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Full Length Research Paper

Differential response to water deficit stress in alfalfa (*Medicago sativa*) cultivars: Growth, water relations, osmolyte accumulation and lipid peroxidation

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The present study was fixed as objective to compare the response to water deficit (33% of field capacity, FC) stress of eight cultivars of *Medicago sativa*, originating from the Mediterranean basin. Comparison was performed on some key parameters such as growth, relative water content, leaf water potential, MDA tissue content, electrolyte leakage and proline and soluble sugar tissue concentrations. In all cultivars, water deficit stress reduced the whole plant growth, increased the root/shoot DW ratio and led to a significant decrease in leaf relative water content and leaf water potential. In water-stressed plants and for the majority of cultivars, proline and soluble sugar concentrations increased significantly. The Tamantit cultivar originating from Algeria showed the better tolerance to water deficit stress. Some criteria are concomitant with this tolerance: (1) a high biomass production under stress conditions when compared to other cultivars (2) the root preferential development, (3) the better ability to accumulate proline and soluble sugars, and (4) the aptitude to protect its photosynthetic apparatus against the oxidative stress generated by the water deficit stress. Ecotipo Siciliano, originating from Italy was the most sensitive cultivar. The increase of proline and soluble sugars concentrations upon water deficit stress particularly in the most tolerant cultivar suggested their involvement in the osmotic adjustment.

Key words: *Medicago sativa*, intraspecific variability, water deficit stress, osmotic adjustment, MDA.

INTRODUCTION

Environmental constraints such as drought, flooding, extreme temperature, salinity, heavy metals, photon irradiance and nutritional disturbances represent the most limiting factors for agricultural productivity and play a major role in the distribution of plant species across different types of environments (Ashraf, 2010). It has been estimated that two-thirds of the potential yield of major crops are usually lost due to adverse growing environments (Chaves et al., 2009). Water shortage is the major constraint affecting fodder production and yield stability in most arid and semi-arid regions (Shao et al., 2009). The situation worsens by the fact that most climate change scenarios predict a worldwide increase in arid areas. When faced with this climatic change and increasing water demand for agriculture, the selection of plants resistant to water deficit stress and particularly the identification of physiological proprieties used by Fabaceae species to cope with drought are of paramount importance (Erice et al., 2010; Sambatti and Caylor, 2007). Improvement of plant drought resistance becomes urgent and should integrate conventional breeding and biotechnological approaches (Shao et al., 2006, 2009).

Due to their capacity of symbiotic nitrogen fixation, leguminous plants, like *Medicago sativa*, are often used to improve soil organic fertility and nitrogen economy (Erice et al., 2010; Yousfi et al., 2010). Most leguminous trees and shrubs having deep roots and fix nitrogen are able to resist drought and can thrive on poor soils with little nutrients.

Some plants have evolved various protective mechanisms allowing them to survive and grow in harsh

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environments and respond to water deficit stress through multiple physiological, biochemical and molecular mechanisms (Anjum et al., 2011). Plant responses to water deficit stress generally vary with varieties, severity as well as with the duration of water shortage. Among these responses, osmotic adjustment (OA), which is defined as the lowering of osmotic potential in plant tissue due to net accumulation of organic and/or mineral solutes, is a key of plant osmo-tolerance (Yang et al., 2011). Compatible solutes involved in OA may act as cytoplasmic osmolytes facilitating water uptake and retention but also serves as protection in plants suffering from oxidative stress by the detoxification of reactive oxygen species and stabilizer of macromolecules and cellular structures (Szabados and Savouré, 2010). These beneficial impacts have been reported for proline, glycinebetaine, soluble sugars and polyols. Common with other abiotic stresses, drought causes increased production of activated oxygen species (ROS) that inactivate enzymes and damage cellular components (Shao et al., 2007, 2008). Oxidative stress occurs when the defence capacity of plants is broken by the formation of free radicals. Malondialdehyde (MDA) is an end product of membrane lipid peroxidation; it has been used extensively as an indicator for free radical production and membrane injury under various abiotic stress conditions (Hermandez et al., 2001). An over-accumulation of proline in transgenic tobacco subjected to osmotic stress reduced free radicals levels, assessed by MDA production (Paranova et al., 2004).

Since water availability is usually the main factor affecting productivity in dry regions, strategies aiming at improving sustainable use of water and plant drought tolerance are urgent (Erice et al., 2010). In this context, exploration of the variability in osmotic stress responses would permit not only to identify some tolerant varieties, but also to determine useful criteria for genetic improvement of osmotic stress tolerance. As part of this approach, the present study aimed to investigate the differential response of eight *Medicago sativa* cultivars originating from the Mediterranean basin to water deficit stress.

