

Chapter 1.3

DROUGHT AND SALINE STRESS

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This chapter examines the response of L. japonicus plants to different types of osmotic stress in the context of the changes most commonly noticed in other plant species. Up to 12-fold proline accumulation was detected in L. japonicus plants in relation to drought and salt stress treatments. In parallel to the accumulation of proline as an osmolyte, the above mentioned stress conditions produced in L. japonicus an increased level of oxidative damage as evaluated through measurement of thio barbituric acid reactive substances (TBARS). Characterisation of the L. japonicus response to osmotic stress is a prerequisite for the use of this plant as a model legume to assist in applied projects aimed to improve salt and drought tolerance of other closely related Lotus species. Different Lotus species are currently used to improve both pastures and hay quality wherein other forage legume species are not suitable (see chapter 1.2). However, many of these Lotus species are not well adapted to high temperature, salt, or drought.

INTRODUCTION

Drought and salinity are the major limitations on crop productivity world-wide. One third of the earth surface is classified as arid or semiarid, and in most of the humid region, wherein much of world food is produced, the crops are subjected to periods of severe drought. Moreover, nearly 40% of the world land surface can be categorised as having potential salinity problems (Boyer, 1982). Other abiotic stresses such as atmospheric pollutants, presence of heavy metals, low temperature, or soil acidity can also affect plant productivity and quality (Figure 1). Drought, high salinity, and low temperature induce common metabolic effects because all of them decrease water activity inside the cell. Once stress is set, highly complex biochemical and physiological mechanisms are switched on in order to protect major

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process such as cell respiration, photosynthetic activity and nutrient transport. Also, stress tolerance responses are induced to preserve cell organelles and tissue structures such as meristems (Kramer, 1983).

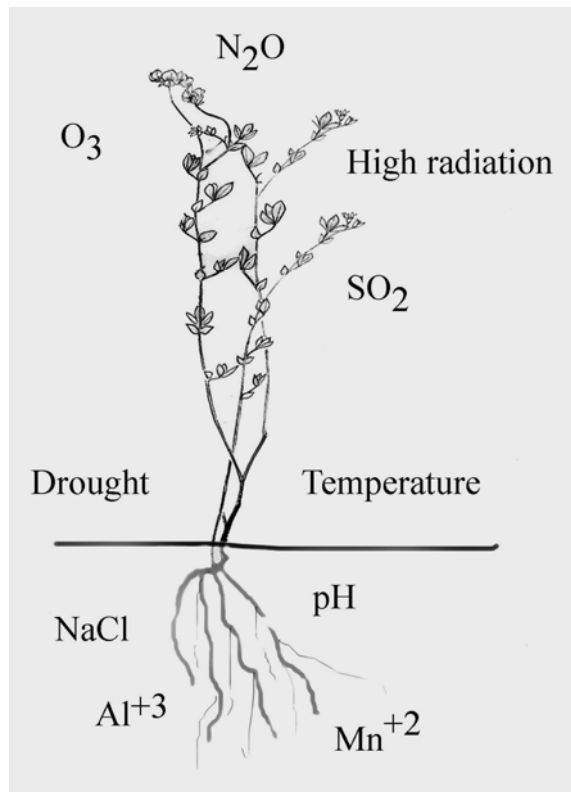


Figure 1. Main types of plant abiotic stresses that may affect growth and productivity of *Lotus* plants.

Animal production in the temperate region is based principally on pastures composed by grasses and legumes. These plants species are subjected to different abiotic stresses that joined with biotic stress decrease growth rate and dry matter production affecting dramatically the plant survival in the field.

Legumes are one of the most important components of the pasture because they are the main crude protein source in the animal food. Amongst the genera used, the most frequently found are *Medicago*, *Trifolium*, and *Lotus*. The last of these species is currently used to improve both pastures and hay quality wherein other forage legume species are not suitable (Papadopolous and Kelman, 1999). Actually, a number of *Lotus* species and cultivars exhibit a wide and different degree of tolerance to a compromised edaphic condition such as low pH, salinity, and high aluminium soil content. Moreover, considering these conditions, almost all *Lotus* species are more tolerant than lucerne or trefoils (Wheeler *et al.*, 1992). However,

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unlike lucerne, *Lotus* species are not well adapted to high temperature, salinity, or drought (Blumenthal and McGraw, 1999).

