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Different Morphological, Physiological and Biochemical Responses to Drought Stress in Cutleaf Medic (*Medicago laciniata* (L.) Mill)

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Abstract: A number of morphological, physiological and biochemical characteristics are proposed as selection criteria for drought tolerance in plants. Shoot and root dry weight, plant height, leaf area, water relationships, organic and inorganic solute accumulation and osmotic adjustment mechanism were studied in two ecotypes of Cutleaf medic (*Medicago laciniata* (L.) Mill) with different drought tolerance levels to different water stress levels. Two ecotypes 50 days after sowing were treated in four levels of water stress included -0.1, -0.2, -1 MPa as low, medium and high stress levels respectively, as well as normal condition (FC = -0.03 MPa) for 10 days. The tolerant ecotype in lower soil water potential was able to produce a higher shoot DW, root DW, shoot / root ratio (Sr) and leaf area (LA) and it exhibited better osmotic adjustment (OA) together with higher relative water content (RWC), while its leaf water potential (ψ_W) and leaf osmotic potential (ψ_S) was lower than the sensitive ecotype. This experiment showed that this plant species utilized K^+ as its main osmolyte for OA, while other osmolytes acted as osmoprotectants. However, the results suggest that tolerant ecotype used different morphological, physiological and biochemical characteristics to cope with prolonged drought stress.

Abbreviations: FC: Field Capacity; MPa: Mega Pascal; DW: Dry Weight; Sr: Shoot / root ratio; LA: Leaf Area, Ht: Height; OA: Osmotic Adjustment, RWC: Relative Water Content; ψ_W: Water potential; ψ_S: Osmotic potential; K^+: Potassium; Ca^{2+}: Calcium; Mg^{2+}: Magnesium; Zn^{2+}: Zinc;

Key words: Cutleaf medic · Drought stress · Water relations · Osmotic adjustment · Organic and inorganic solute

INTRODUCTION

Medicago laciniata (L.) Miller (cutleaf medic) is widely distributed throughout the semi-arid southern and southwestern provinces of Iran with an annual rainfall, which occurs during winter and early spring, lower than 200 mm. Cutleaf medic occurs mainly on sandy-surfaced red earths in a wide variety of vegetation communities [1]. The species is thought to be native to the North African countries that border the Mediterranean Sea. Its natural habitat is dry sandy or stony desert environments, where it is often the only *Medicago* species that survives [2]. Badri *et al.* [3] suggested that it was a most promising species for range improvement in the subdesert regions of northwestern Tunisia. Ghanavati *et al.* [4] collected 32 accessions of cutleaf medics from 12 provinces of southern and southwestern and western provinces of Iran. Moradi [5] reported that, among the 32 collected accessions, the most tolerant ecotype belonged to the Bosheher province (south of Iran) and the most sensitive ecotype belonged to the Lorestan province (west of Iran), with average annual rainfalls of 173 mm and 530 mm, respectively. Young *et al.* [1] reported that dry matter and seed production are usually less than that of *Medicago truncatula* Gaertner under ideal conditions and *M. laciniata* yields may be greater when the season is short, which is a very common condition in the southern provinces of Iran.

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One of the most important factors limiting the growth, development, productivity and dispersion of plants in the biosphere is desiccation induced by decreasing environmental water potential (ψ_W) [6]. Drought is one of the main causes of crop yield reduction for most agricultural regions throughout the world [7]. Thus, it is important to study the mechanisms of drought response in tolerant ecotypes of *M. laciniata*.

In general, the leaf water deficit that develops as a consequence of soil water depletion affects many physiological processes, with eventual consequences for biomass and seed yield. Conservation of high relative water content (RWC) during water stress conditions is usually well correlated with biomass production and grain yield [8].

Many of these changes represent adaptive responses by which plants cope with water stress. The mechanisms that developed as survival strategies include tolerance and avoidance of tissue water stress [9]. Generally, stress avoidance involves stomatal closure, hydraulic conductance and root growth patterns. Stress tolerance usually includes osmotic adjustment (OA) and changes in tissue elasticity [10]. In particular, OA, which is the lowering of osmotic potential by net solute accumulation in response to dehydration, assists the maintenance of turgor at lower water potentials and has been considered a beneficial drought tolerance mechanism during both vegetative and reproductive phases of plant growth [11]. In fact, many physiological processes, such as cell expansion, photosynthesis, gas exchange and enzyme activity are dependent on cell turgor. Turgor maintenance by OA is an important physiological adaptation for minimizing the detrimental effects of water deficits [12]. Conservation of RWC may be attained through OA, more efficient soil water extraction by roots (increase in root length), or reduced transpiration. Selecting for this last characteristic is of limited interest since it implies a reduction in stomatal conductance and leaf area, which would negatively affect the carbon balance [9].

