids; Prot: proteins; DML: dry matter leaves; K^+: potassium; Na^+: sodium

	TSS	AA	PROL	PROT	K^+	Na^+	DML	k/Na	k/ (Na^+K)
TSS	1	-0.67	0.5	-0.15	**0.56****	0.07	**-0.37****	**0.6****	**0.37****
AA		1	**0.82****	**-0.65****	**-0.68****	0.06	0.17	**-0.64****	**-0.73****
PROL			1	**-0.83****	**-0.69****	**-0.24****	**0.56****	**-0.83****	**-0.95****
PROT				1	**0.76****	**0.79****	**-0.69****	**0.69****	**0.87****
K+					1	**-0.84****	**-0.56****	**0.96****	**0.93****
Na+						1	**0.82****	**-0.81****	**-0.91****
DML							1	**-0.51****	**-0.69****
k/Na								1	**0.89****
k/(Na+K)				.					1

Significantly different at 5% level (*), 1% level (**)

Results showed that drought affect significantly DM of aboveground (leaves) and belowground organs (roots) ($p < 0.001$). Significant differences were also observed between the three studied populations, Gannouch exhibited the highest dry matter production under all stress levels and Tebelbou exhibited lowest values.

Relationships between parameters

The correlation analysis for leaves shown in Table 2 indicated that a significant negative correlation ($p < 0.05$) between PROL content and respectively PROT, K^+ contents and K^+/Na^+, $K/(Na^+ + K^+)$ ratios (respectively $R^2 = 0.83$; 0.65; 0.83 and 0.95); While, With AA content and DM, these

Table 3
Correlations between parameters in *Medicago sativa* roots showed by Pearson index
Prol: proline; TSS: total solubles sucres; AA: amino acids; Prot: proteins; DML: dry matter leaves; K^+: potassium; Na^+: sodium

	TSS	AA	PROL	PROT	K^+	Na^+	DMR	k/Na	k/ (Na⁺K)
TSS	1	**0.96****	**0.88****	**0.88****	-0.24	0.1	0.69	-0.25	-0.09
AA		1	**0.8****	**0.85****	-0.29	0.09	**0.61***	-0.17	-0.32
PROL			1	**0.83****	-0.29	0.19	**0.63***	-0.98	0.28
PROT				1	0.01	0.39	**0.88****	0.51	0.21
K+					1	0.27	0.34	0.55	0.15
Na+						1	0.36	0.44	0.41
DMR							1	0.33	0.3
k/Na								1	**0.65***
k/(Na+K)				.					1

Significantly different at 5% level (*), 1% level (**)

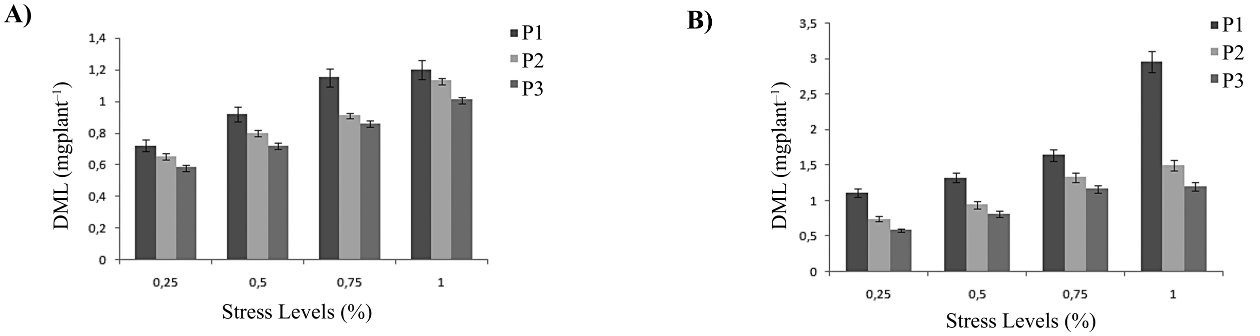

Fig. 7. Dry matter leaves (DML) and roots (DMR) production under different water stress levels (25, 50, 75 and 100% of field capacity) in *Medicago sativa* leaves and roots for the three studied populations (Chenini, Gannouch and Tebelbou).

correlations are positive ($R^2 = 0.82$ and 0.56 respectively). A strong relationship between Na^+/K^+; $K/(Na^+ + K^+)$ ratios and K^+ content (respectively 0.96 and 0.93) was observed. Significant negative correlation existed between DML, PROT and K^+ contents. ($R^2 = 0.69$; 0.56 respectively).

In roots, Table 3 showed that significant positive correlations were established between PROL content and respectively PROT, TSS, AA contents (r $R^2 = 0.85$; 0.88; 0.8). Significant correlations existed also between DMR and respectively PROL, AA, PROT and K/Na contents ($R^2 = 0.63$; 0.61; 0.88 and 0.65).