MATERIALS AND METHODS

Growth conditions

Experiment was carried out on 8 *M. sativa* cultivars originating mainly from Mediterranean basin: Mamuntanas, Sardi 10, Ecotipo Siciliano (Italy), Ameristand 801S (USA), Gabes 2355 (Tunisia), Rich 2 (Marocco), Magali (French) and Tamantit (Algeria). Plants were cultivated in 25 L plastic pots (30 cm of depth). Every pot was filled with 25 kg of soil from the Centre of Biotechnology at the Technopark of Borj Cedria (CBBC) parcel. It is a limono-sandy soil containing 0.25, 0.95, 0.65 and 0.05 meq 100 $g^{-1}$of dry soil of Na^+, K^+, Ca^{2+},Cl^-, respectively and 0.24 and 0.45 g kg^{-1} of dry soil of P_2O_5 and total N, respectively. The pH and the electrical conductivity of the aqueous extract (1/10) were 6.65 and 0.05 mmhos cm^{-1}, respectively. Culture was conducted in semi-controlled conditions in greenhouse in CBBC. Seeds were sown at a rate of 8 seeds per pot. The obtained seedlings were subjected to a phase of one month pre-treatment during which soil was maintained at 100% field capacity (irrigation was carried out with tap water). At the end of this phase of pre-treatment, the plants of each cultivar were divided into two batches, control plants (100% FC) and of the stressed ones (33% FC). Regular weightings (every 2 days) enabled to restore the moisture of soil at 100 or 33% FC. The plant weight was neglected. Experiments were carried-out in greenhouse with a 14 h photoperiod (photosynthetic photon flux density, PPFD: 1000 µmol m^{-2} s^{-1}). Mean temperature and relative humidity were, respectively 30±5°C, 55±5% day and 16±2°C, 90±5% night. At the end of one month of treatment, a final harvest was carried out and plants were separated into shoots and roots.

Growth, water relation measurements and leaf water potential

Fresh weights (FW) of plant samples (roots and shoots) were determined upon harvesting. Dry weight (DW) was obtained after oven drying the samples at 60°C until a constant weight was reached.

Relative water content (RWC) was measured in the third youngest fully expanded leaf harvested in the morning. RWC were determined using the following equation (Schonfeld et al., 1988):

$$RWC\ (\%) = 100 \times (FW - DW)/ TW - DW$$

FW was determined within 2 h after harvest. Turgid weight (TW) was obtained after soaking leaves in distilled water in test tubes for 12 h at room temperature (about 20°C), under low light condition of laboratory. After soaking, leaves were quickly and carefully blotted dry with tissue paper in preparation for determining turgid weight. Dry weight (DW) was obtained after oven drying at 60°C until a constant weight was reached. For Ψs, leaves were quickly collected, cut into small segments, then placed in Eppendorf tubes perforated with four small holes and immediately frozen in liquid nitrogen. After being encased individually in a second intact Eppendorf tube, they were allowed to thaw for 30 min and centrifuged at 15,000 × g for 15 min at 41°C (Martínez-Ballesta et al., 2004). The collected sap was analysed for Cs estimation. Osmolarity (C) was assessed with a vapour pressure osmometer (Wescor 5500) and converted from mosmoles kg^{-1} to MPa according to the Van't Hoff equation:

$$\psi_s = -R\ T\ M$$

R is the universal gas constant, T is the temperature (K) and M is the osmolality

Lipid peroxidation

The extent of lipid peroxidation was assessed by determining the concentration of malondialdehyde (MDA) (Draper and Hadley, 1990). Leaf material was homogenized in 0.1% (w/v) TCA solution. The homogenate was centrifuged at 15,000 × g for 10 min and 1 ml of the supernatant obtained was added to 4 ml 0.5% (w/v) TBA in 20% (w/v) TCA. The mixture was incubated at 90°C for 30 min, and the reaction was stopped by placing the reaction tubes in an ice water bath. Samples were centrifuged at 10,000 × g for 5 min, and the absorbance of the supernatant was read at 532 nm. The value for non-specific absorption at 600 nm was subtracted. The concentration of MDA was calculated from the extinction coefficient 155 mM^{-1} cm^{-1}.

Electrolyte leakage

The leaf and root samples (0.2 g) were placed in test tubes containing 10 ml of double distilled water. The leaves were cut into discs of uniform size (5 mm length). The tubes were incubated in a water bath at 32°C for 2 h and the initial electrical conductivity of the medium (EC1) were measured. The samples were autoclaved at 121°C for 20 min to release all the electrolytes, cooled to 25°C and the final electrical conductivity (EC2) were measured. The electrolyte leakage (EL) was calculated by using the formula:

$EL = (EC1/ EC2)*100$ (Dionisio-Sese and Tobita, 1998).

Proline and soluble sugars determination

Free proline was quantified spectrophotometrically using the method of Bates et al. (1973), while the soluble sugars were determined by the anthrone reagent method according to Yemm and Willis (1954).

Proline was determined following the ninhydrin method, using L-proline as a standard. Leaf samples (100 mg FW) were homogenized in 1.5 ml of 3% (w/v) aqueous sulfosalicylic acid and centrifuged for 30 min at 14,000 g. To the supernatant (500 µl), 2 ml of acid ninhydrin and 2 ml of glacial acetic acid were added and the mixture was boiled for 1 h. After extraction with toluene, the free proline was quantified (λ = 520 nm) from the organic phase using an Anthelie Advanced 2, SECOMAN spectrophotometer. Proline in the test samples was calculated from a standard curve prepared against L-Proline (5 to 30 µg, from MERCK KGaA):

$y = 0.059x – 0.014, R^2 = 0.99$.