Water stress causes a wide array of biochemical and physiological changes, beginning with a decrease in osmotic potential (ψ_S) at the cellular level [13]. Osmotic adjustment is the decrease in ψ_S by the active accumulation of organic as well as inorganic solutes within the cells. High concentrations of inorganic ions become detrimental to cellular metabolism and must be sequestered in the vacuole. In order to maintain osmotic balance, specific types of organic molecules (such as soluble sugars, betaines, polyols, proline, etc.) accumulate in the cytoplasm. These compounds are termed compatible solutes because they can be accumulated at high concentrations without impairing normal physiological functions. Typically, compatible solutes are low molecular weight compounds that are polar, highly soluble and have a larger hydration shell than denaturing molecules but that are not highly charged [14]. The accumulation of compatible solutes is achieved in different ways depending on the type of molecule. For example, the increase in soluble sugars in response to water stress can be attributed to decreased translocation from the leaf, slower consumption due to decreased growth and other changes such as starch hydrolysis [15].

As reported by Morgan [14] a number of osmotically active substances, both organic and inorganic solutes, play a role in the osmotic adjustment phenomenon. However, conflicting results have emerged regarding the nature and quantitative contribution of solutes and ions such as sugars, amino acids and potassium, mainly because of the methods used for the stress induction.

Osmoregulation and the role of osmolytes in the physiology of stressed plants have been investigated in a number of crop species [16, 17]. Because there are large differences among and within species in the degree of adaptation to water deficit, it is important to investigate the metabolic changes involved. In fact, knowledge of plant adaptive strategies to water stress and their physiological basis can aid in formulating plant breeding and management strategies adapted to semi-arid environmental conditions.

Plants subjected to water deficit stress accumulate proline in their cells, resulting in decreased ψ_S. This reduction in ψ_S and consequent maintenance of water absorption and cell pressure potential (ψ_P), might contribute to the maintenance of physiological processes such as stomatal opening, photosynthesis and expansion growth [18]. In addition, proline might be involved in the protection of cellular structures against oxidative damage by scavenging free radicals [19] and serving as a carbon and nitrogen reserve for growth after stress relief [20]. Proline biosynthesis may also be associated with the regulation of cytosolic pH or production of $NADP^+$ for stimulation of pentose phosphate pathway [20]. The accumulation of proline represents a general response to stress in many organisms, including higher plants, exposed to environmental stresses such as water deficit, high salinity, high temperature, freezing, UV radiation and heavy metals [21]. The beneficial role of proline during plant stress tolerance was suggested by earlier related studies demonstrating that proline could increase the tolerance of plants to abiotic stress [22]. However, in these studies using transgenic plants overproducing proline [23], the magnitude of the increase in proline was

too low to be significant for overall OA, despite the possibility that there might be higher levels of proline in specific cell types or subcellular compartments [24]. Soluble sugars [25] and proline [26] have repeatedly been shown to increase under water stress and they are potentially important contributors to OA.

Changes in potassium may contribute substantially to osmoregulation [27] and may occur in concert with changes in sugars and amino acids [28]. In some cases, however, changes in sugars, amino acids, or organic acids are not accompanied by changes in potassium [12].

In the present work, we evaluated the effect of water deficit on water relationships, osmotic adjustment and the role of osmolytes in drought stress tolerance in Iranian cutleaf medics (*Medicago laciniata* (L.) Mill).

MATERIALS AND METHODS

Plant Material and Growth Conditions: Certified ecotypes of cutleaf medic (*Medicago laciniata* (L.) Miller), 'drought tolerant' and 'drought sensitive', were kind gifts from National Plant Gene Bank of Iran. The two ecotypes were classified as drought tolerant or sensitive according to the water stress period. About 500 seeds per ecotype were surface sterilized for 30 s in 97% ethanol (v/v), followed by treatment with 0.8% (v/v) formaldehyde for 40 min and 5% (w/v) calcium hypochlorite for 20 min and rinsing three times with sterile deionized water. Seeds were germinated on two layers of Whatman no. 41 filter paper moistened with 10 ml of sterile deionized water in the dark at 25°C. After 24 h, the seeds were transferred to a mixture of compost and sand (1/1) in a growth chamber for 7 days at 25/20°C (day/night) with a photoperiod of 16 h. Illumination was provided by Sylvania fluorescent tubes (F36 W/ 133-T8/CW) at a photon flux density of 1000-1500 μmol m^{-2} S^{-1}. Seven-day-old seedlings of uniform size were transferred and acclimated in a greenhouse at 25/20°C (day/night) under a photoperiod of 8 h consisting of natural daylight supplemented with Philips mercuric lamps (HPLN 400 W) in order to reach a minimum photon flux density of about 750 μmol m^{-2} S^{-1}. Daytime humidity was about 50%. Twenty days after sowing, the young seedlings were individually transferred to polyethylene pots (15×10 cm^2), each containing 1.7 kg of dry sandy loam soil (70 % sand, 12 % silt and 18 % clay) and gravimetric water content at a field capacity of 19.7%. The soil surface was covered with a 2 cm gravel layer to avoid water loss by evaporation. No water restrictions were applied to the plants for about 40 days after sowing. During this period, the plants were watered with Hoagland nutrient solution once a week. Subsequently, two ecotypes were randomly distributed within four groups that received different water treatments (15 pots per treatment). After 10 days, at which time the soil water potential reached -0.03, -0.1, -0.2 and -1 MPa, sampling was performed.

