Relationships between phenotypic variation in osmotic adjustment, water-use efficiency, and drought tolerance of seven cultivars of *Lotus corniculatus* L.

Luis Inostroza[1*], Hernán Acuña[2], and Gerardo Tapia[1]

Lotus corniculatus L. is a perennial forage legume species highly-adapted to growth under drought conditions. However, the genetic and physiological mechanisms involved in its adaptive capacity have not been elucidated. The role of osmotic adjustment (OA) and water-use efficiency (WUE) on the drought tolerance of *L. corniculatus* was studied in a greenhouse experiment. Seven cultivars of different origin were subjected to two contrasting treatments of available soil water: No water stress (NWS) and with water stress (WWS). Xylem water potential (Ψ_x), osmotic potential (Ψ_π), pressure potential (Ψ_p), relative water content (RWC), stomatal conductance (g_s), shoot DM production, water transpiration (T), and WUE (shoot DM/T) were measured. Water treatments significantly (P < 0.05) affected plant water status, which was reflected in reduced Ψ_x, RWC, g_s, and transpiration rate in the WWS treatment compared with the NWS treatment. All cultivars showed a high capacity for OA under WWS treatment because Ψ_π decreased by approximately 60% and Ψ_p increased by approximately 30%, compared with the NWS treatment. Cultivars with a higher solute accumulation (low Ψ_π value) had the lowest DM production under WWS treatment. In contrast, WUE varied greatly among cultivars and was positively associated ($R^2 = 0.88$; P < 0.01) with DM production under drought conditions.

Key words: Drought acclimation, genetic variability, physiological traits, transpiration rate.

INTRODUCTION

Drought is the most important abiotic stress that curtails plant growth and crop productivity (Cobb et al., 2013). This has prompted the urgent need to understand the genetic and physiological mechanisms that induce plants to tolerate water deficit and to transfer this knowledge for increasing crop productivity in environments where water resources are a limiting factor.

Plants have developed a series of physiological mechanisms to tolerate drought (Cattivelli et al., 2008). Many of these only temporarily protect against dehydration and/or help plant survival and their role is controversial (Turner et al., 2007). Only a few mechanisms have been demonstrated to stabilize crop yield under drought conditions (Rebetzke et al., 2002; Kraakman et al., 2004): Osmotic adjustment (OA) and water-use efficiency (WUE) are two important physiological mechanisms to maintain the growth and productivity of plants under drought conditions (Babu et al., 1999; Richards et al., 2002). Osmotic adjustment

involves actively accumulating solutes at the cellular level in response to a decline in plant water potential. As a result of this accumulation, the cell osmotic potential decreases, which in turn attracts water into the cell and maintains turgor pressure. This allows the conservation of physiological processes that depend on cellular turgor, such as expansive growth and stomatal control (Babu et al., 1999; Cattivelli et al., 2008). Furthermore, accumulated compatible solutes can also protect specific cellular functions either by stabilizing proteins or by scavenging reactive oxygen produced as a response to drought (Sanchez et al., 2008). In plant breeding, WUE has been one of the few physiological mechanisms that have allowed the selection of high production genotypes under drought conditions (Condon et al., 2004; Sinclair et al., 2004). From the physiological perspective, WUE is defined as the assimilation of CO_2 per unit of transpired water (Condon et al., 2004). However, from an agronomic point of view, WUE is an econometric term derived from the marginal productivity theory: production (biomass) per unit of input (water); however, it encompasses a series of physiological processes, given that the physiological WUE depends on the balance between stomatal conductance (g_s) and the photosynthetic capacity of the plant (Earl, 2002; Condon et al., 2004; Inostroza and Acuña, 2010).

In drought-prone environments, some species of the *Lotus* genus have been suggested to be a promising genetic resource, given their high forage yield potential and high capacity to adapt to marginal environments (Striker et al.,

2005; Teakle et al., 2006; 2007; Real et al., 2008; Garcia et al., 2008; Manzur et al., 2009; Teakle et al., 2010; Striker et al., 2012). *Lotus corniculatus* is a perennial forage legume that originates in the Mediterranean Basin and North Africa (Kirkbride, 1999) and has been widely used in southern Europe, North and South America, and New Zealand. It is established as a monocrop for hay or accompanied with grass when used for grazing. It is also used for soil remediation and erosion control. It is a particularly suitable legume for marginal soils and extensive grassland management systems, where it outperforms alfalfa (*Medicago sativa* L.) and white clover (*Trifolium repens* L.), the most popular temperate climate forage legumes (Carter et al., 1997).

Despite the well-known adaptive attributes of *L. corniculatus*, research is limited concerning the physiological mechanisms that cause drought tolerance. Only four studies have dealt with drought tolerance of the species. Carter et al. (1997) used *L. corniculatus* as a representative model of perennial legume species to study the effects of climatic change (temperature, CO_2, and drought) on plant growth and productivity. The results of these studies showed that the species has the capacity to respond to drought by modifying its growth rate, leaf area, and biomass allocation. Neal et al. (2011) characterized WUE of 15 perennial forage species under field conditions in New South Wales, Australia, and showed that *L. corniculatus* reached a higher WUE than white clover and alfalfa under restrictive soil moisture conditions. Borsani et al. (2001) studied the effects of drought on *L. corniculatus* at the molecular level and demonstrated that drought induces oxidative stress in *L. corniculatus* leaves. As a consequence, total superoxide dismutase activity increases in plants as an antioxidant protector, after a 4-h drought treatment. Although this study is interesting, its scope is limited because it represents a short-term application of extreme water stress. This does not occur in the field and neither does it represent the dynamics of perennial forage species that suffer the consequences of stress at an advanced development stage; they are also subjected to cutting and animal grazing, where regrowth capacity plays a fundamental role in plant acclimation to drought. Sanchez et al. (2012) studied *L. corniculatus* drought tolerance from the perspective of its metabolism. Their results show that the accumulation of compatible osmolytes at the cellular level in response to drought is a tolerance mechanism of *L. corniculatus* and other agronomically and genetically important *Lotus* species. They also demonstrated that most synthesized metabolites are species-specific and fulfill different roles within each species. However, the study only has a metabolic scope and does not explore the role of the mechanisms on plant productivity. The study by Sanchez et al. (2012) is the first evidence of the ability of *L. corniculatus* to perform accumulation of solute.

