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Strategies for Selecting Drought Tolerant Germplasm in Forage Legume Species

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1. Introduction

The growing demand throughout the world for good quality soils to expand more profitable productive systems (crops, fruits and vineyards) is displacing forage crop production to marginal environments that often have soils with low fertility, pH problems, poor drainage and subject to periods of drought. The situation is similar in Chile, but the problem is exacerbated by the effects of global climate changes, with an estimated decline in precipitation of 40 percent and an increase of 2-4ºC in continental temperature by the end of this century. In this context grasses are increasingly being cultivated beyond their limit to adapt in areas where the ability to tolerate environmental stresses is an essential characteristic for success.
The breeding of forage *Lotus* species and other perennial forage legumes has historically presented low rates of genetic progress because of their genetic complexity. The majority of forage legume species are polyploid, cross-pollinated and self-incompatible. These characteristics make it necessary to use plant-breeding methodologies that are inefficient and very time-consuming. The most popular selection strategies have been masal and recurrent phenotypic selection. Neither strategy considers progeny tests, because they are not efficient in the selection of characters of low inheritability, such as dry matter production. As well, commercial cultivars are the products of many genotypes that generate heterogeneous combinations, which hinders evaluation and prediction of genetic merits. Consequently, strategies are essential to improve our understanding of the genetic components that determine the expression of phenotypic traits of agronomic and adaptive interest. The objectives of this review are to describe the work carried out by the National Institute for Agricultural Research (INIA) of Chile, and the methodologies and results of its work in selecting naturalized populations of forage legume species to develop a broad genetic base for breeding.

2. The use of naturalized and introduced genetic resources

The collection and conservation of germplasm of vegetal species developed in recent decades from around the world and in particular from Chile has provided abundant genetic material for improving cultivars to increase food production on soils with increasing

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limitations, such as water. The INIA-Chile germplasm banks maintain collections of forage species of the genera *Lotus*, *Trifolium* and other legumes and grasses, the majority of them naturalized in the country, which come from areas with limited rainfall or irrigation and intense competition for water resources with high-yield crops. This germplasm was collected in the 1990s and in the last decade have been characterized in relation to abiotic stresses, in particular water stress. This review focuses on *Trifolium repens* and three species of *Lotus* (*L. corniculatus*, *L. tenuis* and *L. uliginosus*) given their current economic importance despite their low efficiency in the use of water resources, or the potential of these species in marginal areas with limited availability of water, respectively.

During 1990, Ortega *et al.* (1994) collected 51 naturalized Chilean white clover (*Trifolium repens*) accessions in 26 sites between the 38º15'S and 42º 45'S covering the La Araucanía, Los Rios and Los Lagos Regions (Figure 1). Each accession included 15 plants that were propagated to 9 clones for agronomic and morphological characterization using two levels of soil water availability and two levels of soil phosphorus, under field and greenhouse conditions. Most of the accessions corresponded to medium-leaved white clover. Only one accession, 2-3-X, was classified as ladino or large-leaved white clover. No relations were observed between growth and the soil phosphorus level of the collection sites. However, there was a tendency associated with accessions to show higher response to phosphorus application when the original soil phosphorus level was higher. Two accessions, 2-3-X and 7-1-X, showed higher responses to irrigation and phosphorus application.

Fig. 1. Geographical distribution of collecting sites for white clover accessions (Ortega *et al.*, 1994).

The project "Collection of forage germplasm in the Andean-Patagonian forest of Argentina and Chile" was carried out from 1994 to 1996 for INIA-Chile, INTA (National Institute for Agricultural Technology)-Argentine and the National University of Comahue (Argentine) with the objective of collecting forage germplasm of the genera *Bromus, Trifolium, Elymus* and *Agropyron*. The project covered the Andean-Patagonian region of both countries from 39º S, approximately, to Beagle Chanel (Zappe *et al*., 1996). The project was co-funded by the Cooperative Program for the Technological Development of the Agro-Food Sector in the Southern Cone (PROCISUR). Only seed were collected. The white clover accessions collected (31) were regenerated during 2007 and phenologically and morphologically characterized during 2008-2009 (Photo 1). Growth and seed production were also measured. Superior genotypes with desirable characters for breeding programs were identified.

Photo 1. White clover (*Trifolium repens*) characterization under field conditions. INIA, La Araucanía Region, Chile, 2008 – 2009 (Seguel *et al*., 2010)

The forage *Lotus* spp. germplasm available at INIA-Chile germplasm banks were introduced and collected through the FONDECYT (Chilean National Fund for Scientific & Technological Development) project "Evaluation, collection, and characterization of varieties of *Lotus* spp. in different environments in Central South and Southern Chile" from 1998 to 2001. The project considered the introduction in the country of *L. corniculatus* cultivars from North America, as well as the collection of *L. tenuis* and *L. uliginosus* germplasm in the zones of the country where these species are naturalized, together with a phenological, morphological, and agronomic characterization that included nitrogen fixation in field conditions and condensed tannin concentration in foliage (Acuña *et al*., 2002a, 2002b, 2004, 2008).