The content of total soluble carbohydrates in the studied samples was determined according to Mc Cready et al. (1950) and Staub (1963), using glucose as a standard. Twenty-five milligram (DW) leaf samples was homogenized with 5 ml methanol 80% and boiled while shaking at 70°C for 30 min. The homogenate was centrifuged for 15 min at 6,000 g. After decanting, the residue was resuspended in 5 ml of the extraction solution and centrifuged at 6,000 g for 10 min. The supernatant was decanted and combined with the original extract. For measurement of total soluble carbohydrates, anthrone–sulfuric acid assay was used. An aliquot of 250 µl was added to 5 ml of anthrone– sulfuric acid solution. The mixture was shaken, heated in a boiling water-bath for 10 min and cooled at 4°C. The absorption was determined by spectrophotometry (Anthelie Advanced 2, SECOMAN) at 640 nm. A standard curve was prepared using different concentrations of glucose (0 to 100 µg, from MERCK KGaA). From the standard curve, the concentrations of soluble carbohydrates in the test samples were calculated ($y = 0.0095x – 0.0299, R^2 = 0.979$).

Statistical analysis

Standard analysis of variance was done (AV1W procedure of MSUSTAT 4.12, Richard E. Lund, Montana State University (Lund, 1989).

RESULTS

Growth

As shown in Figure 1, under 100% FC, Sardi 10, Tamantit and Rich 2 are the most productive cultivars and Mamuntanas the less productive one. The other cultivars occupied the intermediate position. Under water deficit stress, biomass production was significantly decreased in all cultivars. However, a large variability was observed. Shoot DW was reduced by 55% in Tamantit and up to 75% in Ecotipo Siciliano. This DW reduction was ranged between 64 and 70% in the other cultivars. It is important to note that upon water deficit stress, Tamantit, which showed the lowest DW reduction compared to its control, exhibited the highest biomass production under these conditions (2.5 fold biomass when compared to Ecotipo Siciliano).

Relative water content and osmotic potential

The analysis of the water relations (Figure 2A) showed that relative water content was ranged between 30 and 40% for plants submitted to non limiting water supply and between 10 and 23% for water deficit stressed ones. Thus, a significant decrease was observed in RWC values in all cultivars when submitted to water deficit stress. Tamantit, Sardi 10 and Ameristand, preserved also the highest RWC values in their leaves, whereas Ecotipo Siciliano showed the lowest RWC values in leaves.

Leaf osmotic potential decreased significantly in *M. sativa* cultivars subjected to water deficit stress (Figure 2B). However, the difference between the values of this parameter in plants subjected to the water deficit stress compared to their respective control is more important in Tamantit (reduction by 70%) than in Magali, Ameristand and Sardi (53%) and particularly Ecotipo Siciliano (38%).

Dry matter partitioning between organs

The root /shoot ratio assessed on the basis of dry matter did not exceed 1.6 for well watered plants. However, it increased significantly in all the cultivars subjected to water deficit stress (Figure 3). A large variability was observed. Tamantit showed significantly the highest value of this ratio and Ecotipo Siciliano exhibited the lowest root /shoot ratio value.

Proline accumulation

Plants submitted to 100% FC accumulated proline (Figure 4) at low levels not exceeding 0.8 µmol g^{-1} FW and no significant differences were observed between cultivars upon appropriate water supply. The water deficit stress led to a significant increase in leaf proline concentrations in all cultivars except Mamuntanas cultivars (Figure 4). A large variability was also observed at this criterion level.

The most tolerant cultivar identified on the basis of growth and water relation, Tamantit, is characterized by the highest proline concentrations, about 6 µmol g^{-1} FW. Indeed, this cultivar accumulates at least twice more proline that the sensitive one.