Recently, the use of molecular techniques, such as genetic markers and other biotechnological approaches are providing new tools to traditional plant breeding programs. In the near future, these approaches jointly with traditional breeding could make considerable progress in obtaining new improved cultivars with higher tolerance to water and saline stresses.

The knowledge of the biochemical and molecular basis of plant responses to stress are required to successfully generate improved stress-tolerance forage legumes. Among all forage species, *Lotus* is a privileged candidate to be improved for higher persistence and productivity in regions where other legumes are no suitable to be sown.

L. japonicus is closely related taxonomically to *L. corniculatus* (the most agronomically important *Lotus* species), and offer a number of advantages for genetic studies. This specie can be seen as an excellent model to understand and improve stress tolerance in *Lotus* spp. In this chapter we make a little background of the main plant responses to drought and salt stress in order to analyse afterwards some of these responses in the genus *Lotus* and, in particular, in the model legume *L. japonicus*.

PLANT RESPONSES TO DROUGHT STRESS

In broad terms, drought can be permanent, seasonal, or random (Kramer, 1983). Permanent drought is present in desert areas wherein lack of rainfall is very common; seasonal drought occurs when either winter or summer seasons are regularly dry. Finally, random drought occurs in humid regions subjected to occasional variation in amount and distribution of rainfall. In addition, another type of drought to be considered is non-apparent drought. It is observed on hot summer days when high temperature or wind conditions induce an increase in transpiration rate that exceeds water root absorption rate. Consequently, despite adequate soil water content, plants can show drought symptoms (Sánchez-Díaz y Aguirreolea, 1993).

Plants have different strategies to avoid the deleterious effects of drought and these can be classified as follows:

1. Drought escapes i.e. short-life annual plants that develop their life cycle exclusively during the year period when soil water is available.
2. Drought tolerance i.e. plants cannot avoid the water stress; consequently they develop a number of adaptation mechanisms aimed to increase drought tolerance.

Both anatomical and morphological strategies are developed in plants to resist dehydration, such as deep and branched root systems or presence of thick cuticle in leaves and stems. All the strategies play an important role in drought tolerance by either increasing the water input by absorption or by reducing water output by transpiration (Kramer, 1983). In addition to these strategies, plant tolerance to

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drought is also mediated by metabolic responses that will be mentioned later in this chapter.

Lotus species in the geographical zones where they are sowed could be exposed to random or non-apparent drought and they are adapted to temperate and humid environments, wherein these types of drought are frequent. Therefore, they show mechanisms to tolerate the drought and not to avoid it.

PLANT RESPONSES TO SALT STRESS

Salt stress caused by high salt levels in the external solution of plant cells produces several deleterious consequences, such as ionic imbalance (Niu *et al.*, 1995;Zhu *et al.*, 1997). Also, when salinity results from an excess of NaCl, by far the most common salt stress, the increased intracellular Na⁺ and Cl⁻ levels became deleterious to cellular systems (Serrano *et al.*, 1999). In addition, not only Na⁺ and Cl⁻ homeostasis is disturbed, but also K⁺ and Ca²⁺ (Hasegawa *et al.*, 2000, Rodríguez-Navarro, 2000, Serrano *et al.*, 1999). As a result, plant survival and growth are dependent on adaptations that re-establish ionic homeostasis, thereby minimising the duration of cellular exposure to ion disequilibrium. Moreover, high concentrations of salt impose a hyperosmotic shock by decreasing the chemical activity of water and causing loss of cell turgor. This negative effect in the plant cell is thought to be similar to the effects caused by drought.

Plants respond to salt stress, as well as drought, at three different levels, i.e., cellular, tissue and whole plant level. Cell-based mechanisms of ion homeostasis and the synthesis of osmoprotectants are essential determinants for salt tolerance.

Salt tolerance mechanisms strategies can be classified as those that function to minimise osmotic stress or ion unbalance, or alleviate the consequent secondary effects caused by the stresses. Some of these mechanisms are similar to drought mechanisms. Other strategies are different, such as ion homeostasis and ion transport systems. The cytotoxic ions in saline environments, typically sodium and chloride, are compartmentalised into the vacuole and used as osmotic solutes (Borsani *et al.*, 2003).