Table 1 indicates the origin of *Lotus corniculatus* cultivars introduced in the country. Germplasm considered representative of the global genetic diversity of the species was requested from institutions that had available seeds. *Lotus tenuis* was collected (11 accessions) in the 1998-1999 spring-summer season between the Metropolitan and Biobío

Regions, from 33°S to 38°S (Figure 2). *Lotus uliginosus* was collected in 1991 in La Araucanía and Los Rios Regions (10 accessions), and in 1999 in Los Lagos and Aysén Regions (11 accessions), from 38°S to 45°S, covering a wide range of agroecological conditions (Figure 3). Thirty plants of each accession were taken from each collection site. They were extracted from the soil, transported in polyethylene bags, and provided with an environment that ensured their conservation during transport. A record was kept of the collection sites with georeferenced location data. Similarly, a soil sample (0-15 cm) was taken from each site to conduct a complete chemical analysis (Table 2).

Fig. 2. Geographic distribution of collecting sites for *Lotus tenuis* accessions

The germplasm characterization revealed that there are cultivars of high value in the introduced germplasm of *L. corniculatus* that can be recommended for different environments of the central zone of Chile, as well as for local breeding programs. Information obtained from *L. tenuis* and *L. uliginosus* accessions showed genetic variability in both species. *L. tenuis* germplasm is adapted to clay, medium-textured, or sandy soils with water restrictions and phosphorous deficiency. Therefore, characterized accessions could be used to breed cultivars for low input production systems in constrained environments. *L. uliginosus* accessions include genotypes collected in sites with acidic soils and variable tannin content in plant tissues that may be promising genetic materials for breeding programs with the objective of producing cultivars for animal production systems for the damp and acidic soils in southern Chile.

Cultivar	Origin
Quimey	INIA, Chile
Upstart	Pickseed Canada Ltd., Canada
Norcen	Crops Breeding Association, Minnesota, USA
Dawn	University of Missouri, USA
AU-Dewey	University of Missouri, USA
Steadfast	University of Missouri, USA
Georgia - 1	University of Missouri, USA
Ganador	INIA, Uruguay
San Gabriel	INIA, Uruguay
Ges-5	CSIRO, Australia
Granger	USDA, Oregon, USA
Viking	Cornell University, New York, USA
Empire	Cornell University, New York, USA

Table 1. Cultivars of *Lotus corniculatus*

Fig. 3. Geographic distribution of collecting sites for *Lotus uliginosus* accessions

				mg kg^{-1}		cmol kg^{-1}				
Accession	**Collecting site**	**pH**	**OM%**	**N**	**P**	**K**	**Ca**	**Mg**	**Na**	**Al**
Lt 1	Cabrero	6.6	3	20	11	0.26	5.56	2.26	0.16	0.01
Lt 3	Yumbel	6.0	4	21	15	0.32	2.30	1.91	0.15	0.01
Lt 4	San Javier	6.1	2	12	2	0.17	5.06	2.46	0.73	0.01
Lt 5	Parral	5.2	6	25	5	0.17	7.58	2.45	0.52	0.11
Lt 6	Parral	5.7	7	19	8	0.33	8.44	3.89	0.60	0,01
Lt 7	Cato	5.9	5	22	11	0.70	8.74	2.33	0.30	0.01
Lt 8	Coihueco	5.2	6	18	11	0.38	5.45	2.01	0.25	0.10
Lt 11	Itahue	6.3	14	9	6	0,36	5.69	1.26	0.33	0.01
Lt 12	Villa Alegre	7.0	7	30	4	0.20	20.14	4.63	0.74	0.02
Lt 14	Melipilla	7.6	4	27	9	0.49	22.03	2.59	0.80	0.02
Lt 15	Las Cabras	6.9	3	12	2	0.22	8.87	1.13	0.54	0.01
Lu 1	Piedra Azul	5.2	33	28	11	0.14	1.41	0.21	0.16	0.72
Lu 2	Caleta Puelche	5.3	6	25	3	0.28	2.24	0.70	0.24	0.75
Lu 3	Contao	5.4	8	21	5	0.34	3.25	1.54	0.36	0.16
Lu 4	Hornopirén	6.2	2	17	3	0.09	1.58	0.52	0.14	0.01
Lu 5	Chaitén	5.2	20	21	4	0.17	0.85	0.48	0.26	0.36
Lu 7	Chaitén	5.2	11	27	6	0.21	0.24	0.21	0.19	0.48
Lu 8	Sur Chaitén	5.4	3	25	7	0.51	3.88	0.86	0.25	0.33
Lu 9	La Junta	5.4	15	28	5	0.12	0.25	0.12	0.16	0.41
Lu 10	Lago Verde	5.8	17	26	3	0.15	1.39	0.45	0.18	0.13
Lu 11	La Junta	5.5	9	47	5	0.10	1.00	0.20	0.10	0.49
Lu 12	Coyhaique	n.o.	n.o.	n.o.	n.o.	n.o.	n.o.	n.o.	n.o.	n.o.
Lu P4	Villarrica	5.2	22	65	7	0.19	2.04	0.43	0.26	0.31
Lu P11	Pto. Saavedra	6.0	6	52	8	0.42	5.03	5.58	0.69	0.01
Lu P23	Vilcún	5.4	25	82	25	0.37	6.57	1.73	0.64	0.10
Lu P32	Liucura	6,0	7	20	6	0.29	5.82	1.03	0.22	0.01
Lu P36	Pitrufquén	5.3	25	84	39	0.45	5.20	1.92	0.69	0.16
Lu P48	Quellón	5.1	27	74	8	0.29	1.22	0.48	0.38	0.87
Lu P52	Castro	5.2	20	73	8	0.31	3.01	1.01	0.44	0.29
Lu P54	Castro	5.0	27	85	14	0.78	2.98	2.17	0.42	0.71
Lu P58	Puerto Varas	5.6	21	67	34	0.33	7.54	1.68	1.44	0.9
Lu P63	Riñihue	5.3	19	61	11	0.59	5.41	1.09	0.24	0.17

Table 2. Soil chemical analyses of collecting sites of *L. tenuis* and *L. uliginosus*. OM : organic matter; n.o.: not observed.