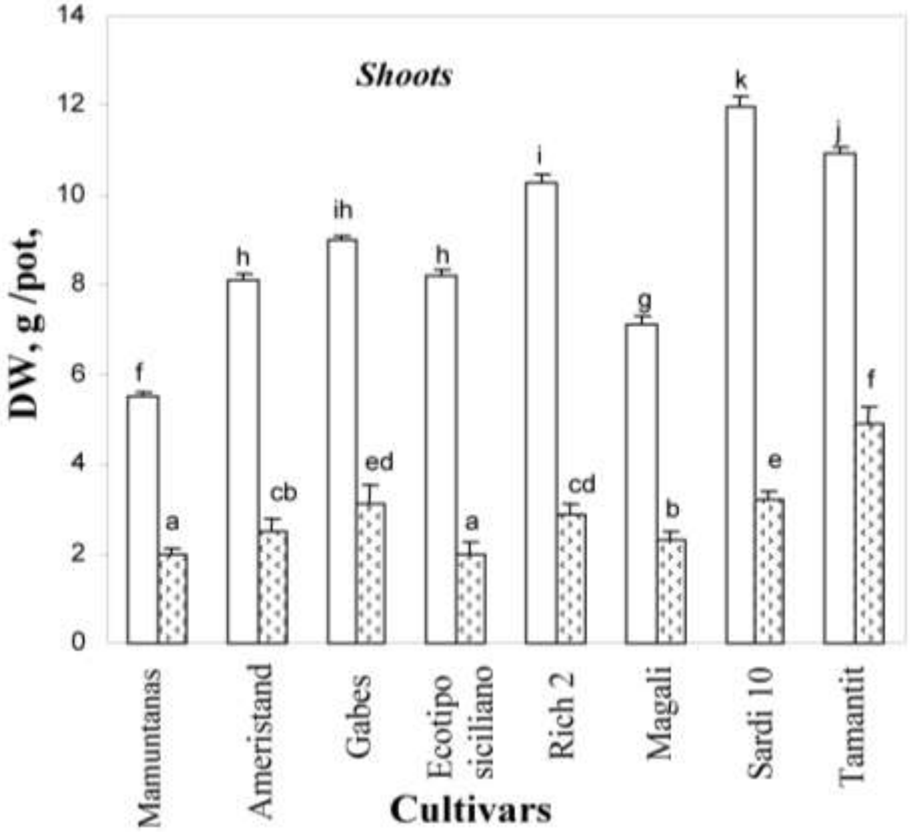

Figure 1. Changes in shoot dry matter production in eight cultivars of *Medicago sativa* during one month of treatment with appropriate water supply (open columns): 100% field capacity and 33% field capacity (dotted columns). Values are the means of ten replicates, vertical bars are SE. Values sharing a common latter are not significantly different at P<0.05%.

Carbohydrate content

The water deficit stress led to a significant increase in the soluble sugar leaf concentration in Rich 2, Sardi 10 and Tamantit. In other cultivars, no significant differences were observed between water deficit subjected plants and control ones (Figure 5). Tamantit water deficit stressed plants showed a substantial soluble sugars content increase when compared to control, followed by Sardi 10 and Rich 2.

MDA content and electrolyte leakage

In well watered plants, Ecotipo Siciliano exhibited higher values of leaf MDA content when compared to other cultivars (Figure 6A). Exposure of plants to water deficit stress increased significantly this parameter only in this cultivar which accumulated 21% more MDA in their leaves when compared to Sardi 10, Tamantit and Rich 2 upon water deficit stress conditions. As shown in Figure 6B, a large variability was observed under limiting or not limiting water supply in leaf electrolyte leakage. Indeed in control plants, this parameter was the highest in Tamantit. In stressed plants, this parameter increased significantly in Ecotipo Siciliano, Mumantanas, Ameristand and Rich 2. However, electrolyte leakage was not affected by water deficit stress in other cultivars.

DISCUSSION

Our results show that biomass production was reduced in all cultivars of *M. sativa* by water deficit stress. Despite this sensitivity, a large intraspecific variability is highlighted. Thus, contrary to Ecotipo sciciliano, Tamantit cultivar maintained a relative high biomass production upon water deficit stress and showed the lowest reduction when compared to its control (100% FC). Some morphological and physiological parameters are associated with this intraspecific variability.

Our results show an increase in root/shoot dry matter ratio, under water deficit stress conditions in all cultivars, a very great variability was observed. Indeed Tamantit and Magali showed the highest values in root/shoot dry matter ratio contrary to Ecotipo Siciliano and Ameristand.