The objectives of this experiment were to describe the role of OA and WUE on *L. corniculatus* drought tolerance and biomass productivity. The study analyzes the effect of gradual soil moisture loss and the application of drought treatment on plant regrowth; this is a relevant condition for perennial forage species; genetic variability is explored in terms of these physiological mechanisms in seven *L. corniculatus* cultivars with different origins.

MATERIALS AND METHODS

The experiment was carried out in pots under greenhouse conditions at Instituto de Investigaciones Agropecuarias, INIA Quilamapu, Chillán, Chile. The treatments included all factorial combinations of seven *L. corniculatus* cultivars and two levels of soil water availability. During May 2008, a number of seeds of each cultivar were individually sown in 27-cm^3 capacity seedbeds containing peat as a substrate (Biolan, Kauttua, Finland). Seedlings were inoculated, 1 wk after emergence, with a *Mesorhizobium loti* suspension. In winter (June and July), plants were grown in a greenhouse with heating and artificial light. At the beginning of spring (August) they were moved to a shed.

On 10 November 2008, when plants reached the five-leaf fully expanded developmental stage, they were transplanted into 3.5 L pots (25 cm diameter) containing soil derived from volcanic ash (medial, thermic, Humic Haploxerands, Andisol, according USDA soil taxonomy) of silty loam texture, as a substrate, and were moved to the greenhouse. All the pots contained 3 kg of soil homogenized by sieving without fertilizer application. Five plants of each cultivar were established in each pot. Temperature and relative humidity were controlled with a forced-air cooling system. These variables were recorded at 15-min intervals throughout the experiment with an automatic sensor (Hobo pro series, Onset Computer Corporation, Bourne, Massachusetts, USA). The greenhouse remained at a mean temperature and relative humidity of 23/16 ºC (day/night) and 50/75% (day/night), respectively.

Lotus corniculatus cultivars
Six *L. corniculatus* cultivars introduced from USA ('Norcen', 'Empire', and 'Steadfast'), Australia ('Ges-5'), Brazil ('San Gabriel'), Uruguay ('Ganador'), and one Chilean cultivar ('Quimey') were used in the study. 'Quimey', 'Ganador', and 'San Gabriel' have an erect growth habit, whereas the others are semi-erect (Acuña et al., 2002).

Growth environment and water stress treatments
Two soil water treatments were established according to Inostroza and Acuña (2010). Treatments were: no water stress (NWS) and with water stress (WWS), where soil moisture remained at 54% and 25% (dry soil basis) and a water potential of -0.01 and -0.5 MPa, respectively. To

maintain moisture levels the weight of pots with soil and water was determined for each water treatment, by daily weighing and the replacement of transpired water. All pots were watered after transplantation, until the NWS treatment weight was reached, to ensure plant survival. A 3-cm layer of expanded polystyrene beads was also added to each pot to prevent soil water evaporation. The substrate weight in the root of each plant and the weight of the expanded polystyrene sheet were added to the total pot weight in each treatment. Subsequently, pots in the WWS treatment were not watered until they reached their corresponding weight (Figure 1). By recording the pot weight before and after irrigation, the appropriate amount of water transpired by the plants was then replaced daily (between 08:00 and 10:00 h).

The experiment was conducted in two periods. The first period was 30 d after planting (DAP), during which the effectiveness of the water treatments was evaluated by determining the relative water content (RWC), xylem water potential in stems (Ψ_x), osmotic potential (Ψ_π), stomatal conductance (g_s), and daily transpiration rate. At the end of this period, plants were cut 3 cm above the soil surface to evaluate shoot DM production (leaves + stems). One replicate was used destructively after this cutting to quantify the gain in root weight. This information was used to correct the pot weight during the second experimental period. The second period began after the cutting and lasted for 27 d. The following traits were evaluated during this stage: RWC, Ψ_x, Ψ_π, g_s, specific leaf area, and water transpiration (T). Finally, shoot and root DM (57 DAP) production was quantified. Water-use efficiency was estimated during both periods as the relationship between shoot DM and accumulated T during each experimental period.

Evaluation methodologies
In each pot, g_s was evaluated with a porometer (AP4, Delta-T Devices, Cambridge, UK) in the central leaflet of three fully expanded leaves located in the top third of the

plant. One stem from each pot was evaluated for Ψ_x by wrapping the stem with plastic film and covering it with aluminum foil 2 h before the measurement. Subsequently, the stem was removed by cutting the plant with a scalpel to determine Ψ_x with a Scholander type pressure pump. The osmotic potential was determined in a sample of eight leaves at a similar developmental stage in each pot. Leaves were extracted from the plant, immediately frozen in liquid nitrogen, and stored at -40 °C until evaluation. A 20-μL aliquot of cell liquid was later extracted using pressure. The osmolarity of the solution was determined with an osmometer (Model 3320, Advanced Instruments, Norwood, Massachusetts, USA) and Ψ_π was calculated by van't Hoff law: $\Psi_\pi = -n \times T \times R$, where n is osmolarity, T is the absolute temperature, and R is the gas constant. The pressure potential (Ψ_p) was then calculated as $\Psi_x - \Psi_\pi$.