Halophytes have a particular ability to grow under high concentrations of NaCl, unlike halophytes most crop species are sensitive or hypersensitive to salt presence in the growth medium. It seems clear that the NaCl tolerance of halophytes is not the result of adaptive mechanisms or metabolic processes that are unique to these plants (Glenn *et al.*, 1999). On the contrary, it seems that the biochemical mechanisms leading to salt tolerance in halophytes plants, are regulated in such way that allow a more successful response to salt stress than in other plants (Hasegawa *et al.*, 2000). The question to be addressed now is whether the same mechanisms employed by halophytes in salt stress tolerance could be employed in glycophytes without a loss in productivity.

BIOCHEMICAL RESPONSES TO OSMOTIC STRESS

Salinity and drought induce osmotic stress and have similar biochemical responses, that include the accumulation of compatibles osmolytes and increase of reactive oxygen species (ROS) levels (Zhu *et al.*, 1997). According to this, and from a practical point of view, salt stress can be imposed in laboratory settings more easily and precisely by applying controlled osmotic stress.

For several years there has been known that plants respond to drought and salt stress by undergoing biochemical adaptive processes such as ion transport and by accumulating different compounds, named compatible solutes (Hanson and Hitz, 1982; Smirnov and Stewart, 1985). Solute known that accumulate under osmotic stress include carbohydrates, organic acids, polyamines, and amino acids. These molecules can accumulate to high concentration without interfering with cell metabolism (Bray, 1993).

Stressful conditions such as drought or salt stress, associated with high light intensity are able to promote ROS production and oxidative stress. Water and salt stress induced physiological changes such as decrease of the cell water potential and stomata closing. These events, in turn, result in lower CO₂ availability to the green tissues favouring the Mehler reaction that result in superoxide production. Moreover, during drought, plant glycolate oxidase activity is induced generating hydrogen peroxide. The increased activity of antioxidant enzymes such as superoxide dismutase (SOD), peroxidase, and ascorbate peroxidase during stress has been reported in several organisms. Oxidative stress is associated with osmotic stress damage to membrane lipids and nucleic acids. The levels of thio barbituric acid reactive substances (TBARS) can be used to evaluate this damage. Another ROS that are produced in stress conditions are the hydroxyl radical and singlet oxygen. These molecules are inactivated by non-enzymatic systems. Osmolytes such as proline and polyols must act as scavengers (Smirnov and Cumbes, 1989; Alia *et al.* 2001. Hong *et al.*, 1999) and could play a protective role from oxidative damage to macromolecules such as proteins.

***L. JAPONICUS* RESPONSE TO DROUGHT STRESS**

Few reports are available concerning the biochemistry and molecular responses to osmotic stress (drought and salt stress) in *Lotus* spp. While most of reports referred to *L. corniculatus* response to drought (Olsson *et al.*, 1996; Carter *et al.*, 1997; Borsani *et al.*, 1999; and Borsani *et al.*, 2001), none study has been carried out in the biochemistry of *L. japonicus* response to different stress conditions.

The drought imposition form determines the types of biochemical response that the plant will induce. In plants the drought can be imposed in two ways: a) fast drought that can mimic the named non-apparent drought wherein evaporation rate is higher than the water absorption, b) slow drought similar to seasonal and random drought wherein the water loss is slowly imposed. Experimentally the form of drought imposition will determine the biochemical response to be studied.

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L. japonicus plants show a well-developed growth in hydroponic assemblies (Borsani *et al.*, 1999; Orea *et al.*, chapter 7.3, this book) fed with Hornum nutrient solution (Handberg and Stougaard, 1992). When these plants were subjected to fast drought for 12 h, withholding the nutrient solution, the relative water content (RWC) decreased from 90 to 60%. However, when *L. japonicus* plants were grown in plastic pots filled with a soil-type support (vermiculite or montmorillonite-based terragreen^R) the RWC dropped to 50% when they were subjected to slow drought for 4 days withholding the irrigation.

Working with 30 days-old *L. japonicus* plants, fast and slow drought periods should not exceed 12 hours and 4 days, respectively, since for longer drought periods the plants were unable to rehydrate irreversible drought. In these experimental conditions, for both fast and slow drought assays, *L. japonicus* plants recovered 90% of RWC at 6-8 h after re-establishing the water supply.

During the above mentioned drought treatments, the plants showed slight wilt symptoms but no change in pigment contents was observed (Table 1). Therefore, the photosynthetic apparatus appears not to have been seriously damaged by water stress. For this reason, changes observed in other metabolic pathways in response to water stress should not be associated with photosynthesis alterations.