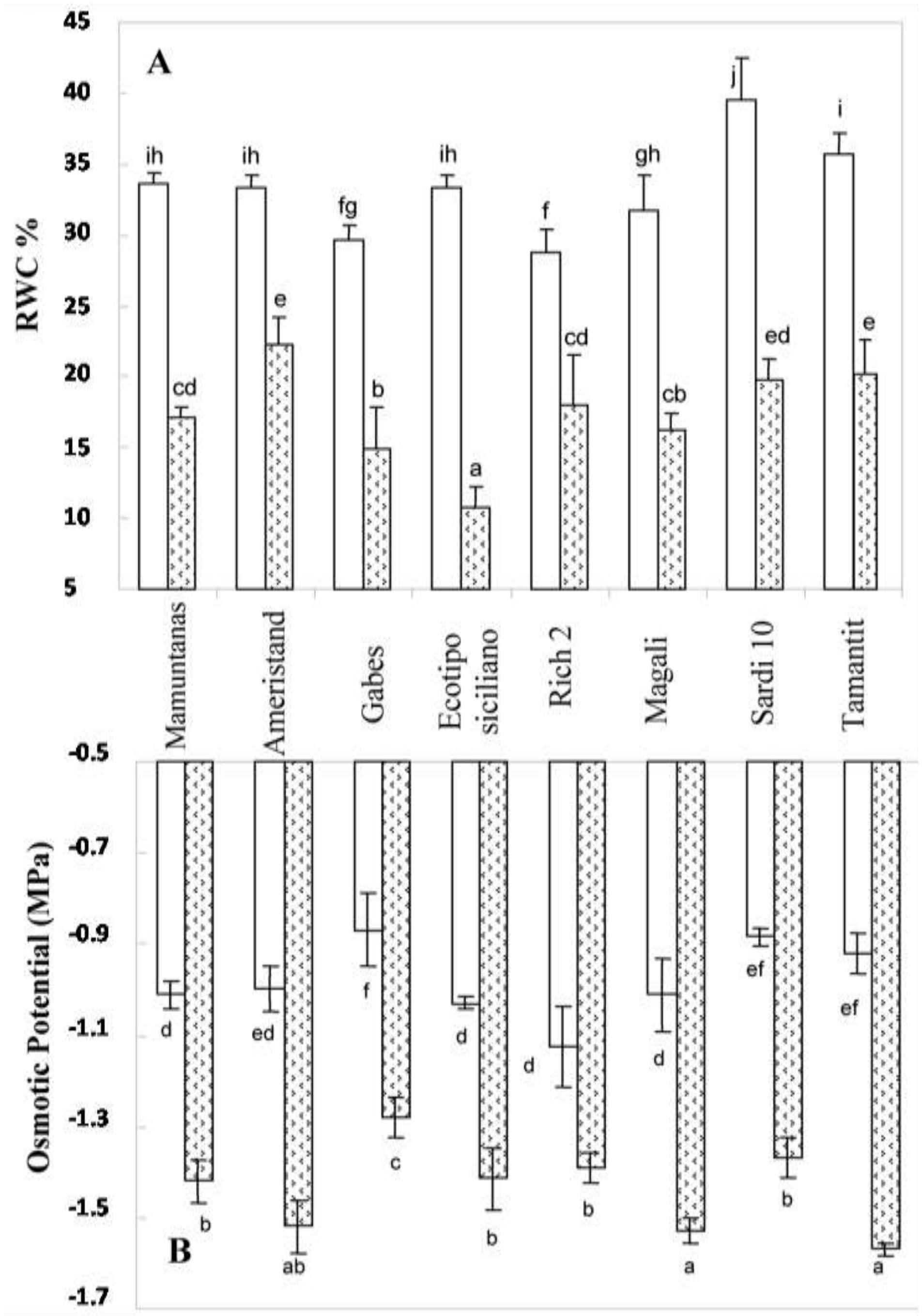

Figure 2. Changes in the leaf relative water content (A) and leaf osmotic potential (B) in eight cultivars of *Medicago sativa* during one month of treatment with appropriate water supply (open columns): 100% field capacity and 33% field capacity (dotted columns). Values are the means of ten replicates, vertical bars are SE. Values sharing a common latter are not significantly different at P<0.05%.

This morphological plasticity which resulted in a preferential development of the roots is often concomitant with the tolerance to water deficit stress (Slama et al., 2006; Yousfi et al., 2010). One of the well known mechanisms reported by Serraj and Sinclair (2002) for beneficial crop yield responses to water deficit stress was the maintenance of root development in order to reach water that may be available in the deeper soil profiles. Hydraulic and chemical signals sent from drying roots to the shoot are involved in the regulation of these responses. Abscisic acid (ABA) plays a central role in the protection of plants from various environmental stresses.

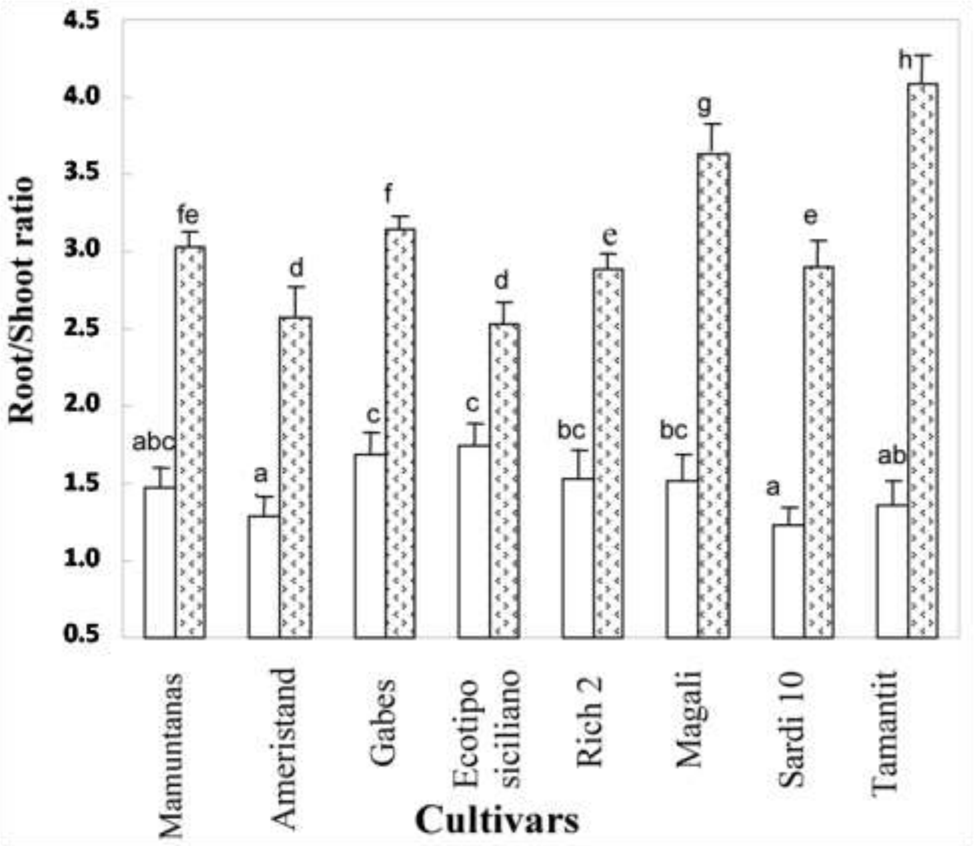

Figure 3. Changes in Root / Shoot DW ratio in eight cultivars of *Medicago sativa* during one month of treatment with appropriate water supply (open columns): 100% field capacity and 33% field capacity (dotted columns). Values are the means of ten replicates, vertical bars are SE. Values sharing a common latter are not significantly different at P<0.05%.