The RWC was determined according Inostroza and Acuña (2010) by the following expression:

$$RWC = (FW - DW/FTW - DW) \times 100 \; RWC$$

where FW and DW are the fresh and dry weight, respectively, and FTW is the weight at full turgor. After determining FTW, leaf area was measured with a leaf area meter (CI-202, CID Bio-Science Inc., Camas, Washington, USA). Specific leaf area was calculated based on this information (SLA = leaf area/leaf DM). All the evaluations were performed on clear days between 12:00 and 14:00 h.

Dry-matter production was determined by drying tissues (leaves, stems, and roots) in a forced-air oven at 65 °C until constant weight was reached. Roots were separated from the substrate by washing them under running water in a 27-mesh sieve. The sum of shoot DM measured in the two periods was considered total shoot DM, and using this value, the root/total shoot DM ratio was determined. The leaf-weight ratio (LWR = Leaf DM/ Shoot DM) and leaf-area ratio (LAR = SLA × LWR) were calculated using the DM production data for the second period. The transpiration rate (g water d^{-1}) was the slope of the linear regression between transpired water and time (DAP).

Experimental design and statistical analyses
A randomized complete block design with a factorial arrangement (two water levels and seven *L. corniculatus* cultivars) and four replicates was used. Data were analyzed by ANOVA and means were compared by the Least Significant Difference (LSD) test. The relationship among variables was analyzed by correlation and linear regression analysis with SAS (SAS Institute Inc., Cary, North Carolina, USA).

RESULTS

Water treatments and plant water status
The experiment began with the same level of water availability (NWS treatment) in all pots, to promote
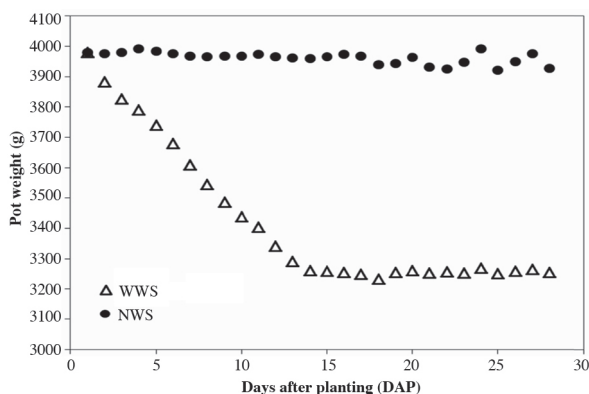

Figure 1. Weight loss of pots exposed to treatment with water stress (WWS) compared with no water stress (NWS) treatment. Values are means of seven *Lotus corniculatus* cultivars.

plant establishment and allow a gradual transition to the WWS treatment. Figure 1 shows the kinetics of water losses in the WWS treatment which reached the expected soil water deficit, at approximately 14 DAP. The transpiration rate showed differences between water treatments from 5 DAP, being 15% lower in the WWS treatment compared with the NWS treatment (Figure 2). At 14 DAP, the difference increased to 40% (Table 1). The accumulated T during the first experimental period showed a highly significant Cultivar × Water treatment

interaction (P < 0.001). Only 'Quimey' and 'Empire' were different in the WWS treatment (P < 0.05), exhibiting the highest and lowest accumulated T, respectively. In contrast, in the NWS treatment, the accumulated T of the South American cultivars ('Quimey', 'Ganador', and 'San Gabriel') considerably exceeded that of the other cultivars (Table 1). The accumulated T in the second experimental period also showed a significant Cultivar × Water treatment interaction (P < 0.05). Accumulated T in the NWS treatment was 25% higher in the first period, and in the WWS treatment it was the same in both periods (Table 1). The reduction in accumulated T caused by the WWS treatment during the second experimental period was more severe than in the first one, with fluctuations between 57% ('Ganador') and 72% ('Empire'). 'Quimey' and 'Empire' showed the highest and the lowest accumulated T, respectively, in both WWS and NWS treatments (Table 1).

The transpiration rate during the first experimental period also showed a highly significant Cultivar × Water treatment interaction (P < 0.001, Table 1) and fluctuated in the NWS treatment between 65.4 ('Empire') and 148.6 ('San Gabriel') g water d^{-1}. During the second period there was not significant interaction Cultivar × Water treatment and a highly significant effect was observed in the soil-water treatments and cultivars (P < 0.001). The transpiration rate was approximately 9% and 28% higher in the first experimental period in the WWS and NWS treatments, respectively. 'San Gabriel' and 'Quimey' had the highest transpiration rate, whereas 'Empire' had the lowest one.