Principal component analysis (PCA) is a method of choice used to determine differences between used parameters and to identify the main osmolyte responsible for osmotic adjustment. Results were based on the first two axes. Figure 8 showed the contribution of different significant variables to each axix. These axes represented 77% of the total variation. The first axis represented 48% and it is correlated positively to PROT content and negatively with amino acids and proteins content. The second axis represented

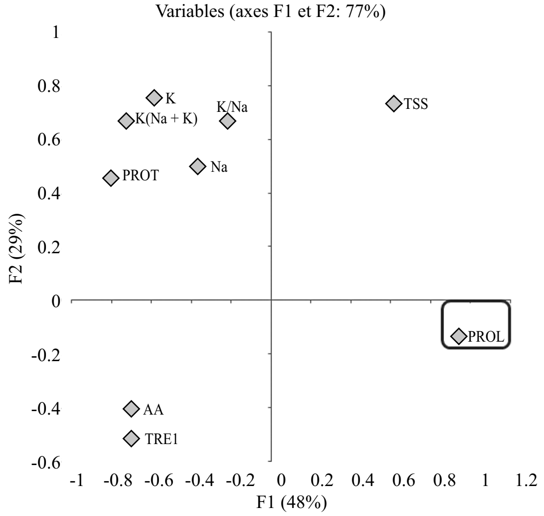

Fig. 8. Principal component analysis (PCA) of different variables(PROL, TSS, AA, PROT, K, Na, K(Na+K), K/Na) in leaves and roots of *Medicago sativa* plants grown under water stress.

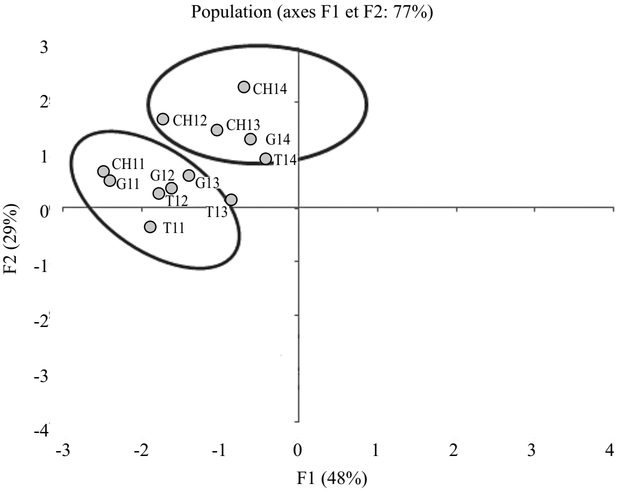

Fig. 9. Principal component analysis (PCA) of 3 populations (CH: chenini; G: gannouch and T: tebelbou) grown under water stress

29.14% of total variability and it is correlated positively with TSS, K and Na contents.

For populations, results were based on the first two axes. Figure 9 showed that the two axes represented 77% of the total variation. This analysis presented the classification of Chenini population as the most tolerant population under stress conditions (CH14: 25% of field capacity), CH13: 50% of field capacity and CH12: 75% of field capacity). This is in relation with PROT accumulation.

Discussion

The accumulation of soluble carbohydrates in plants has been widely reported as a response to salinity or drought. In this study, it is very similar; results showed that alfalfa plants accumulate the common amino acid proline in response to progressive water stress. It constitute less than 5% of free amino acids but it is among the most widespread stress metabolites and its accumulation in many plants represents a general response to drought-stress Garcıa Sanchez et al. (2007). In agreement with our results, PROL increased in *Medicago truncatula* and *Medicago laciniata* populations under water deficit yousfi et al. (2010), in wheat and maize plants (Ahmed and Hasan, 2011) and in Pea exposed to water deficit Sanchez et al. (1998). However, as compared to nodulated alfalfa witch accumulated highest proline rates in response to drought especially in roots (Yang et al., 2011), highest accumulation was detected in *Medicago sativa* leaves than roots. This difference can be explained by the different proline uses under stress conditions. It helps to maintain osmotic adjustment (Kishor et al., 2005), is a free radical scavenger and antioxidant (Sharma; Dietz, 2006). In addition, this amino acid can serve as an energy supply for growth and survival of parts with active metabolism (leaves especially) and to be a carbon and nitrogen reserve to trigger growth after defoliation (Silveira et al., 2003). It is shown also that PROL accumulation is due to proteins degradation and it is related to carbohydrates levels (Roy et al., 2009), highest and negative correlations between PROL and PROT, TSS contents respectively reinforce this idea (r^2 = 0.83 and 0.5 respectively). The same result is observed in *physiolus vulgaris* and chickpea (*Cicerarietinum*) plants subjected to water stress (Yadav et al., 1999).

This study showed that TSS increased in response to drought. These results corroborate with results of (Irigoyen et al., 1992) witch pointed that TSS increased in alfalfa leaves and nodules and it is the same in annual alfalfa plants (Yousfi et al., 2010). Iannucci et al. (2002) indicated that TSS contribute the most to the osmotic adjustment and seems to be associated with drought tolerance in many plant species such as Pea (Sanchez et al., 1998), sugar beets (Choluj et al., 2008) and black poplars (Regier et al., 2009).TSS increase was more marked in leaves than roots, it was demonstrated that this increase was due to a drought-induced starch hydrolysis, a restriction in sucrose translocation from leaves and to the reduced assimilates uses in leaf and root induced by water stress (Sturm and Tang, 1999).