3. Phenotypic characterization

In marginal environments drought and salinity are the abiotic stresses that most limit plant growth and the crop productivity (Reynolds *et al.*, 2001). Drought is defined as the situation in which the water potential and turgor of the plant falls below a threshold that affects its normal functioning (Kramer, 1983). Blum (1996) defines it as the water deficiency of the plant that occurs when the evaporative demand of the atmosphere on the leaves exceeds the capacity of the roots to extract water from the soil. The development of drought tolerant and/or greater water-use efficient (WUE) genotypes is currently a global challenge, owing

to the continued growth of the world population and the reduction of water resources destined to agricultural use (FAO, 2008).

The response of plants to water deficit can be studied through the systematic identification of morphological, physiological and biochemical characteristics that provide the ability to tolerate stress. Many of these have been associated with drought tolerant genotypes (Acuña *et al*., 2010; Araus *et al*., 2002; Condon *et al*., 2004; Inostroza & Acuña, 2010; Poormohammad *et al*., 2007;Reynolds *et al*., 1999; Richard *et al*., 2002), but not all are expressed by the same genotype. To determine which characters confer adaptive advantages under water stress conditions, it is necessary to know the environment in which these genotypes will be established. Donald (1968) proposes determining selection traits through the theoretic conception of a preconceived model plant (ideotype), which must express phenotypic traits that confer adaptation to a specific environment. The use of morphological and physiological traits as indirect selection criteria for forage yield is an alternative breeding approach. However, the limited success of this approach may be due to a lack of understanding of the physiological factors most directly involved in determining yield, in addition to the absence of proper methods for evaluating them in a rapid and routine manner. In this section, some physiological traits related to drought tolerant genotypes will be discussed.

3.1 Plant water status

There are two basic parameters used to describe plant water status, these being (i) the water content and (ii) the energetic state of the water in the plant. The water content is normally expressed based on the water content in full turgor and the term used is the relative water content (RWC). The energetic state is expressed as the water potential of the plant (Ψ_W).

The RWC is the most commonly used expression to measure the level of water in plant tissue, given that it is related to Ψ_W, because the Ψ_W and its components ($\Psi_W = \Psi_P + \Psi_\pi$), the pressure potential (Ψ_P) and the osmotic potential (Ψ_π), are in function of the water volume of the protoplasm (Jones, 2007). The leaf RWC is defined as: RWC = (fresh weight – dry weight) / (turgid weight – dry weight); whereas Ψ_π is measured with a Scholander pump. More antecedents about these traits were broadly reviewed by Jones (2007).

3.2 Stomatal conductance and canopy temperature

Given that water stress affects the photosynthetic capacity of plants because of stomatal closing, genotypes that present greater stomatal conductance under water stress conditions show a greater adaptability to water stress (Medrano *et al*., 2002).

The water state of the plant and its rate of transpiration determine its thermal state (Reynolds *et al*., 1994). It has been demonstrated that the temperature of the leaves can be lower than the air temperature. The degree of cooling reflects the rate of evapotranspiration occurring on the surface of the canopy (Ayneh *et al*., 2002; Jones *et al*., 2002; Möller *et al*., 2007). The temperature of a canopy can be measured with an infrared thermometer and is generally expressed as the difference between the temperature of the canopy and the air, the term assigned to this difference is canopy temperature depression (CTD). The CTD has been strongly correlated with stomatal conductance. Furthermore, increases in grain yields of eight wheat genotypes liberated by CIMMYT between 1962 and 1988 were associated with greater stomatal conductance and cooler canopies (Ficher *et al*., 1998).

The advantage of evaluating stomatal conductance through CTD over direct measurement by porometer is that CTD evaluates a canopy whereas porometry can only be applied to a

leaf from an individual plant. For genetic improvement programs it is recommended to make selections using CTD in large populations. Once the number of individuals in the population is reduced, selection by porometry is recommended.

3.3 Spectral vegetation indices

The yield of a crop during a given period of time and under particular growing conditions is determined by three major process or integrative traits: the interception of incident solar irradiance by the canopy, the conversion of the intercepted radiant energy to potential chemical energy, and the harvest index. The first depends on the photosynthetic area of the canopy, while the second relies on the overall photosynthetic efficiency of the crop. These two processes, which are responsible for the overall crop biomass, are more affected than the harvest index by drought and other related stress typical of Mediterranean growing conditions (Bort *et al*., 2001). In principle, the spectra reflected by crop canopies at different wavelengths through the photosynthetically active radiation (PAR) and near infrared radiation (NIR) regions of the electromagnetic spectrum provide rapid, nondestructive, simultaneous estimations of the two processes determining crops biomass at the canopy level (Peñuela & Filella, 1998). Such estimation requires spectral reflectance indices, which are formulated based on simple mathematical operations among reflectance levels at given wavelengths (ratios or differences).

Nowadays, spectroradiometric indices are used to assess characteristics associated with the development of the photosynthetic area of the canopy. The most widespread spectral vegetation index is the normalized difference vegetation index (NDVI) followed by the simple ratio (SR) (Bort *et al*., 2001).