The value of Ψ_x evaluated during the first experimental period (23 DAP) was almost six-fold less in the WWS treatment than in the NWS treatment (P < 0.001), but no significant differences were observed among cultivars (Table 2). In the same period, RWC showed a significant Cultivar × Water treatment interaction (P < 0.05; Table 1). The RWC of 'Empire', 'Steadfast', 'Ges-5', and 'Norcen' decreased by approximately 13% in the WWS treatment compared with the NWS treatment. 'San Gabriel' was
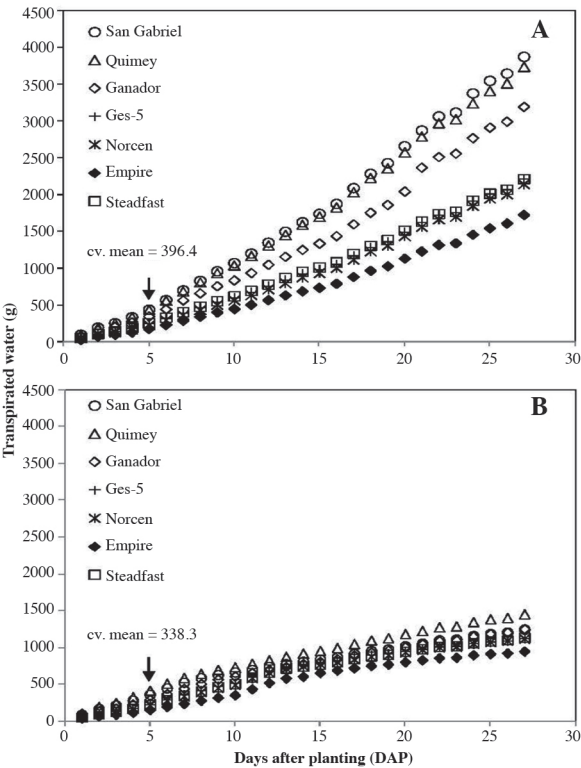

Figure 2. Daily accumulated transpiration of seven *Lotus corniculatus* cultivars exposed to treatments with water stress (A) or no water stress (B) under greenhouse conditions during the first experimental period.

Table 1. Accumulated water transpiration (T), transpiration rate, relative water content (RWC), and stomatal conductance (g_s) of seven *Lotus corniculatus* cultivars exposed to two water treatments (NWS and WWS, without or with water stress, respectively) under greenhouse conditions.

| | Accumulated T | | | | | | Transpiration rate | | | | RWC | | g_s | |
| | 14 DAP | | Exp. Period I | | Exp. Period II | | Exp. Period I | | Exp. Period II | | | | | |
Cultivars	NWS	WWS	NWS	WWS	NWS	WWS	NWS	WWS	NWS	WWS	NWS	WWS	NWS	WWS
	g water per pot						g water d^{-1}				%		mmol m^{-2} s^{-1}	
San Gabriel	2093	912	3876	1244	4342	1338	148.6	42.2	166.2	52.4	0.90	0.83	436.8	78.0
Quimey	2036	1047	3739	1453	4262	1605	143.1	49.6	163.1	63.2	0.89	0.85	601.7	75.8
Ganador	1601	890	3196	1249	3570	1547	121.4	44.6	138.4	60.5	0.84	0.86	630.0	159.0
Empire	897	719	1740	946	3438	946	65.4	38.5	133.0	35.9	0.84	0.75	530.4	161.9
Steadfast	1201	842	2214	1134	3482	983	84.4	43.4	135.2	36.8	0.88	0.71	447.5	266.0
GES-5	1174	863	2219	1122	3403	1078	84.3	43.0	132.6	41.3	0.91	0.78	507.9	133.4
Norcen	1122	839	2145	1118	3672	1170	81.9	43.0	142.8	45.5	0.86	0.78	459.6	221.8
Significance	***		***		*		***		ns		*		*	
LSD	182.6		362.8		385.9		14.3		16.4		0.072		153.70	

Least significant difference (LSD) of the Cultivar × Water treatment interaction. Significant at *P < 0.05, **P < 0.01, and ***P < 0.001.
DAP: Days after planting.

Table 2. Xylem water potential (Ψx), osmotic potential (Ψπ), and pressure potential (Ψp) of seven *Lotus corniculatus* cultivars exposed to two water treatments (NWS and WWS, without or with water stress, respectively) under greenhouse conditions, evaluated 23, 49, and 56 d after planting (DAP).

Cultivars	23 DAP			49 DAP			56 DAP		
	Ψx	Ψπ	Ψp	Ψx	Ψπ	Ψp	Ψx	Ψπ	Ψp
					MPa				
San Gabriel	-0.66	-1.43	1.37	-0.38	-1.49	1.11	-0.43	-1.72	1.29
Quimey	-0.48	-1.47	1.42	-0.39	-1.45	1.06	-0.39	-1.65	1.26
Ganador	-0.48	-1.45	1.40	-0.47	-1.54	0.92	-0.51	-1.73	1.22
Empire	-0.43	-1.67	1.63	-0.37	-1.63	1.17	-0.50	-1.89	1.39
Steadfast	-0.55	-1.35	1.30	-0.34	-1.54	1.20	-0.39	-1.63	1.23
GES-5	-0.56	-1.51	1.46	-0.39	-1.55	1.16	-0.32	-1.82	1.50
Norcen	-0.49	-1.57	1.53	-0.32	-1.62	1.30	-0.37	-1.81	1.43
Significance	ns	ns	ns	ns	*	ns	ns	*	ns
LSD	ns	ns	ns	ns	0.122	ns	ns	0.175	ns
Water treatments									
NWS	-0.15	-1.09	1.08	-0.26	-1.21	0.95	-0.29	-1.43	1.14
WWS	-0.89	-1.90	1.81	-0.51	-1.88	1.37	-0.54	-2.07	1.53
Significance	***	***	***	***	***	***	***	***	***
LSD	0.170	0.095	0.100	0.103	0.065	0.129	0.126	0.094	0.153

Significant at *$P = 0.05$, **$P = 0.01$, and ***$P = 0.001$; ns: nonsignificant; LSD: least significant difference.

the only South American cultivar that showed a decrease in RWC in the WWS treatment (8%), whereas 'Quimey' and 'Ganador' did not show any significant differences in RWC between either water treatments (Table 1). Stomatal conductance (g_s) also showed a significant Cultivar × Water treatment interaction ($P < 0.05$; Table 1). Not all cultivars adjusted g_s to the same degree; for example, it decreased more than 80% in 'San Gabriel' and 'Quimey', but only by 40% in 'Steadfast' in the WWS treatment compared with the NWS treatment (Table 1). 'Quimey' showed one of the highest g_s values in the NWS treatment, whereas it had the lowest g_s in the WWS treatment (Table 1).