Soil Water Content and Water Stress Treatment: Water stress treatments included -0.1, -0.2 and -1 MPa soil water potentials as low, medium and high stress levels, respectively, as well as a control condition (-0.03 MPa). The water stress regimens were applied 50 days after sowing (the usual time of water deficit in the real habitat) by weighing each pot and adding water to the weight calculated for the desired water regimen [29]. Soil gravimetric water content was defined as θ = Ww / DWs ×100 where Ww is the weight of the water contained in a soil sample and DW is the dry weight of the sample [30] and the pots were weighed twice a day (0800 and 1600 hours). Water loss was replaced by top watering. Evaporation from the soil surface and transpiration water loss, as well as increases in plant weight was estimated by weight differences between pots without plants and the experimental pots.

Plant Growth, Morphological Traits: Morphological traits were estimated by measuring the plant heights in 1, 4, 7 and 10 days after imposing water stress. Leaf area (LA) per plant was measured with leaf area meter (LICOR, model LI3050A, USA). Plant growth was also measured on the basis of shoot dry weight (shoot DW) and root dry weight (root DW) per plant. Shoot and root DW (after 48 h in an oven at 80°C) were determined using 6 plants per treatment.

Leaf Water Relationships, Osmotic Adjustment: Leaf water potential (ψ_w) was evaluated immediately after sampling using the pressure chamber method [15]. Relative water content (RWC) was estimated using the following formula:

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

where FW= weight of freshly collected material, TW= weight after rehydration for 20–24 h at 4°C in the dark and DW= weight after drying at 80°C for 72h. Both ψw and RWC were determined for three plants per replication.

For osmotic potential (ψ_S), leaves were quickly collected, cut into small segments, placed in eppendorf tubes (one leaf per tube) perforated with four narrow holes and immediately stored at - 20°C. The frozen samples were then allowed to thaw for 30 min. Each

eppendorf tube was then encased in a second intact tube and centrifuged at 13000*g* for 15 min. The osmolarity (C) of the collected sap was assessed for a given leaf of the different plants using a vapor pressure osmometer (Wescor 5520, USA) and converted from mosmol $kg^{-1}$to MPa using the formula: ψ_s (MPa)= - C (mosmol kg^{-1})× 2.58×10^{-3} according to the Van't Hoff equation.

To measure osmotic potential at full turgor (ψ_S^{100}), leaves of stressed and control plants were rehydrated in demonized water for 24 h at 4°C in the dark. Turgor pressure (ψ_P) was calculated as $\psi_P = \psi_W - \psi_S$ [15]. Total OA (OA^{tot}) was calculated as the difference in osmotic potential at full turgor between the control ($\psi_{S\ C}^{100}$) and water stress ($\psi_{S\ S}^{100}$) treatments for each population [31]: $OA^{tot} = \psi_{S\ C}^{100} - \psi_{S\ S}^{100}$.

Organic Solute Analysis: Proline content was estimated by the method of [32]. Briefly, the plant material was homogenized in 3% aqueous sulfosalicylic acid and the homogenate was centrifuged at 10000 rpms. The supernatant was used for estimation of proline content. The reaction mixture consisted of 2 ml of supernatant, 2 ml of acid ninhydrin and 2 ml of glacial acetic acid and it was boiled at 100 °C for 1 h. After termination of the reaction in an ice bath, the reaction mixture was extracted with 4 ml of toluene and the absorbance was read at 520 nm [33].

Soluble sugars were extracted in 80% ethanol from 1 g of fresh leaf tissue. After centrifugation for 10 min at 8000g, the pellet and supernatant were stored for analysis. Total soluble sugar content was determined in the leaves of five plants per replication by the classical anthrone method [34] using a spectrophotometer (Varian-Cary 300, Australia).