Leaf area duration affects the total photosynthetic area and thus the radiation accumulated by the canopy, especially in Mediterranean environments where forage crops must confront summer drought that reduces leaf area duration and halts the developments of new photosynthetic area. Early senescence of photosynthetic tissues is characterized by chlorophyll degradation, with increases in the ratios of total carotenoids to chlorophyll *a* and chlorophyll *b* to *a*. In this context, spectroradiometric indices able to provide information on the relative changes in these pigments may be appropriate indicators of the duration of photosynthetic organs (Bort *et al*., 2001).

3.4 Chlorophyll fluorescence

Photosynthesis is an essential process to maintain crop growth and development. Chlorophyll is one of the major chloroplast components for photosynthesis, and relative chlorophyll content has a positive relationship with the photosynthetic rate (Guo & Li, 1996). Furthermore, focusing on the stay-green trait, in effect the ability of the plant to maintain high chlorophyll content for long periods of time, has been proposed as a strategy to increase crop production, particularly under water-limited conditions (Guo *et al*., 2008).

In addition, photosystem II (PSII) is an important component of plant photosynthesis that is particularly sensitive to water deficit conditions (Lu and Zhang, 1999). Drought-induced decrease in photosynthesis has been associated with perturbations of biochemical processes (Graan & Boyer, 1990) and photodamage of PSII reaction centers (He *et al*., 1995). While chlorophyll fluorescence is widely accepted as an indication of the energetic behavior of photosynthetic system, it is emitted mainly by PSII in the range of 680–740 nm spectral region and can be considered an intrinsic indicator of the destination of excitation energy (Dau, 1994).

Several fluorescence parameters, such as initial fluorescence (Fo), maximal fluorescence (Fm), variable fluorescence (Fv=Fm-Fo) and maximum/potential quantum efficiency of PSII (Fv/Fm), have been widely used for investigations into various plants species under diverse growth conditions (Araus *et al*., 1998; Guo *et al*., 2008).

3.5 Water use efficiency

In environments where water availability limits growth and output, crop yields (CY) can be expressed using the following identity: $CY = WU \times WUE \times HI$, where WU is the total of water used by the crop (evapotranspiration), WUE is the ability of crop to produce biomass per unit of water evapotranspirated and HI the crop harvest index (commercial biomass/total biomass). If the three terms are independent, an increase in any of them implies an increase in CY. For many decades the genetic improvement of forage legumes has been oriented to increasing forage production and persistence. However, there is recognition of the need to address the adaptability of plants to climatic change (Humphreys, 2005; Taylor, 2008).

Water use efficiency is defined as the fraction of accumulated biomass, expressed as the rate of CO_2 assimilation (A), or total biomass production per volume of water consumed, which can be expressed as the transpiration rate (T), evapotranspiration or the total of water taken into the system. The time scale to define WUE can be an instant, a day or a growth season (Sinclair *et al*., 1984). There are few genetic improvement programs oriented to increasing WUE owing to the scarcity of evaluation methodologies. These are generally very time-consuming, costly and require a large number of plants under uniform climatic conditions (Teulat *et al*., 2001).

On the other hand, theoretical (Farquhar *et al*., 1982) and empirical approximations (Farquhar & Richards, 1984) show that ^{13}C isotopic discrimination ($\Delta^{13}C$) can provide an indirect measurement of WUE. This has stimulated research into the potential use of $\Delta^{13}C$ as a selection criterion in genetic improvement programs. Experimental evidence currently shows a linear and negative relationship between $\Delta^{13}C$ and WUE in crops such as wheat, barley, peanuts, tomatoes, and cowpeas (Acevedo *et al.,* 1997). As well, linear and positive relationships have been observed between $\Delta^{13}C$ and yields for different crops such as wheat, barley, beans, peanut, cowpeas and tomatoes (Acevedo *et al*., 1997). The main advantage of using $\Delta^{13}C$ in genetic selection programs is its high inheritability, which is mainly due to the low level of genotype x environment interaction (Teulat *et al*., 2002).

3.6 Theoretical relationship between $\Delta^{13}C$ and WUE

The WUE at the level of the leaf is defined as the assimilation of CO_2 per unit of transpirated water and is represented with the following equation (Condon *et al*., 2002): $EUA = 0.6C_a(1 - C_i/C_a) / (W_i - W_a)$, where the factor 0.6 corresponds to the diffusivity coefficient between CO_2 and water vapor in the air, C_a and C_i are the concentration of CO_2 in the atmosphere and in the vegetal cell, respectively, and W_i-W_a is the gradient water vapor concentration between the interior of the vegetal cell (W_i) and the atmosphere (W_a). If the W_i-W_a gradient is considered as an independent variable, the equation indicates that WUE is a negative function of the C_i/C_a ratio. As well, the C_i/C_a ratio depends on the balance between stomatal conductance (g_s) and the pohotosynthetic capacity of the plant. The supply of CO_2 to the interior of the leaf is influenced by g_s, while the photosynthetic capacity determines the demand for CO_2. On the other hand, theoretical and empirical

evidence shows that ^{13}C isotopic discrimination ($\Delta^{13}C$) is an indicator, in direct proportion, of the C_i/C_a fraction and is consequently an estimator of WUE (Condon *et al*., 2002; Farquhar *et al*., 1989). As noted above, WUE depends on the balance between stomatal conductance (g_s) and the photosynthetic capacity of the plant. If a higher WUE is the product of an increase in the photosynthetic capacity of the plant, it results in higher DM production. On the other hand, if higher WUE is a product of an increase in gs, it results in lower DM production.