Xylem water potential ($\Psi_x = \Psi_\pi + \Psi_p$) and its osmotic ($\Psi_\pi$) and pressure ($\Psi_p$) components were evaluated three times during the experiment (Table 2). For all potentials (Ψ_x, Ψ_π, Ψ_p) and evaluation dates, a highly significant effect was observed for the soil-water

treatments ($P < 0.001$). Significant differences were observed only between cultivars in Ψ_π values at 49 and 56 DAP (Table 2). Xylem water potential values stabilized at about -0.28 and -0.52 MPa in the NWS and WWS treatments, respectively, during the second experimental period (49 and 56 DAP) (Table 2).

Dry matter production and partitioning

Dry matter production evaluated during the first and second experimental periods showed a significant Cultivar × Water treatment interaction ($P < 0.05$; Table 3). In the first period DM production in 'Quimey' decreased by 60% in the WWS treatment compared with in the NWS treatment, whereas it decreased by approximately 80% in 'Empire'. The DM production of the South American cultivars in the NWS treatment was considerably higher ($P < 0.05$) than DM production in all the other cultivars.

Table 3. Shoot dry mater production during the first and second experimental periods; root DM/shoot DM ratio, leaf weight ratio (LWR), specific leaf area (SLA), and leaf area ratio (LAR) evaluated during the second experimental period in seven *Lotus corniculatus* cultivars exposed to two water treatments (NWS and WWS, without or with water stress, respectively) under greenhouse conditions.

Cultivars	Shoot DM production				Root DM/Shoot				
	Exp. Period I		Exp. Period II		DM				
	NWS	WWS	NWS	WWS	NWS	WWS	LWR	SLA	LAR
		g pot⁻¹						cm² g⁻¹	
San Gabriel	5.47	1.67	7.36	1.68	0.52	0.70	0.49	238.1	116.48
Quimey	5.52	2.12	7.19	2.05	0.49	0.73	0.48	228.9	108.25
Ganador	4.32	1.33	5.58	2.13	0.40	0.66	0.52	223.1	116.44
Empire	2.30	0.54	5.36	0.91	0.44	0.83	0.52	248.4	130.74
Steadfast	2.76	0.78	5.72	1.16	0.49	1.00	0.54	223.4	119.62
GES-5	3.06	0.92	5.42	1.24	0.41	0.74	0.48	222.6	107.91
Norcen	2.92	0.81	5.84	1.32	0.61	0.95	0.57	248.9	142.68
Significance	***		*		*		***	ns	*
LSD	0.73		0.80		0.132		0.037	ns	23.06
					Water treatments				
					NWS		0.50	274.4	139.0
					WWS		0.53	189.6	100.4
					Significance		*	***	***
					LSD		0.020	20.47	12.40

Significant at *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$; ns: nonsignificant; LSD: least significant difference.

The situation was similar for the WWS treatment, but only DM production in 'Quimey' and 'San Gabriel' was significantly different from all other cultivars (Table 3). The decrease in DM production during the second experimental period for the WWS treatment was similar to values observed in the first period. 'Quimey' and 'San Gabriel' in the NWS treatment significantly outperformed the other cultivars for DM production. 'Quimey' and 'Ganador' had the highest DM production in the WWS treatment and were significantly different from the cultivar with the lowest DM production ('Empire').

Soil water availability affected biomass partitioning of all cultivars under study. The root/shoot DM ratio showed a significant interaction among cultivars and soil water treatments (P < 0.05; Table 3). All cultivars showed a significant increase in root/shoot DM in the WWS treatment compared to in the NWS treatment. The leaf-area ratio (LAR = LWR × SLA) varied significantly between water treatments and among cultivars (Table 3). The LAR decreased by almost 30% in the WWS treatment compared to in the NWS treatment. Variations in LAR were caused only by the different capacity of the cultivars to partition biomass to the leaves (LWR), because no significant differences were observed in SLA (P > 0.05; Table 3).

Osmotic adjustment and water use efficiency
Xylem water potential was evaluated three times during the experiment and decreased almost six-fold at 23 DAP and by more than 90% at 49 and 56 DAP in the WWS treatment compared with the NWS treatment. The decrease in Ψ_x was not lethal for the plant but affected its transpiration rate and biomass production and partitioning (Table 3). However, under drought conditions (WWS), all the *L. corniculatus* cultivars showed a significant difference in Ψ_x components (P < 0.001), according to the conceptual definition of OA. On average, Ψ_π decreased

by 60% on the three sampling dates, but Ψ_p increased by approximately 30% in the WWS treatment compared with the NWS treatment (Table 2). These results lead to the conclusion that *L. corniculatus* strongly induces OA under drought conditions. However, the decrease in Ψ_π and increase in Ψ_p are significantly related (P < 0.05) to a decrease in DM production (Figure 3).