Cellular ABA levels are precisely controlled to elicit adaptive responses in reaction to a changing environment (Chaves and al., 2010).

Our results show that tissue water status was also significantly impaired by water deficit stress. Several studies have reported the decrease of RWC under severe water deficit stress conditions (Mohsenzadeh et al., 2006; Slama et al., 2008; Nunes et al., 2008; Yousfi et al., 2010; Gorai et al., 2010). Contrary to severe stress conditions, mild drought has no effects on RWC (Nunes et al., 2008). In addition to the severity of stress, plant response to water deficit stress was variety- dependent. Indeed it has been reported that osmotic stress induced by PEG had no effects on RWC in tolerant *Phaseolus acutifolius,* while RWC in sensitive *P. vulgaris* decreased (Türken et al., 2005). Our results show considerable variation for drought tolerance among the cultivars, Tamantit and Sardi 10 preserved the highest RWC values when compared to other cultivars, suggesting the ability of these cultivars to avoid relatively tissue dehydration as consequence of osmotic adjustment. Indeed, leaf osmotic potential decreased in all cultivars submitted to water deficit stress. At the end of the drought treatment, Tamantit, Ameristand and Magali water-stressed plants exhibited significantly the lowest leaf osmotic potential when compared to other cultivars. Thus, El Jaafari (1993) showed that the osmotic adjustment is a criterion of selection to characterize the tolerant varieties of durum to water deficit stress. This adaptive mechanism includes traits, which promote the maintenance of high tissue water content, as well as those for promoting tolerance to low water availability (Moinuddin et al., 2005; Cattivelli et al., 2008; Chaves et al., 2010). In order to keep osmotic balance, specific types of organic molecules (such as proline) are accumulated in the cytoplasm. These compounds are termed compatible solutes, because they can be accumulated in high concentrations without perturbing the normal physiological functions (Safarnejad et al., 1996). Our results show an increase in leaf soluble sugars under water deficit stress with large variability. The superiority in leaf soluble sugar accumulation was observed in Tamantit, the most tolerant cultivar. Therefore, soluble sugars could be implied in osmotic adjustment in *M. sativa* cultivars submitted to water deficit stress. Our results are in agreement with other data

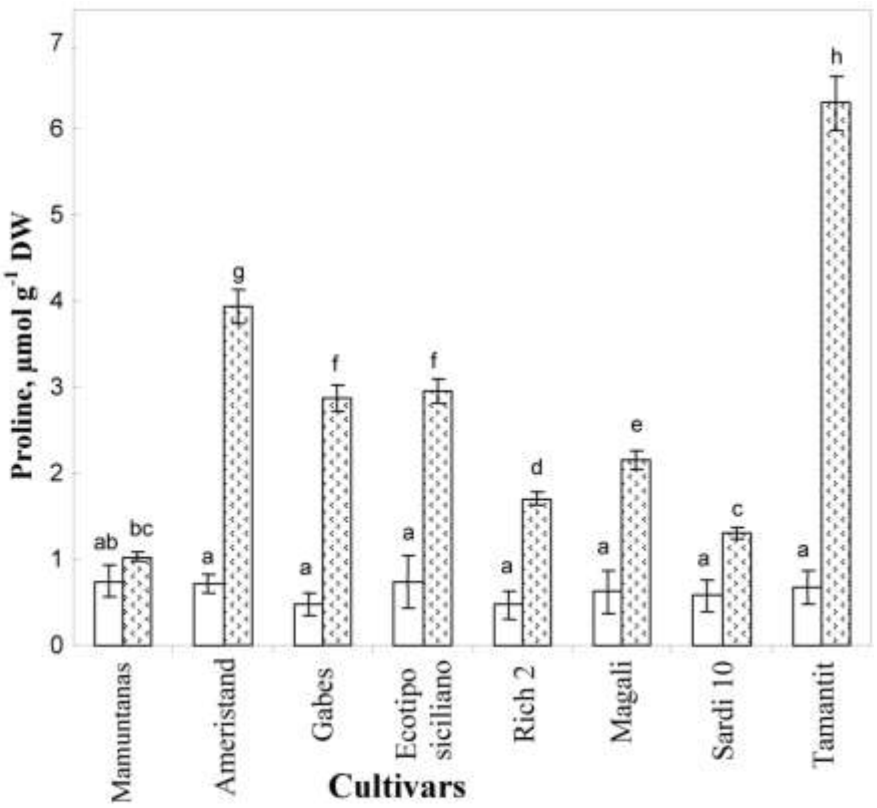

Figure 4. Changes in leaf proline concentrations in eight cultivars of *Medicago sativa* during one month of treatment with appropriate water supply (open columns): 100% field capacity and 33% field capacity (dotted columns). Values are the means of ten replicates, vertical bars are SE. Values sharing a common latter are not significantly different at P<0.05%.