Leaf soluble protein concentrations were determined by the Bradford method [35]. Since water stress had a significant effect on the RWC of the plants, proline and sugar contents were adjusted to the RWC of unstressed plants (Z) according to X × Y/Z, where X is the solute content and Y is the RWC of the stressed plants [30].

Inorganic Solute Analysis: Inorganic solutes, including Ca^{2+}, Mg^{2+} and Zn^{2+}, were determined in mature and fully expanded leaves by atomic absorption spectroscopy using a spectrophotometer (Perkin-Elmer model 3110, USA). Sample leaves (~500 mg FW) were dried at 70°C for 3 d and digested in a 3:1 nitroperchloric mixture (nitric acid 70%: perchloric acid 70%) by gradual warming to 250°C until a clear extract was obtained, which was then diluted in water and read. Potassium (K^+) was estimated flame photometerically (Corning 410, USA) based on [36].

Statistical Analysis: The experiment was conducted using a factorial arrangement in Randomize Complete Block Design with three replications. Statistical analysis of the data was carried out using a SAS (Ver. 9.2) package program and data were subjected to analysis of variance. The mean values were compared using Least Significant Different (LSD) test and the differences were considered significant at $p \leq 0.05$. All data presented are means ± SE.

RESULTS AND DISCUSSION

Drought had a significant effect on the growth and development of treated plants. Changes in plant growth and structure in response to progressive drought stress exhibited the primary responses to drought adaptation.

Table 1: Shoot dry weight, root dry weight, shoot to root ratio and leaf area of two contrasting cutleaf medic genotypes in different soil water potentials 10 days after exposure to the treatments

Soil water potential (Mpa)	Shoot dry weight (g)		Root dry weight (g)		Shoot / Root Ratio		Leaf Area (cm^2)	
	Sensitive	Tolerant	Sensitive	Tolerant	Sensitive	Tolerant	Sensitive	Tolerant
-0.03	1.05±0.02	0.94±0.02	0.13±0.01	0.13±0.01	7.51±0.33	7.34±0.05	160±1.7	154±2.8
-0.1	0.63±0.01	0.83±0.02	0.12±0.01	0.13±0.02	5.34±0.22	6.23±0.21	105±4.5	118±1.4
-0.2	0.51±0.02	0.75±0.01	0.11±0.02	0.12±0.02	4.48±0.23	5.91±0.24	69±2.3	115±1.3
-1	0.48±0.01	0.64±0.04	0.10±0.01	0.11±0.01	4.84±0.07	5.5±0.32	53±1.7	97±2.8
Mean	0.67	0.79	0.11	0.12	5.54	6.24	97	121
ANOVA (main effects)								
Stress (S)	***		***		***		***	
Genotype (G)	**		***		***		***	
S*G	*		***		*		***	
LSD 0.05								
Stress (S)	0.008		0.048		0.048		5.53	
Genotype (G)	0.006		0.034		0.34		3.91	
S*G	0.071		0.011		0.772		8.68	
C.V	5.5		5.3		6.6		4.1	

Data indicate means ± SE of three replications.
, * significant at P < 0.01 and 0.001, respectively. ns = non significant.
LSD least significant difference at P ≤ 0.05.

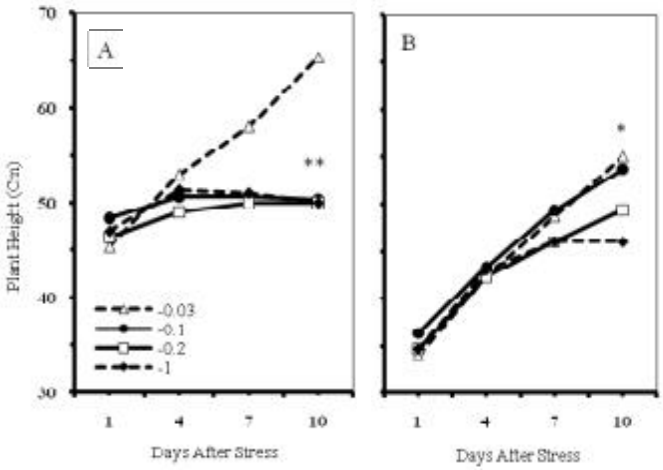

Fig. 1: Shoot height (Cm) of sensitive (A) and tolerant (B) ecotypes of cutleaf medic under different soil water potentials (-0.03, -0.1, -0.2 and -1 MPa). Measurements performed during 1, 4, 7 and 10 days after imposing water stress. * and ** indicate significance P=0.05 and 0.01, respectively, at day 10 using LSD multiple range comparison tests