4. Strategies for selecting germplasm

4.1 Selection of contrasting genotypes

In regions with Mediterranean climates, like central Chile, the annual rainfall is concentrated in winter (approximately 80%) when lower temperatures limit plant growth. On the other hand, water limitations for perennial forage species during spring and summer are strong and affect their productivity and persistence. Therefore, genotypes tolerant to water shortage for long periods are required for these environments, where water for irrigation is scarce and there is a competition for this resource between crops and pasture.

The aims of germplasm characterization at the first stages has been to identify genotypes that show contrasting degrees of tolerance to water stress for their survival, growth and productivity, in effect, to identify tolerant and sensitive materials relevant for genetic and physiological studies of physiological traits, quantitative trait locus (QTL) and genes that modulate drought tolerance or sensitivity in forage species. This information will allow for increasing the efficiency of selection in breeding programs aimed at developing cultivars better adapted to conditions of limited moisture without affecting their productivity or forage quality.

In this sense, an experiment was carried out as part of the project 'Lotus adaptation and sustainability in South American soils' (LOTASSA; http://www.lotassa.org/online/site/) funded by the EU. The growth and plant water status of eleven populations of *L. tenuis*, naturalized in Chile, and a cultivar of Argentinean origin of the same species, were evaluated to select contrasting genotypes (Acuña *et al*., 2010). The cultivars were grown in a greenhouse under different levels of soil water availability. The relative rate of stem elongation (RRSE), DM production, RWC and specific leaf area (SLA) all showed significant reductions in the treatment with the highest water restriction (10% of soil water availability). There were significant differences among genotypes in RRSE and DM production means, but not in RWC and SLA. The drought sensitivity index (DSI) varied broadly among genotypes, from 0.49 to 1.34, and was correlated negatively with DM production under water stress. It was concluded that the *L. tenuis* populations showed genetic variability in water-stress tolerance, with accessions Lt14 and Lt4 at the extremes of tolerance and sensitivity, respectively (Acuña *et al*., 2010). These findings will permit to identify chromosomal regions associated with drought-tolerant genotypes and accelerate the development of cultivars adapted to water-restricted environments. Figure 4 shows the two contrasting populations in terms of their genotype x environment interaction. The population Lt14 showed a regression coefficient (b) greater than one, which means that this population has a high response capacity to the environment. As growth conditions improve, the population Lt14 responds with increased DM shoot growth. In contrast, the population Lt4 showed a b-value of less than one (0.51).

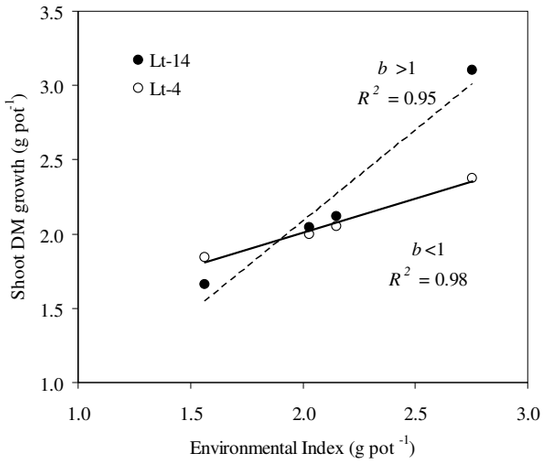

Fig. 4. Response of contrasting *L. tenuis* populations to soil water treatments as calculated by the Finlay & Wilkinson (1963) methodology. Coefficients of regression (b) and determination (R^2), (Acuña *et al*., 2010).

4.2 Selection of high water use efficiency genotypes

In contrast to the *Lotus* species, white clover is a highly sensitive species to water deficits owing to its shallow root system (Hart, 1987). As well, under drought conditions it does not reduce stomatal conductance, as many other forage species do (Karsten & MacAdam, 2001). Consequently, in central Chile, where a Mediterranean climate predominates, white clover is cultivated exclusively under irrigated conditions. In this sense a high water use efficiency cultivar is necessary to increase water-use productivity.

On the other hand, white clover populations naturalized in Chile represent an abundant source of genetic diversity that can be used in the search for genotypes that express high efficiency in water use. Work was carried out in 2009 (i) to evaluate the WUE of the nine populations collected and selected by Ortega *et al.* (1994), and (ii) to identify morphological and physiological characteristics associated with genotypes with high WUE, to assist in the selection of plants for the genetic improvement of the species for this trait (Inostroza & Acuña, 2010). The work arrived at several conclusions. First, the evaluated white clover populations showed wide differences in their WUE, which can be attributed to the intrinsic ability of each genotype to regulate stomatal opening and changes in DM partition induced by water stress. Higher WUE in white clover is associated with genotypes that show less investment of DM in transpirant surface and favour the growth and development of stolons. In this sense, the 9-1-X population was the most efficient in water use. Second, it was concluded that under the experimental conditions, the RWC, the stem water potential and ^{13}C isotopic discrimination are good indicators of the water state of the plant, but do not allow for identifying differences among populations and do not show an association with WUE. Third, the crop water stress index (CWSI) calculated in the treatment without stress, the fraction of intercepted photosynthetically active radiation (FIPAR), the leaf weight ratio, LWR (LWR=leaf DM/shoot DM), and the leaf-area ratio, LAR (LAR= specific leaf area (SLA) x LWR), together form integral physiological traits to estimate WUE in the studied white clover populations. Additionally, this work identified a naturalized white clover population that is highly efficient in water use and reaches the same levels of DM

production as commercial cultivars (Huia and Will) with 25 % less water used. High white clover WUE was also associated with genotypes that can regulate the water loss through the stomata and modify its biomass partitioning under water stress conditions.