Imposition of water deficit conditions induced an enhancement on amino acids (AA) content in *Medicago sativa* leaves and roots. Similarly, many studies showed that these compounds are involved in osmotic adjustment (Irigoyen et al., 1992; Abranches et al., 2005). However, these results showed that this increase was observed especially in alfalfa roots. In opposition, some studies indicating that AA content increased highly in the leaves (Ashraf and Iram, 2005). This result can be explained by the storage of Carbon and Nitogen reserves in roots to ensure regrowth after defoliation (Avice et al., 2003). In this study, it seems that the hypothesis indicating that amino acid increase was due to protein hydrolyze was supported; in fact, results showed that there is a negative and elevated correlation between the two parameters ($p < 0.01$; $r^2 = 0.653$). In addition, protein rates decreased highly in response to progressive water stress in *Medicago sativa* leaves and especially in roots. This is underlined the crucial role of roots in maintaining osmotic regulation. Further for alfalfa which is a perennial forage legume of great agronomical interest, a majority of the roots are distributed preferentially in deep soil in order to enhance the plant to tolerate water deficit stress. This morphological plasticity is often concomitant with the tolerance to water deficit stress (Slama et al., 2006; Yousfi et al., 2010). This idea is supported by these results showing that there is a greater decrease in dry matter production exhibited by the aboveground components. This reduction especially observed in leaf DM may allow these cultivars to maintain relative water content under drought conditions by decreasing the size of transpiring organs. Comparative results had been obtained for a number of plants (Navas and Garnier, 2002; Lei et al., 2006; Markesteijn and Poorter, 2009).

Furthermore, significant correlations were detected between DML and PROL, TSS, PROT, K^+ contents indicating the close relationships between osmotic adjustment and dry matter production. Similar results were detected in Pea (Sanchez et al., 2004) and chickpea (*Cicerarietinum* L.) plants (Krouma, 2009) subjected to drought and salinity.

The potassium content increased under progressive drought level treatments in both roots and leaves. Leaf tissues showed higher potassium contents than roots. Further, K^+ ion play a crucial role in osmotic adjustment for alfalfa. The same finding was detected in annual Medicago Yousfi et al. (2010), in water-stressed maize plants (Premachandra et al., 1989) and in *Aeluropus lagopoides* (Mohsenzadah et al., 2006).This accumulation may occur in concert with changes in sugars and amino acids (Pérez-Pérez et al., 2009). Highest correlation coefficients between K^+ and TSS, AA and Prol contents supported this idea ($r^2 = 0.56$; 0.68 and 0.69 respectively)

This increase in potassium content in the three populations is due also to the good selectivity of “Gabesien variety” for K^+ (Mezni et al., 2001) as compared to Na^+. This conclusion corroborate with our results indicating that Na^+ content was not significantly altered in leaves and roots by any water-deficit treatment, hence, the Na^+ ion did not contribute as much to the osmotic adjustment in both organs in all treatments. This is indicated that alfalfa plants excluded Na^+ from their organs in order to avoid its toxcitiy. This mechanism was shown in many spaces as common behavior of tolerant plants (Marshner, 1995).

This is led to increased K/Na and K/(K+Na) ratios and highest correlations coefficients between these ratios and K^+ and Na^+ contents (Table 2). Furthermore, the ratio K/Na is an index of Na^+ toxicity and for some species, it is more important than maintaining lowest concentrations of Na^+ (Cuin et al., 2003). Such behavior has been observed in maize (Botella et al., 1997) and rice plants Garcia et al. (1997). In the same context, many authors (De Lacerda et al., 2005) reported that tolerant plants use to maintain an elevated K^+ / Na^+ ratio in their aerial parts as a result of the reduced absorption of K^+ and Na^+ by roots and their transport to aerial parts (Al-Zahrani; Al-Toukhi, 2012).

According to Table 2 and 3 and to PCA analysis, significant and higher correlations coefficients were detected between PROL and others osmolytes. We can note that proline is the main osmolyte in the alfalfa osmotic adjustment process followed by K (Na+K) and K/Na ratios and can be used as the most important criterion in studying water stress resistance in alfalfa plants. Chenini population is the most tolerant to drought in these oases as it accumulates highest contents of varied osmolytes. This difference can be explained by the difference of using climatic conditions.

Conclusion

The strategy of an effective osmotic adjustment mechanism in leaves and roots tissues by accumulating different compatibles solutes employed by *Medicago sativa* plants is a common method in tolerance-avoidance mechanisms for many plants species to survive under drought. Thus, it is very interesting to enhance genetic studies to select more tolerance criteria in order to ameliorate plant development and stress tolerance capacity.

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