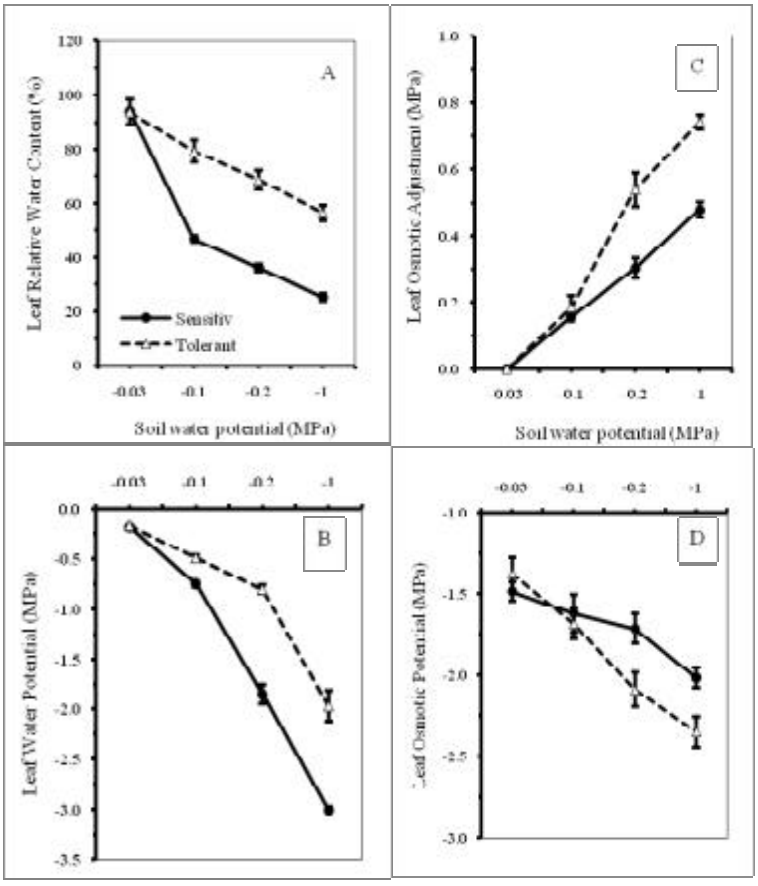

Fig. 2: Leaf relative water content (RWC) (A), Leaf water potential (B), Leaf osmotic adjustment (C) and Leaf osmotic potential (D) of two cutleaf medic ecotypes in different soil water potentials (-0.03, -0.1, -0.2 and -1 MPa). Vertical bars indicate ± SE

Table 2: K^+, Ca^{2+}, Mg^{2+}, Zn^{2+} as inorganic solutes and organic solutes including proline, total sugar and soluble protein concentration in two contrasting cutleaf medic genotypes leaves under different soli water potentials

	Inorganic solutes								Organic solutes					
Soil water potential	K^+ Concentration		Ca^{2+} Concentration		Mg^{2+} Concentration		Zn^{2+} Concentration		ProlineConcentration		Total Sugar Concentration		Soluble Protein Concentration	
	mmol kg^{-1} d.wt								mmol kg^{-1} d.wt				mg g^{-1} f. wt	
(Mpa)	Sensitive	Tolerant	Sensitive	Tolerant	Sensitive	Tolerant	Sensitive	Tolerant	Sensitive	Tolerant	Sensitive	Tolerant	Sensitive	Tolerant
-0.03	730±46	7714±44	3319±2	3320±3	1115±3	1121±1	11.3±0.08	11.4±0.09	227±0.9	226±0.8	5511±19	5532±13	99.1±0.18	99.0±0.19
-0.1	7745±41	8851±33	3337±3	3361±6	1119±1	1127±2	11.2±0.07	11.3±0.03	2238±21	4401±11	5530±16	9929±12	11.5±0.03	44.1±0.08
-0.2	8820±17	9974±87	3355±4	4406±8	1116±2	1135±3	00.7±0.06	00.9±0.03	1153±7	4412±19	5545±13	9927±13	11.2±0.02	33.1±0.06
-1	8873±58	11120±36	4405±4	4426±3	998±3	1133±3	00.5±0.07	00.8±0.06	1136±9	3363±26	4417±10	8816±18	00.6±0.01	22.1±0.04
Mean	7792	9915	3354	3378	1112	1129	00.9	11.1	1138	3301	5501	8801	33.1	44.5
ANOVA (main effects)														
Stress (S)		***		***		**		***		***		***		***
Genotype (G)		***		***		***		**		***		***		***
S*G		**		**		**		n.s.		***		***		***
LSD 0.05														
Stress (S)		58.6		11.2		5.4		0.14		34.6		29.8		0.17
Genotype (G)		41.5		7.9		3.8		0.1		24.4		21.09		0.12
S*G		97.9		16.9		9.01		0.24		45.1		54.5		0.28
C.V		5.5		2.4		3.6		11.1		12.7		3.7		3.6

Data indicate mean ± SE of three replications.