5. Plant perception and gene regulation under drought stress

Unlike other organisms, plants do not have the possibility of physical movement to escape adverse conditions. Therefore, plants have to develop adequate systems of perception and response to confront adverse environmental conditions. The ability to perceive water deficit early on constitutes an advantage and permits a rapid response to new environmental scenarios. This phenotypic plasticity is related to the activation of defined sets of genes (Harb *et al*., 2010).

5.1 Hormonal signals

Plant perception of drought stress is mediated by phytohormonal signals involving auxin, cytokinin, jasmonic acid (JA), salicylic acid, ethylene, gibberellins and abscisic acid (ABA) (Huang *et al*., 2008). However, different plant hormones have been described to regulate similar processes, although exerting distinctive domains over transcriptional response (Nemhauser *et al*., 2006). Processes such as stomatal movement are controlled by interactions among various hormones. These hormonal combinations are specific to environmental conditions, period and species. Some hormones act as antagonists, while others function cooperatively in a common response. The effect of ethylene during drought stress has been described as antagonistic of ABA, thus inhibiting stomatal closure that is induced by ABA. Ethylene probably contributes to maintaining a basal photosynthesis level, but induces leaf senescence (Spollen *et al*., 2000; Tanaka et al., 2005). It has been suggested that hormones, including ethylene, gibberellic acid (GA) or auxin, may modulate drought stress signalling by acting antagonistically to ABA. This is supported by evidence that genes down-regulated by ABA are responsive to these hormones (Huang *et al.,* 2008). Other group of genes are up-regulated or down-regulated by drought with a differential effect of individual hormones. For example, *GASA1* encodes for a related GAST1 gene of Arabidopsis that is gibberellin-regulated (Herzog *et al*., 1995). GASA1 is repressed by brasinosteroids (BR) and drought, but induced by ABA and rehydration (Bouquin *et al*., 2001; Huang *et al*., 2008).

Drought stress is commonly associated with a decrease in cytokinins (CKs) (Davies & Zhang, 1991), which act as antagonists of ABA response. Mediating the expression of P_{sark}::IPT in tobacco plants, Rivero *et al*., (2010) obtained a sustainable photosynthetic system, preventing its degradation by drought. It was a consequence of constant expression of genes coding for photosystem complex proteins (Rivero *et al*., 2010). Additionally, high cytokinin levels caused activation of BR synthesis and repression of ABA signalling response. Higher abundance of proteins involved in photosynthesis, respiration, aminoacid and protein synthesis, antioxidant defence system have been reported by Merewitz *et al*., (2011) in creeping bentgrass expressing an ipt gene. By the other side, jasmonic acid is known by regulate of induced systemic resistance (ISR) which is triggered by plant interaction with beneficial soil-borne microorganism (Van Wees *et al*., 2008). In plants methyl jasmonate (MJ) was described as inductor of ABA biosynthesis in rice, enhancing response to drought stress (Eun *et al*., 2009).

5.2 Hormonal control ABA dependent pathway

One of the better-known processes for ABA signalling is triggered by stomatal closure in guard cells. Most of the ABA signal transduction components have been identified in Arabidopsis and found by classical forward genetics in mutants with high or low sensitivity to ABA (Schroeder *et al*., 2001).

The most important and heavily studied signal responsible for growth and development under drought stress is abscisic acid (ABA). Under drought conditions, ABA accumulates in plants and catabolizes when the stress disappears (Koorneef *et al*., 1998; Taylor *et al*., 2000). The response of plant requires hormonal synthesis and accumulation, which is why ABA-dependent tolerance mechanisms are considered as an adaptive stress response (Bray, 1997; Chandler & Robertson, 1994; Xiong *et al*., 2002; Yamaguchi-Shinozaki & Shinozaki, 2005). ABA accumulation is mediated by both the liberation of biologically active hormones and the activation of their *de novo* synthesis. Conjugated ABA has been suggested constitute a reserved or stored form of ABA. Conjugation reaction is mediated by ABA-glucosyltransferase and compartmentalized in vacuole or apoplastic space as ABA glucose ester (Cutler & Krochko, 1999; Dietz *et al*., 2000; Xu *et al*., 2002). To make ABA immediately available, for example during a drought stress, a β-glucosidase converts inactive conjugated ABA (ABA-glucose ester) into active ABA (Lee *et al*., 2006). Biosynthetic pathway genes are mainly activated by 9-cis-epoxycarotenoid dioxygenase (NCED), which catalyzes limit step for the ABA metabolic pathway (Iuchi *et al*., 2001; Qin & Zeevart, 1999; Thompson *et al*., 2000; Xiong & Zhu, 2003). ABA is transported from roots to leaves by long distance translocation (Wilkinson & Davies, 2002).