Water-use efficiency, calculated for each experimental period, varied significantly between soil-water treatments and among cultivars (P < 0.05). Water-use efficiency in both periods decreased by approximately 30% in the WWS treatment compared to in the NWS treatment and the cultivars with the highest and lowest WUE were 'Quimey' and 'Empire', respectively (Figures 4 and 5). However, WUE was strongly associated with DM production under water stress conditions (WWS) in both evaluation periods (P < 0.05; $R^2 = 0.88$; Figure 4).
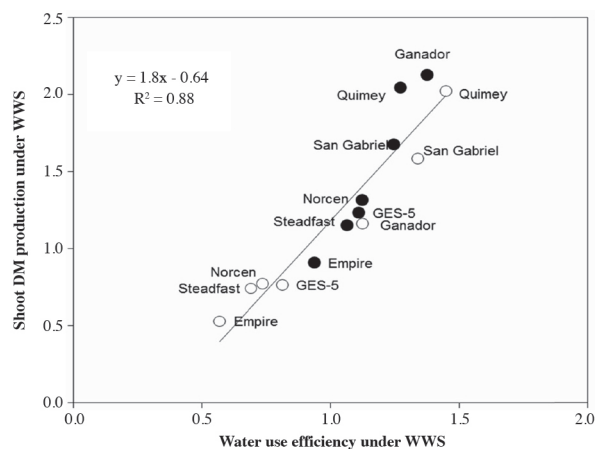

Figure 4. Relationship between shoot dry mater (DM) production and water-use efficiency under water stress conditions (WWS) evaluated during the first (black circle) and second (white circle) experimental periods.
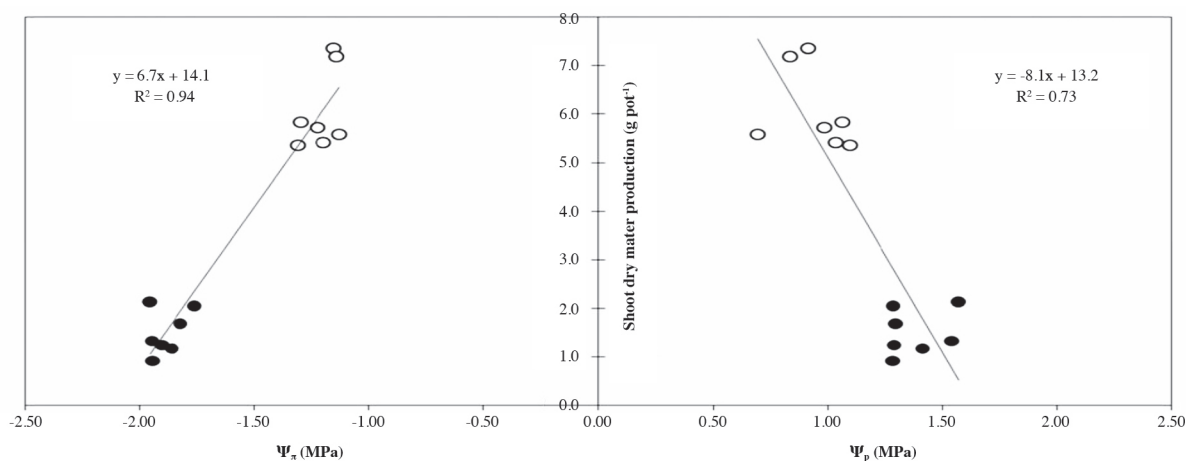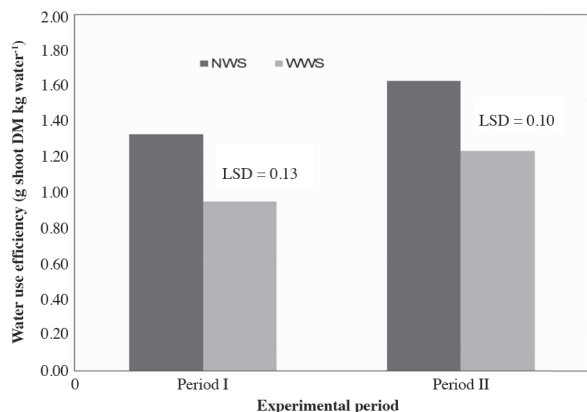

Figure 3. Relationship between shoot dry mater production and osmotic (Ψ_π) and pressure (Ψ_p) potential of seven *Lotus corniculatus* cultivars subjected to two soil water treatments under greenhouse conditions: with water stress (black circle) or no water stress (white circle). Data from the second experimental period.

Period I (from planting to first cutting; 30 d); Period II (27 d after first cutting)

Figure 5. Water-use efficiency evaluated in two experimental periods (period I and period II) of *Lotus corniculatus* subjected to two soil water treatments (NWS and WWS, without or with water stress, respectively). Values are means of seven cultivars. LSD: least significant difference.

DISCUSSION

Drought acclimation

Plant water deficits can occur as a consequence of a seasonal decline in soil water availability in the long term or can result from drought spells. The timing, intensity, and duration of stress episodes are fundamental for determining the effects produced by drought (Chaves and Oliveira, 2004). The present study was therefore designed to simulate drought that was typical of Mediterranean environments and occurs when pastures are already established and the plant is completely developed. Moreover, the perception of stress is gradual and depends on the soil water-holding capacity and atmospheric evaporative demand. The results show this gradual entry to the WWS treatment, where pots took 15 d to attain the water treatment (Figure 1). The seven *L. corniculatus* cultivars began to acclimate within this period, which included a sharp decrease in the plant water status (Ψ_x and RWC), and a rapid decrease in the gas exchange rate (g_s and transpiration rate) for the time-scale of the experiment, because the transpiration rate at 5 DAP decreased by 15% in the WWS treatment compared to that in the NWS treatment (Figure 2) and decreased by 40% at 14 DAP, when all pots entered the WWS treatment (Table 1).