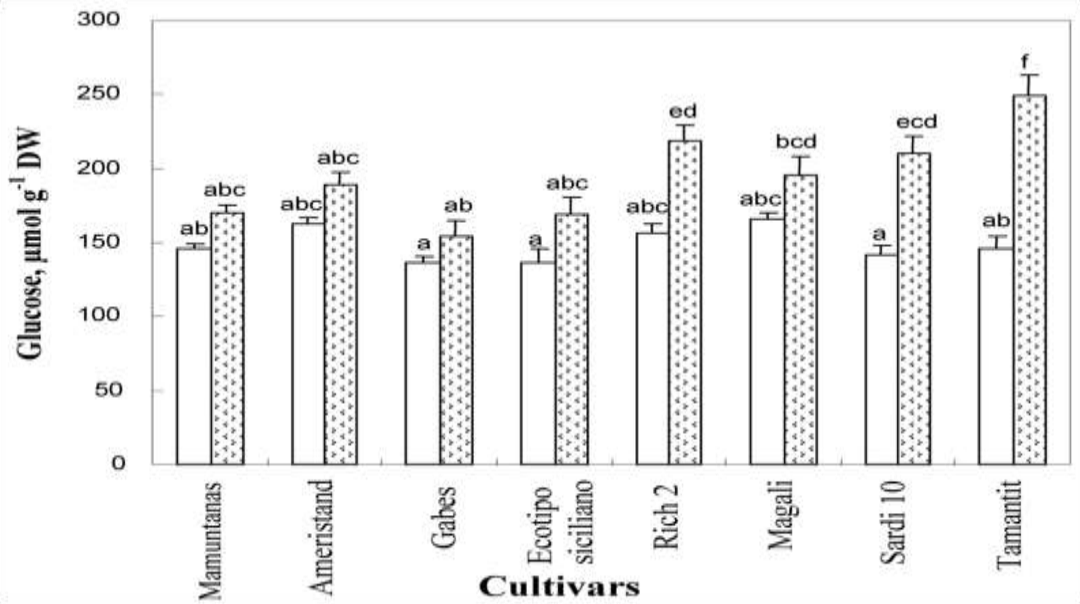

Figure 5. Changes in leaf soluble sugar concentrations in eight cultivars of *Medicago sativa* during one month of treatment with appropriate water supply (open columns): 100% field capacity and 33% field capacity (dotted columns). Values are the means of ten replicates, vertical bars are SE. Values sharing a common latter are not significantly different at P<0.05%.