Several types of proteins, among them phosphatases, kinases and transcription factors, constitute ABA signal transduction pathways. Intracellular receptors have been discovered recently and named PYR/PYL/RCAR (Pyrabactin resistance/PYR-like/Regulatory components of ABA receptors). During signal perception of ABA, these receptors interact with the clade A of protein phosphatases type 2Cs (PP2Cs) and inhibit their action in an ABA-dependent manner, resulting in the activation of several SnRK2-type kinases (Holappa & Simmons, 1995; Kelner *et al*., 2004; Yoshida *et al*., 2002). These proteins modulate the activity of components located downstream, mediating phosphorylation of transcription factors (TFs) AREB/ABF (Johnson *et al*., 2001). Under non-stress conditions, PP2C interacts with and phosphorylates SnRK2s-inhibiting kinase activity (Cutler *et al*., 2010). Transcription factors dependent on the ABA signal transduction pathway interact with *cis* elements present in the promoter region of regulated genes, leading to activation of the expression of different stress-responsive genes (Shinozaki *et al*., 2003; Yamaguchi-Shinozaki and Shinozaki, 2005). Primary *cis* elements that bind AREB/ABF TF are termed ABRE (ABA responsive element), originally identified in the Em gene from *Triticum aestivum* and Rab16 from *Oryza sativa* (Guiltinan *et al*., 1990; Mundy *et al*., 1990). In general, the presence of ABRE motive is not sufficient for efficient induction of gene expression, requiring participation of additional regulatory elements (Shen & Ho, 1995; Shen *et al*., 1996), or repetitions of ABRE motive (Uno *et al*., 2000). Additionally, some genes induced by drought through ABA-dependent signal pathway do not have ABRE sequences in promoters. Activation requires the presence of sites for MYBR and MYCR binding, recognized by transcription factors from the MYB and MYC family, which are also induced by endogenous ABA accumulation (Abe *et al*., 2003).

The ABA-dependent signal pathway activates several groups of genes, in particular genes related to compatible osmolytes and LEA proteins, protection against oxidative stress as

ELIP proteins (early light-inducible proteins) or scavenger enzymes like ascorbate peroxidase, superoxide dismutase and catalase (Apel *et al*., 2004; Finkelstein *et al*., 2002; Gao *et al*., 2004; Zeng *et al*., 2002).

5.3 Redox signals

The strategy that photosynthetic organisms have devised to acclimatize to adverse conditions is to maintain photosynthetic efficiency as high as possible. Environmental changes are related to changes in the redox potentials of electron chain transporters or associated components (Thioredoxin, Glutation), which constitute the primary signals to regulate chloroplast or nuclear gene expression.

Redox regulation of photosynthetic genes occurs at multiple levels of expression, suggesting the existence of a complex network of signals. Chloroplast gene expression is in part modulated by nuclear factors that carry out functions including RNA transcription, editing processing, degradation and translation. Several of them are components of basic expression machinery, while others are required specifically for expression of small gene subgroups from chloroplasts (Pfannschmidt, 2003). Plastidial signals can also regulate transcription of nuclear gene coding of both plastidial and non-plastidial proteins. These signals are related to derivative compounds from photosynthetic chloroplast metabolism, such as porphyrins, reactive oxygen species (ROS), carotenoids and tetrapyrrol (Johanningmeier *et al*., 1988; Rodermel, 2001; Surpin, 2002). An example of these signal compounds is hydrogen peroxide, which is produced in plants under normal conditions. Levels of H_2O_2 are efficiently regulated by a detoxifying system. However, abiotic stress like drought produces an oxidative imbalance, caused by increased H_2O_2 levels, activating the redox signal. Hydrogen peroxide modulates expression of genes coding for detoxifying enzymes and H_2O_2 biosynthesis regulators (Neill *et al*., 2002). H_2O_2 also participates, together with ABA, as a signal for stomatal guard cell movement (Desikan *et al*., 2004). Mutant plants abi1-1 and abi2-1 exposed to high levels of light showed diminished expression of cytosolic ascorbate peroxidase (APX), a demonstrated form of H_2O_2–induced gene expression (Gupta *et al*., 1993). These results suggest that there is crosstalk between ABA and H_2O_2 signal pathways, enhancing the response (Fryer *et al*., 2003). H_2O_2 also induces the expression of superoxide dismutase (SOD) and malic enzyme (ME) (Slesak *et al*., 2003).

In summary, H_2O_2 constitute a part of the redox signal in chloroplasts, regulating nuclear genes related to detoxifying functions that protect protein components in chloroplasts, inducing acclimation of photosynthesis to environmental stimuli (Noctor *et al*., 2000).

5.4 Sugar signals

Sugars play a regulation role, coordinating resource use and localization. Sugars effectively adjust metabolism to confront environmental changes. Sugar signals act in a complementary manner and amplify early signals during drought stress for metabolic control. Although the response is slow at the genic level, sugars allow persistent and sustained intensity of exchange, which is not possible with other types of signal regulation.

Soluble sugars regulate gene expression of several cellular functions and metabolic pathways. However, in general, sugars induce gene expression of proteins related to biosynthesis, utilization and storage of reserve compounds (starch, lipids and proteins), while suppressing expression of enzymes related to photosynthesis and reserve

mobilization (Koch, 1996). Among the genes regulated positively by sugars are storage enzymes like patatin and sporamin in potato, starch and sucrose biosynthesis enzymes like ADP glucose pyrophosphorilase (AGP), invertase, sucrose synthase and genes for protein defence like proteinase II inhibitor (Geigenberg., 200; Jefferson *et al*., 1990; Kim *et al*., 1991). In contrast, numerous genes are negatively regulated by sugars, such as genes coding for alpha-amylase in rice, endopeptidase, sucrose syntethase and asparagine synthase in corn roots or malate synthase and isocitrate lyase in cucumber cotiledons (Koch, 1996).