, * significant at P < 0.01 and 0.001, respectively. ns = non significant.

LSD least significant difference at P ≤ 0.05.

Two ecotypes of *M. laciniata* differed significantly in their morphological properties, i.e., shoot dry weight (shoot DW), root dry weight (root DW), shoot/root ratio (Sr), total leaf area (LA) (Table 1) and shoot height (Ht) (Figure 1), under the four watering regimens (soil water potentials). Ecotypes from the Lorestan province (sensitive ecotype, moderate climate) in the control condition (-0.03, MPa) had greater shoot DW, root DW, Sr and LA than the ecotype from the Bosherher province (tolerant ecotype, dry climate). The watering regimens significantly affected all of these morphological properties (P<0.01).

This experiment showed that, under control conditions (-0.03 MPa), leaf RWC, ψ_W, and ψ_S (Figures 2a, b and d, respectively) demonstrated no significant difference in both ecotypes after 10 days of exposure to water stress. However, in stressed plants, RWC, ψ_W and ψ_S declined markedly in both ecotypes (*P<0.01*), with a greater decline observed for the sensitive ecotype (Figures 2 a, b and d, respectively). The relative water contents of the tolerant ecotype in soil water potentials (-0.03, -0.1, -0.2 and -1.0 MPa) were about 95, 80, 75 and 65%, respectively, whereas they were correspondingly about 95, 43, 35 and 20 % in the sensitive ecotype. In the control treatment, the two ecotypes displayed similar values for leaf water potential (ψ_W= -0.02 MPa), but, with increasing water stress severity, ψ_W decreased in sensitive ecotype much faster than in the tolerant one (Figure 2b). Our results show that, in -0.1, -0.2 and -1.0 MPa, leaf ψ_W declined in the sensitive ecotype to about -0.08, -2.0 and -3.1 MPa, respectively. In the tolerant ecotype, the decline in water potential was much less dramatic about -0.06, -0.08 and -1.9 MPa, respectively (Figure 2b). In spite of the ψ_W and RWC, ψ_S in the tolerant ecotype decreased rather dramatically with increasing water stress intensity (Figure 2d). In the control treatment, there were no significant differences in ψ_S between the two ecotypes before starting the water stress, (1.40 in the tolerant and 1.50 MPa in the sensitive ecotype). Both ecotypes exhibited significant OA (*P<0.01*), with a greater increment in the tolerant ecotype. Our results indicate that OA began at -0.1 MPa (Figure 2c) and the most significant difference was observed when water stress reached -1.0 MPa (0.78 and 0.45 in the tolerant and sensitive ecotypes, respectively).

Inorganic solute (K^+, Ca^{2+}, Mg^{2+}, Zn^{2+}) concentrations for measuring osmolarity in leaf extracts from controls and those under different water stress levels are shown in Table 2. Under the control conditions, the contribution of these solutes was not significantly different in either ecotype. In the leaves of water stressed plants, the K^+ and Ca^{2+} concentrations generally significantly increased (P<0.01, Table 2). The Mg^{2+} concentration increased in the tolerant ecotype but decreased in the sensitive ecotype. Despite the other inorganic solutes, the amount of Zn^{2+} declined in the leaves of both ecotypes (Table 2).

Changes in the tolerant ecotype differed from those of the sensitive ecotype. For instance, the amount of K^+ in the tolerant ecotype in control conditions (0.03 MPa) compared to severe drought stress (1.0 MPa) increased 60% (714 to 1120 mmol kg^{-1} d. wt, respectively), while that of the sensitive ecotype increased only about 28.9% (730 to 873 mmol kg^{-1} d. wt, Table 2). Leaf Ca^{2+} content of tolerant and sensitive ecotypes increased 34.6% and 31.5%, respectively, in the same water levels. However, while the amount of Mg^{2+} in the intolerant ecotype decreased 2.1%, it increased 21.1% in the tolerant ecotype (Table 2). On the other hand, Zn^{2+} concentration in the leaves of both ecotypes declined, but the reduction was about 34.4% in the tolerant ecotype and 53.8% in the sensitive ecotype (Table 2).