	Control	Drought (12h)	Drought (4d)	Salt stress (4d)
Hydric deficit (%)	14.3 ± 2.5	30.1 ± 6.5	49.5 ± 7.0	15.3 ± 2.2
chlorophyll a (mg. g ⁻¹ DW)	6.54 ± 0.58	6.05 ± 1.64	5.68 ± 1.02	5.82 ± 0.97
chlorophyll b (mg. g ⁻¹ DW)	3.14 ± 0.18	2.70 ± 0.74	2.78 ± 0.43	2.87 ± 0.41
a/b ratio	2.14 ± 0.24	2.22 ± 0.11	2.08 ± 0.10	2.12 ± 0.22
Proteins (mg. g ⁻¹ DW)	115 ± 25	99 ± 18	103 ± 17	111 ± 9
Amino acids (μmol. g ⁻¹ DW)	250 ± 62	263 ± 42	233 ± 18	234 ± 76

Table 1. Hydric deficit, chlorophyll a/b ratio, proteins, and amino acids in wild type leaves of *L. japonicus* plants subjected to drought and salt stress. Values are the mean of four replicates ± standard deviations. All parameters were determined according to Borsani *et al.* (1999).

We have studied the ability of *L. japonicus* plants to accumulate proline as osmolyte in response to drought. Three to 12-fold proline accumulation was detected, respectively, in leaves from plants subjected to fast or slow drought (Figure 2), in spite of the fact that leaves in both treatments began with similar RWC. A concomitant increase in proline could be detected during the slow drought process. In fact, a linear relationship could be observed between the proline content and the hydric deficit produced on the plants at different times of this process (Figure 3).

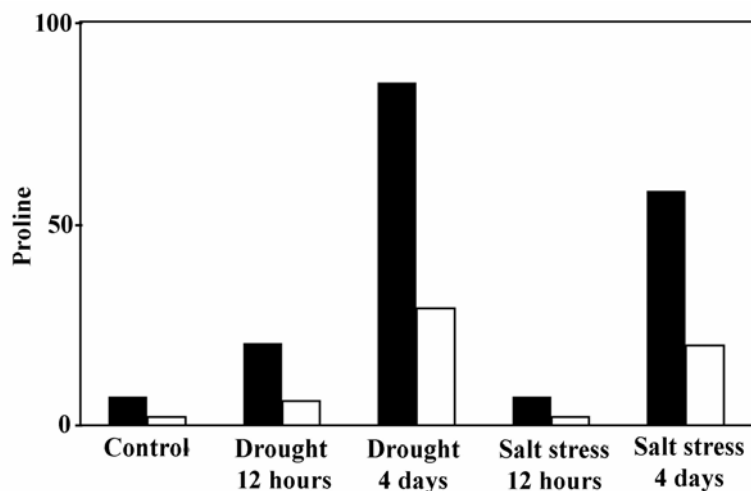


Figure 2. Proline content in *L. japonicus* leaves from plants subjected to different drought and salt stress treatments. Black bars: absolute content of proline (mol per g dry weight). White bars: relative content of proline (% of total amino acid content). Plants were grown as described by Orea et al. (Chapter 7.1, this book). Proline was determined according to Borsani et al. (1999). Other experimental details are given in the text.

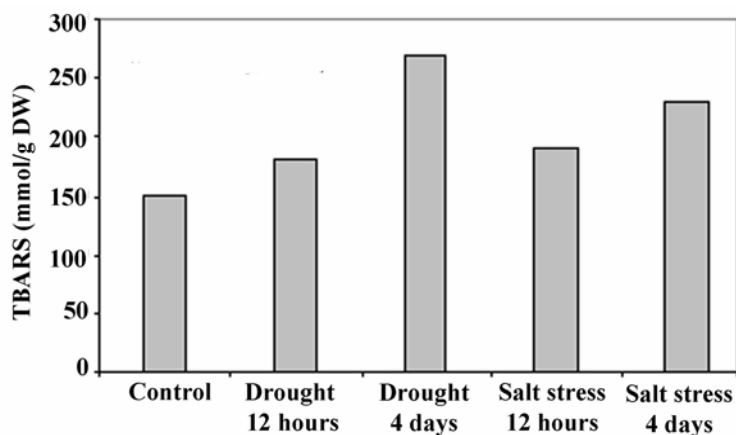


Figure 3. Relationship between Hydric Deficit (HD) and proline content in leaves of *L. japonicus* subjected to different times of slow drought applied by removal of the irrigation to the plants.