One of the main sugars described in photosynthetic gene repression is glucose. Studies of promoters of photosynthetic genes fused to GUS have described repression by glucose and sucrose (Sheen, 1990). Similar conclusions have been obtained by other researchers using modified plants that accumulate high hexoses levels (Dickinson *et al*., 1991). Examples include CAB protein, δ-ATPase and enzymes involved in the glyoxylate cycle, the expression of which is inhibited by sugars (Harther *et al.,* 1993; Krapp *et al*., 1993; Sinha *et al*., 2002).

5.5 Effect of drought stress on legumes nodules

Nitrogen fixation in legumes is severely affected by drought, salinity, defoliation, darkness and chilling, among other factors. Decline in nitrogen fixation has been related to reduced permeability of the diffusion barrier for oxygen flux in the nodule and thus prevents nitrogenase damage (Hunt & Layzell, 1993). Water stress inhibits sucrose synthase activity, which increases sucrose concentration in nodules, as a result of osmotic regulation, but negatively affects bacterial respiration, limiting carbon flux (Arrese-Igor *et al*., 1999; González *et al*., 1995). González *et al*., (1998) did not find differences in the expression of SS during ABA treatment, but did find decreased expression of the leghemoglobin gene (Lb).

Abiotic stress also induces production of ROS in nodules and turns an oxidative stress that could be a cause of biological nitrogen fixation decline. Evidence suggests that the main targets of ROS in nodules are SS and Lb (Marino *et al*., 2006).

Nodule specific genes have been described that are regulated by abiotic stress and play a putative role in response to stress to facilitate the nitrogen fixation. Three cysteine cluster protein (CCP) and LTP genes were found in *A. sinicus* during salt treatments (Chen *et al*., 2007).

5.6 Summary of local research

Studies in *Lotus spp.* have been directed to describing the effects of drought stress and response. It has emphasized the role of nodules in nitrogen fixation. Nitrogen-fixing nodules are formed by the interaction between rhizobia and legume roots through the integration of two processes, infection by rhizobia and initial cell division in the cortex of the roots. The number of nodules is regulated by several factors, including drought stress. The abundance of nodules must be regulated to avoid interfering with nutrient distribution. Early nodulation inhibits further nodulation in young roots (Oka-kira and Kawaguchi, 2006). A chitinase Ltchi7 was recently described (Tapia *et al*., 2011) that is induced during drought stress in *Lotus tenuis,* a drought tolerant *Lotus* species. Ltchi7 is a class III chitinase, which is part of a subgroup of chitinases from legumes with an additional COOH terminal domain. Nodule chitinases are related to lipochitooligosaccharide (LCO) cleavage. LCOs act as signals in nodule organogenesis and are synthesized by bacteria in the initial stages of nodule formation (Mergaert *et al*., 1997). It is suggested that this type of chitinases has a role

in regulating LCO abundance in root proximity during drought stress and contributes to maintaining control in infection and nodule formation (Tapia *et al*., 2011).
On the other hand, research to study the functional role of nsLTPs in *Lotus japonicus* during water deficit has been developed. Expression analysis mediating RNA blotting experiments, in situ hybridization, promoter GUS fusion have demonstrated that nsLTPs are expressed in an epidermis-specific manner, particularly in organs with active cuticle synthesis (leaves, stems and flowers) (Kader, 1996; Arondel *et al*., 2000; Clark *et al*., 1999). Additionally, correlations of wax accumulation with increased LTP expression, especially during abiotic stress, support the hypotheses that these proteins are responsible for the lipid load component in the cuticle (Cameron *et al*., 2006; Sohal *et al*., 1999; Treviño & O'Connell, 1998). Genes coding for nsLTPs in *L. japonicus* have been identified, which were drought induced. One of them is expressed in epidermal cells of leaves and shoots. Studies of 3D modelling show similarities in cavity ligand binding for different nsLTPs and apparent structural redundancy. Future studies could reveal the function of these proteins in cuticle formation and drought tolerance.

6. Conclusions

The results obtained have provided genotypes for INIA-Chile's genetic improvement program for forage legumes, in particular *Lotus* spp. and white clover, for generating cultivars that are tolerant to drought conditions for the Mediterranean climate zone and the rainy southern region of the country.

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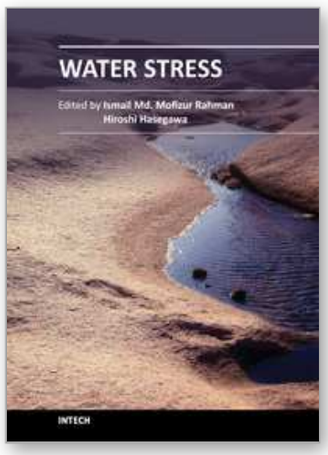

Water Stress

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Plants experience water stress either when the water supply to their roots becomes limiting, or when the transpiration rate becomes intense. Water stress is primarily caused by a water deficit, such as a drought or high soil salinity. Each year, water stress on arable plants in different parts of the world disrupts agriculture and food supply with the final consequence: famine. Hence, the ability to withstand such stress is of immense economic importance. Plants try to adapt to the stress conditions with an array of biochemical and physiological interventions. This multi-authored edited compilation puts forth an all-inclusive picture on the mechanism and adaptation aspects of water stress. The prime objective of the book is to deliver a thoughtful mixture of viewpoints which will be useful to workers in all areas of plant sciences. We trust that the material covered in this book will be valuable in building strategies to counter water stress in plants.

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