As mentioned, the acclimation process to drought was gradually induced in plants from an optimal water condition. Prior to water stress, plants had already developed leaves and stems that slowly modified their physiology to stress, but not their morphology (Table 2). The physiological response to drought (WWS) in this period was the same for all cultivars and consisted of a decrease in Ψ_x of approximately six-fold that observed in the NWS treatment. This was accompanied by an

approximately 90% decrease in Ψ_π and 80% increase in Ψ_p (Table 2). The regrowth process began following cutting and when plants were physiologically acclimated to drought and showed clear morphological modifications caused by alterations in the growth, development and partitioning of shoot biomass (LAR and LWR; Table 3). The interaction between physiological and morphological acclimation that occurred during the regrowth period, generated a phenotype of small and very turgid leaves (Tables 2 and 3). In general, this allowed Ψ_x values to stabilize at about -0.28 and -0.52 MPa in the NWS and WWS treatments, respectively (Table 2); the difference between water treatments was three-fold lower than that observed during the first experimental period (Table 2).

Water relationships ($\Psi_x = \Psi_\pi + \Psi_p$) were determined three times and the same response pattern was observed for all cultivars, which consisted of a decrease in Ψ_x and Ψ_π and an increase in Ψ_p in the WWS treatment compared with the NWS treatment (Table 2). Despite maintaining this response pattern, significant differences were observed among cultivars ($P < 0.05$) for transpiration rate, RWC, and g_s, which reflect specific physiological mechanisms of each cultivar and allow acclimation of its metabolism to drought conditions. For example, 'Quimey' in the WWS treatment showed one of the highest RWC values and the lowest g_s values. Despite this physiological modification, 'Quimey' showed the highest accumulated T value during the first experimental period. This accounts for a water-saving strategy through a conservative gas exchange that allows an increase in its physiological (CO_2/H_2O) and agronomic WUE (Figure 4).

The role of osmotic adjustment and water-use efficiency on DM production

Plant strategies to control water status and resist drought are numerous (Chaves and Oliveira, 2004) and can be grouped into three categories: those allowing escape from drought by shortening the ontogenetic cycle (drought escape); those allowing the maintenance of plant water status and physiology under drought conditions (drought avoidance); and those allowing the maintenance of plant metabolic functions with a depressed water status (drought tolerance). In agronomy, whichever strategy should allow for continued or increased biomass production under water-deficit conditions. Strategies in perennial species such as *L. corniculatus* are concentrated in the second category, for which OA has been one of the most-studied physiological mechanisms in model and cultivated plants (Verslues and Bray, 2004; Blum, 2005; Maggio et al., 2006). The results of the present study are evidence of the high capacity of *L. corniculatus* to induce OA under drought conditions from the initial stage of the acclimation process (Table 1). Under drought conditions (WWS), all *L. corniculatus* cultivars modified the Ψ_x components according to the conceptual definition of OA. On average, Ψ_π was reduced by 60% on the three

sampling dates and Ψ_p increased by approximately 30% in the WWS treatment compared to in the NWS treatment (Table 2). The present study is the first to quantify the capacity to induce OA in *L. corniculatus* in physical terms (water potential). Using a non-targeted metabolomic approach, Sanchez et al. (2012) recently studied the kinetics of accumulating solutes in a model and a forage *Lotus* species subjected to drought. Their results confirm the high capacity for *L. corniculatus* to perform OA and extends this conclusion to all species of the *Lotus* genus that are agronomically important (*L. tenuis* and *L. uliginosus*), since the osmolyte concentration increased in all species under drought conditions.

Similar to *L. corniculatus*, several plant species of agricultural importance have the capacity to carry out OA under drought conditions (Turner, 1990; Morgan, 1995; Babu et al., 1999; Turner et al., 2007). However, the role of OA in conferring productive stability under drought conditions has been controversial to date. For instance, the study by Turner et al. (2007) concluded that differences in OA were not associated with yield benefits in a population of advanced breeding lines of chickpea that were developed from a cross between cultivars with high and low OA, similar results has been reported in barley (Chen et al., 2007; Widodo et al., 2009) and potatoes (Vasquez-Robinet et al., 2008). On the other hand, wheat possesses considerable genetic variability to carry out OA and cultivars with a high OA capacity exhibit higher yield under water stress conditions than cultivars with a low OA capacity (Morgan, 1995; El Hafid et al., 1998; Blum et al., 1999). Under drought conditions, the results of the present study show that the increase in the accumulation of solutes in *L. corniculatus* (lowest Ψ_π value) caused by water stress is associated with a sharp decrease in DM production (Figure 3). The cultivars with the most divergent Ψ_π values on all the sampling dates were 'Quimey' and 'Empire' (Table 2). These cultivars also showed the highest and lowest DM production, respectively, in both experimental periods (Table 3). Accordingly, OA in *L. corniculatus* is a component of its drought tolerance, since it allows the maintenance or even an increase in Ψ_p in acclimated plants under water-deficit conditions. This provides continuity in gas exchange (T and g_s), growth, and biomass accumulation (Tables 1 and 3), but overexpression of the mechanism is associated with a lower DM production under drought conditions (Figure 3 and Table 1). In this respect, the physiological mechanism only supports plant survival and not productivity under drought conditions, which coincides with the results observed in chickpea by Turner et al. (2007).