In response to drought stress (from -0.03 to -0.01 MPa), leaf proline content increased rapidly in both ecotypes and then remained constant regardless of the stress level (Table 2). This sudden increase was very swift in the tolerant ecotype. The same trend was also observed for the concentration of soluble sugars (Table 2). Although the increase in soluble sugar content was remarkable in the tolerant ecotype, no significant change was observed for the sensitive ecotype. In contrast to proline and soluble sugar, a substantial reduction in soluble protein was observed for both ecotypes, although the decline was more distinctive for the sensitive ecotype (Table 2).

The existence of a large number of species and varieties of *Medicago* growing in many diverse habitats of Iran allows for the selection of species and seed sources for almost any environmental condition, including severe drought regions. Physiological adaptation, plant structural modifications and growth pattern adjustments are useful indices of the consequences of water deficit. Differences in drought adaptations among *M. lacinita* ecotypes were demonstrated and attributed to differences in morphological and physiological responses to water availability. Significant differences among ecotypes were found for shoot DW, root DW, Sr, LA (Table 1) and Ht (Figure 1) in response to four water regimens. The effect of drought stress and the interaction between drought and ecotypes were also highly significant with regard to measured physiological and morphological properties. Apparently, several adaptation strategies have evolved in the tolerant ecotype to cope with adverse environmental conditions. The sensitive ecotype demonstrated good performance only in well-watered conditions. Nonetheless, the dry climate ecotype (tolerant), which grows in habitats having a prolonged annual drought, grew better under water stress regimens and, regardless of the leaf osmotic potential, demonstrated higher values for the other measured parameters compared to the sensitive ecotype. All of these responses enabled the tolerant ecotype to overcome prolonged drought conditions. This study provided evidence that plant structural and growth adjustments as well as changes in OA are important mechanisms used by *M. laciniata* to cope with water limitations.

This experiment showed that the reduction in RWC occurs simultaneously with the reduction in shoot DW (Table 1 and Figure 1a). The tolerant cultivar was able to maintain its RWC in all water levels almost twice as well as sensitive the ecotype. It seems that a RWC of about 40% was lethal in this species, which reached this point in -0.1 MPa. Through the use of different strategies, the tolerant ecotype did not reach such a lethal point. Plants that develop low leaf ψ_W can sill partially maintain RWC, adjust osmotically to drought and experience a reduction in ψ_S in their leaf cells. The mechanisms responsible for leaf dehydration are poorly understood. One hypothesis is that a critical RWC exists that can be modulated by certain processes, such as leaf death. The RWC of plant tissue provides a measure of relative symplast (i.e., cell) volume and the plant may respond to changes in volume or, more likely, to changes in turgor pressure, but not dehydration [37]. Ludlow and Muchow [34] reported that the RWC for pigeon pea at which zero turgor occurs in about 80%, while an RWC of 32% could represent a leaf that has suffered a catastrophic irreversible inward collapse of cell wall resulting from a critical level of negative turgor pressure. An alternative hypothesis is that drought-induced leaf death is really a programmed leaf senescence caused by changes in hormonal signals coming from the roots, which are subjected to day-to-day decreases in soil moisture content [38]. In this hypothesis permitting osmotic to leave the cells would result in an adaptive recycling of nutrients from the senescing leaves. The reduction in osmotic potential within the cells would result in a loss of water, negative turgor and a collapse in the cells. It has frequently been suggested that the aim of OA is to maintain growth capacity through turgor maintenance at lower external osmotic potentials [30]. The conservation of RWC in water stress conditions is usually well correlated with biomass production and grain yield [8]. Conservation of RWC may be attained though osmotic adjustment and more efficient soil water extraction either by increase root DW (Table 1), increasing root length (not measured) or reduced transpiration [22]. Selection for the latter characteristic has limited interest

since it implies a reduction in stomatal conductance and leaf area, which would have a negative effect on the carbon balance [39]. However, the effect of water stress on stomatal behavior in this species requires further investigation.

Indeed, in the present study, leaf ψ_S declined consistently in response to water stress in both ecotypes, but the reduction was larger in the tolerant ecotype during the experimental period (Figure 2). Decreased ψ_S are generally considered to be an indicator of OA as a result of the production and/or accumulation of so-called compatible osmolytes, although such decreases could also result from dehydration of the tissue and/or reduced osmotic volume [40]. A dehydration process was revealed by the observed reduction in leaf RWC, but it was insufficient to account for the ψ_S difference existing between both ecotypes. This result is agreement with results on durum wheat [13] and on *Populus davidiana* [41]. The concentrations of a variety of organic compounds are known to increase in some plant tissues subjected to water stress [40].