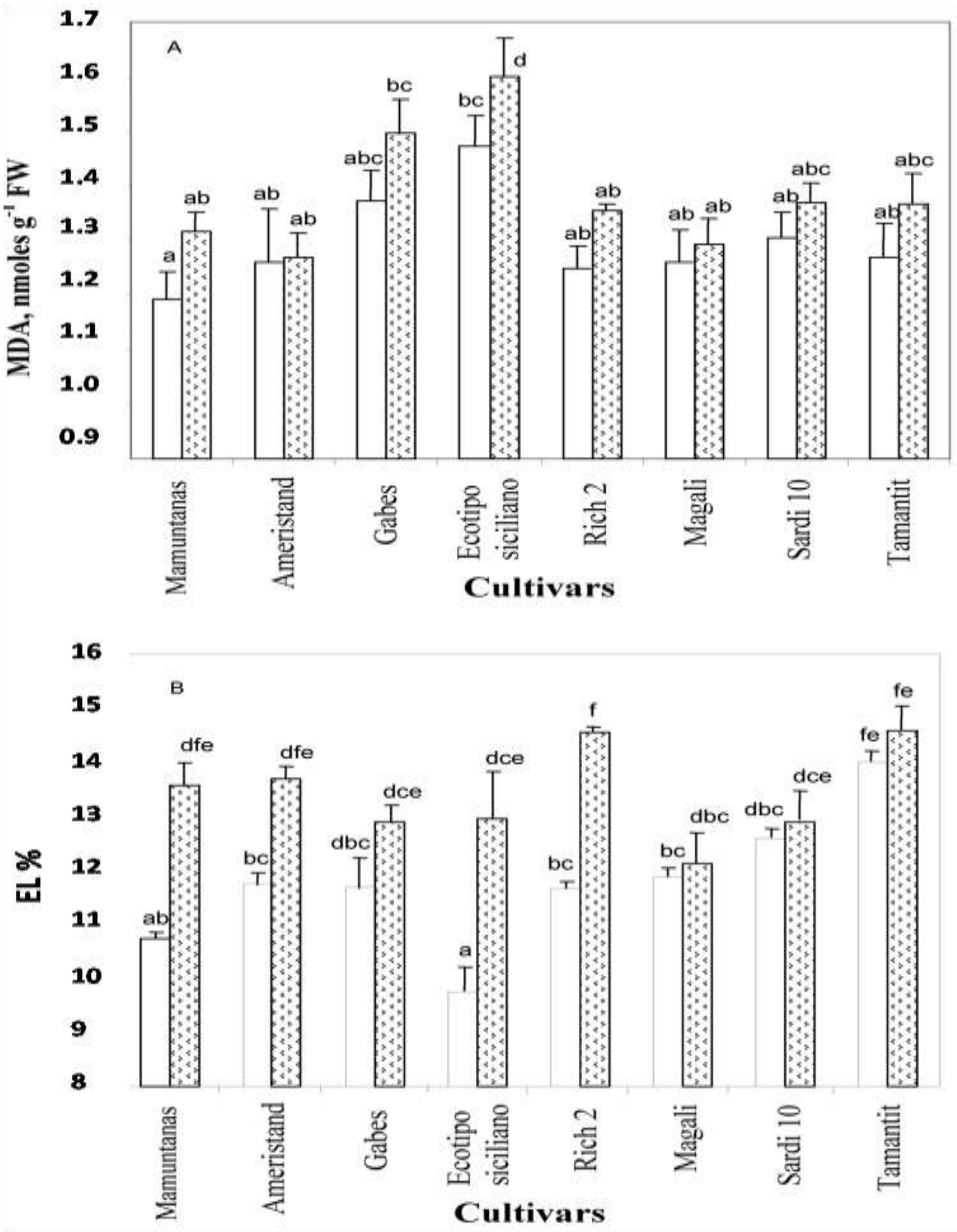

Figure 6. Changes in leaf MDA (malondialdehyde) concentrations (A) and electrolyte leakage (B) in eight cultivars of *Medicago sativa* during one month of treatment with appropriate water supply (open columns): 100% field capacity and 33% field capacity (dotted columns). Values are the means of ten replicates, vertical bars are SE. Values sharing a common latter are not significantly different at P<0.05%.

considering that the increase in the concentration of leaf soluble sugars is a criterion of adaptation to drought (Kameli and Losel, 1995) In addition, it is reported that the sucrose is known as key factor intervening in the stabilization of the membrane and the molecules (Hoekstra and Golovina, 1999). Under water deficit stress, sugars particularly the hydroxyl group replace water associated with the membrane macromolecules and thus, allow the maintenance of the structural integrity of soluble proteins (Chaves et al., 2003). It has been reported that carbohydrate metabolism is very sensitive to changes in plant water status (Penhiro et al., 2001).

Moreover, we found that Tamantit cultivar, the most tolerant cultivar on the basis of growth and water relations, showed the highest concentrations in proline. In the same context, it has been observed that there is higher proline content in drought-tolerant Sorghum as well as in *Phaseolus* species than in sensitive ones (Türken et al., 2005; Zaifnejad et al., 1997). Vendruscolo et al. (2007) showed also that proline concentration reached a high value under water deficit stress in *Durum* plants. The PEG-induced water deficit stress increased proline accumulation in alfalfa particularly in osmotic tolerant accession (Yazdi) suggesting a positive correlation between proline accumulation and osmotic stress tolerance. Proline accumulation differs between cultivars adapted to certain growth conditions or regions, as well as within species more or less tolerant to drought (Heuer, 1994). In maize primary root, for example, the proline level increased as much as a hundred fold under a low water potential (Voetberg and Sharp, 1991). The free proline level also increased from 4 to 40 times in pea in response to water deficit stress (Francisco et al., 1998). A positive correlation between proline accumulation and osmotic stress tolerance has been often established (Ashraf and foolad, 2007). The establishment of the kinetics of proline accumulation and enzyme (δ-OAT, PDH) activities showed that the increase of proline biosynthesis in plants subjected to water deficit was concomitant with a stimulation of the first enzyme and an inhibition of the second one (Slama et al., 2006). The accumulation of the proline is one of the adaptive responses frequently observed at the plants to limit the effects of drought. Proline accumulation under stress protects the cell by balancing the osmotic strength of cytosol with that of vacuole and external environment. In addition to its role as cytosolic osmotica, it may interact with cellular macromolecules such as enzymes and stabilize the structure and function of such macromolecules. Thus, this compound is often proposed as relevant tool for selection of plant species and varieties tolerant to the osmotic constraints (Ashraf and Foolad, 2007).

Water deficit stress induced the generation of reactive oxygen species (ROS) (Shao et al., 2005; Shao, 2008). It is now widely accepted that these cytotoxic ROS are responsible for various stress-induced damages to macromolecules mainly lipid membrane peroxidation which could be determined by measuring malondialdehyde (MDA). The high accumulation of MDA is often concomitant with water deficit stress sensitivity. The increase in lipid peroxidation observed in the leaves of Ecotipo sciciliano could be resulted from the ROS accumulation. The minor MDA variation observed in some *Medicago* cultivars suggests a better protection from oxidative damage. Electrolyte leakage following cellular membrane degradation indicated permeability degree and membrane stability (Prasil and Zambien, 1998). The examination of the variation of electrolyte leakage variations showed that in spite of the high value of this parameter, Tamantit cultivar showed no significant differences when compared to control one. It is important to note that the cultivar showing the highest levels of proline is the least affected by the oxidative stress attributing an antioxidant feature to proline, suggesting ROS scavenging activity and proline acting as a singlet oxygen quencher (Szabados and savoué, 2009).

In conclusion, variability in response to water deficit stress occurred among alfalfa seedlings. The root preferential development, the better ability to accumulate proline and soluble sugars, and the aptitude to protect photosynthetic apparatus against the oxidative stress are the main criteria that are associated with the relative tolerance of some cultivars.

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