The values of Ψ_p showed no significant differences among cultivars in the three evaluations (23, 49, and 56 DAP; Table 2). However, Ψ_p values under water-stress conditions (WWS) were unexpectedly higher than those observed under control conditions (NWS; Table 2).

Thus, *L. corniculatus* had a lower growth and biomass accumulation in WWS, with Ψ_p values higher than those obtained in NWS (Tables 2 and 3). These results contradict the historically accepted theory formulated by Lockhart (1965), who established a first-order relationship between turgor (Ψ_p) and cell growth, which is expressed as $E = m (P - Y)$, where E is expansive growth, m is the cell elasticity coefficient, P is cell turgor, and Y is the turgor pressure at which cell expansion begins. This relationship indicates that any factor that modifies plant water status and reduces turgor pressure (Ψ_p), such as drought or salinity, reduces cell growth. Studies subsequent to the Lockhart equation establish the existence of a linear relationship between plant growth and turgor (Passioura and Fry, 1992). However, background information supporting the most recent plant growth theories indicates that cell elongation no longer depends on turgor (Maggio et al., 2006). Bressan et al. (1990) demonstrated that after an acclimation period to osmotic stress, the relationship between cell division and elongation is altered and is not controlled by turgor or the osmotic status of the cell; this indicates that there must be proactive genetic control of the integrated growth process that can be reset after osmotic stress acclimation and which results in slower growth with equal or higher turgor levels.

Another relevant factor in maintaining and/or increasing turgor (Ψ_p) of *L. corniculatus* under drought conditions was its capacity to modify leaf size. The leaf area ratio decreased by approximately 30% under drought conditions (WWS) compared with the control treatment (NWS), which was associated with a sharp decrease in SLA and a modification in aerial biomass partitioning. The latter is expressed as an increase in LWR in WWS compared with NWS (Table 3). This phenotypic plasticity resulted in smaller-sized, but very turgid leaves under water-stress conditions. Similar results were reported by Inostroza and Acuña (2010) for white clover and Acuña et al. (2010) for *L. tenuis*.

Water-use efficiency was estimated in two growth periods and significant differences were observed in both periods among cultivars and a close relationship between WUE and shoot DM production under water stress conditions was found (Figure 4). Cultivars with divergent WUE were 'Quimey' and 'Empire', with the highest and lowest WUE value and DM production, respectively, under water stress conditions (Figure 4). Unlike species such as wheat (Condon et al., 2004) and white clover (Inostroza and Acuña, 2010), the WUE of *L. corniculatus* decreases significantly ($P < 0.001$) under WWS conditions compared to the control (NWS; Figure 5). Carter et al. (1997) evaluated WUE in *L. corniculatus* with a methodology similar to the one used in the present study and observed that it was not affected by water treatment. Both findings are of great importance in the breeding of cultivated species and provide information concerning the recent controversy over the relationship

between WUE and yields under water-stress conditions. Blum (2005; 2009) argued that high yield potential and high yield under water-limiting conditions are generally associated with reduced WUE, mainly because of high water use (transpiration). Accordingly, any phenotypic trait that increases water-use under drought conditions will also increase yield under stress. In contrast, phenotypic traits linked to low yield potential, such as smaller plants or a short duration of growth, have a high WUE because they reduce water use and yield. Blum's (2005; 2009) argument contradicts current views, which is strongly supported by theoretical and empirical studies (Richards et al., 2002; Condon et al., 2004), where a high WUE is associated with highly productive genotypes under drought conditions; this coincides with the results obtained in this study (Figure 4). The cultivar with the highest water use and DM yield under drought conditions (WWS) was 'Quimey', and this coincides with Blum's (2005 and 2009) arguments. 'Quimey' was also the cultivar with the highest WUE. Given our results, the water use and DM yield of *L. corniculatus* sharply decreases under drought conditions compared with the control treatment, but yield reduction was higher (-75%) than the water-use reduction (-65%). For this reason, WUE was lower in WWS than in NWS conditions (Tables 1 and 3; Figure 5). Considerable variability in WUE and its components was observed (water use and DM yield) under drought conditions (WWS) among the seven cultivars under study; those that captured more water achieved a higher yield and thus a higher WUE (Figure 4).

CONCLUSION

In conclusion, *Lotus corniculatus* exhibits a high capacity to induce osmotic adjustment (OA) under drought conditions. This is an intrinsic characteristic of the species since all the cultivars evaluated in the present study, originating from five countries on two continents, showed the same ability. Those cultivars that showed a higher accumulation of solutes under drought conditions (lower Ψ_π) were negatively affected in their DM production. In this respect, OA only accomplishes a relevant role in the plant's survival capacity under stress conditions. On the other hand, under water stress (WWS) conditions, cultivars that had the highest water-use efficiency (WUE) produced the highest DM yield due to an acclimation strategy with the objective of maintaining water status (Ψ_p) and ensuring continuous gas exchange (T or water use). The strategy is based on saving water, where stomatal conductance (g_s) plays a fundamental role, but is not the only factor, and also includes a decrease in the transpiration area (specific leaf area and leaf-area ratio), a reduction in gas exchange (g_s and transpiration rate), a modification of aerial biomass partitioning (leaf-weight ratio), and the maintenance of cell turgor (Ψ_p) by OA. Together, these generate a phenotype with small and very turgid leaves that allow the maintenance of plant growth under drought conditions.

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