In the studied ecotypes, the carbohydrate concentration increased in plants subjected to -0.1 MPa and then plateaued in all other stress levels. However, this result indicates that the first signals of drought stress were probably enough to upregulate OA mechanisms and/or genes mediated by soluble carbohydrates (Table 2). These observations indicate that sugar accumulation plays an important role in OA. In addition, carbohydrates have been shown to significantly contribute to osmotic adjustment in the growing regions of leaves [40], stems [6] and roots [13]. Iannucci *et al.* [40] reported that the leaves of all clovers studied synthesized more reducing sugars and proline when subjected to water stress as compared to well-watered plants. It is not possible to assign an exact osmotic contribution to the soluble sugar accumulation during stress without knowing the proportions of monosaccharides, disaccharides and oligosaccharides. Nevertheless, it is possible to assign a range if all carbohydrates are present as mono or disaccharides. The contribution of the average soluble sugar content to ψ_S of cultivars ranged from 38% in control conditions (-0.03 MPa) to 57% during severe water stress (-1.0 MPa, data not shown), suggesting an appreciable role in the OA process. Furthermore, a decrease in non-reducing sugar concentrations under drought conditions has also been reported for other legumes and ascribed to the inhibition of photosynthesis due to turgor loss [42]. Differences in proline accumulation during water stress treatment were also found among these species.

Although the concentration of proline in the leaves was significantly increased ($P<0.01$) in response to water stress (Table 2), its concentration was still too low to have a strong effect on ψ_S and its contribution to the total change in osmotic potential was insignificant. It is well known that the free proline level increases in response to drought. Sánchez *et al.* [43] reported that, while accumulation of proline in *Pisum sativum* L. increased in response to drought stress from 4 to 40 times, its concentration and contribution to ψ_S was small. However, the significant relationships indicated in the present study are in agreement with the results of Iannucci *et al.* [40] who proposed that the metabolic differences between species may reflect differences in the water status achieved rather than metabolic differences at a given water status. This seems to indicate that the role of proline in OA is not important. Leigh *et al.* [44] observed that proline is predominantly confined to the cytoplasm, which could suggest that proline affects OA in certain organelles. However, no such evidence has been found. Our results indicate that proline does not play an appreciable role in OA. However, proline can act as an osmoprotector of cytosolic enzymes and cellular structures [45].

Among the inorganic solutes, Potassium appears to be present at a sufficient concentration for playing an important role in OA (Table 2). In the tolerant ecotype, the contribution of K^+ to ψ_S increased from 15% (control) to 35 % during severe drought stress (-1.0 MPa; Table. 2 and Figure 2d), although its effect on OA was less pronounced (Figure 2c). Nevertheless, its contribution to the effects observed in the sensitive ecotype was much less. However, among the ions in the natural habitat of this species in Iran, K^+ is one the most abundant and plants have evidently used this ion as an osmoregulator for adaptation to harsh environments. Potassium ions are known to be quite soluble and to play a key osmoregulatory role in guard cells and in turgor maintenance [46]. Nonetheless, plant species differ in their primary osmolytes [12]. In the work of Alves and Setter [47] with mature and expanding cassava leaves, K^+ salts were the major contributors to total leaf osmolyte concentration, accounting for approximately 60% of the osmotic potential. In contrast, sugars accounted for <25% of the osmotic potential and their concentrations decreased during water stress. Thus, they proposed that cassava might be classified among the species that use K salts as their primary osmolyte. Moreover, potassium is the main solute in both expanding and fully expanded

leaves in sunflowers [28] and chickpeas [48], whereas sugar is the main substance in both elongating and elongated regions of the leaf in wheat [13]. Both potassium and sugars contribute to the changes in osmolyte concentration induced by water deficit in soybeans [49] and sorghum [50], while Bajji *et al.* [13] showed that inorganic solutes do not seem to play an important role in OA in durum wheat despite their high proportion among total solutes.

CONCLUSION

The results obtained in this experiment indicate that the tolerant ecotype used a combination of different morphological and physiological strategies for survival in dry climes. Reductions in plant height, shoot DW and leaf area could reduce water loss and increases in root DW and probably root length might increase water uptake by the plant. Ecotypes with slower growth duration in harsh environments have the advantage of decreased water demand and prevention of depleted soil water reserves in a short period of time. The contribution of various solutes to osmotic adjustment and the production of osmoprotectants help plants maintain a higher RWC, which prevents cell shrinkage and cell death in dry conditions. Complementary experiments are required to determine the impact of the root system, cell wall properties and antioxidant system in resistance to water stress in this type of cutleaf medic.

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