Although the amount of proline produced was lower in the fast drought compared to the slow drought, it is important to say that the actual rate of proline accumulation was always higher in the fast drought than in the slow drought if considering the length of time applied in both processes. This result is easy to explain considering that proline accumulation may result from both induction of proline biosynthesis

and/or inhibition of its oxidation (Hong *et al.*, 2000). Moreover, there are previous reports that activation of pyrroline-5-carboxylate synthetase (an enzyme from proline biosynthesis) as well as inhibition of proline dehydrogenase (an enzyme from proline degradation), are both faster in fast drought than in slow drought. An additional explanation considered the oxidation of proline to glutamate through the proline dehydrogenase pathway during the dark period of slow drought treatment (Sanada *et al.*, 1995). Leaf proline accumulation in response to drought seems a peculiar characteristic of *Lotus* since it has been reported in other in other species such as *L. corniculatus* cv. San Gabriel (Borsani *et al.* 1999) and *L. uliginosus*, *L. glaber*, and *L. subbiflorus*. In addition, proline content was higher in ammonium than nitrate fertilized *Lotus* plants suggesting that nitrate could be used as osmolyte instead of proline under water stress conditions in the last case (Diaz *et al.*, manuscript in preparation).

The level of oxidative stress associated with drought in *L. japonicus* has been also examined. Figure 4 shows that the level of TBARS increased up to 80% during drought, being much higher during the slow than in the fast drought.



Figure 4. TBARS content in *L. japonicus* leaves from plants subjected to different drought and salt stress treatments. TBARS were measured as described by Borsani *et al.* (2001).

This result indicates that water loss is able to generate an important extent of oxidative damage in *L. japonicus* leaves. Similarly, in *L. corniculatus* leaves it was

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also shown a drought increased oxidative stress and total SOD activity. Additionally, accumulation of Cu-ZnSOD transcripts as well as the presence of a new Cu-ZnSOD protein were detected in water stressed *L. corniculatus* leaves (Borsani et al., 2001). This enzymatic response may constitute in plants an advantageous adaptation to water stress by protecting the plant cell from oxidative reactions (Bowler et al., 1992). For example, glutamine synthetase purified from *L. corniculatus* leaves was effectively protected against inactivation from in vitro hydroxyl production by addition of proline, sorbitol, and mannitol. According to the scavenger index in decreasing order, the ranking was sorbitol, mannitol, and proline (unpublished data). Molecules as polyamines and nitric oxide could act as non-enzymatic defences scavenging superoxide radical (Bors et al., 1989; Radi et al., 2001).

THE *L. JAPONICUS* RESPONSE TO SALT STRESS

L. japonicus salt stress studies were carried out in similar conditions than for previously described drought studies. Thirty day-old *L. japonicus* plants were subjected to salt stress by irrigating with Hornum NaCl supplemented solution for 4 days until reaching final concentration of 250 mM. Neither wilt symptoms nor changes in pigment contents were observed in this stress conditions. Whether short time salt tolerance of *L. japonicus* plants is due to salt exclusion or compartmentation in vacuole is unknown. However, when plants were subjected to salt stress, but in a lower relative humidity (RH) around 50%, the plants showed a dehydrated phenotype. This fact may indicate that low RH increases transpiration rates and consequently rises in the water and Na⁺ input in *L. japonicus* tissues.

We have studied the extent of accumulation of proline because of application to *L. japonicus* plants of different concentrations of NaCl and for different lengths of time. Figure 5A shows that concentrations higher than 100 mM of NaCl were required to produce significant accumulation of proline after 4 days of salt treatment. Using 250 mM NaCl, this increase in proline was observed at 3-4 days of treatment (Figure 5B). In these conditions the maximum level of proline increased by 7-fold as a result of the salt treatment. This increase in proline happened both in absolute terms, reaching values around 50 µmol per g DW, as well as in its relative proportion with regard to total amino acid content (Figure 2). However, these increases were lower than those observed in *L. japonicus* plants subjected to drought at the same time (4 days, Figure 2). A similar behaviour in proline accumulation was observed in *Triticum durum* subjected to salt stress and drought (Mattioni *et al.*, 1997).

A 50% increase in TBARS species was detected in *L. japonicus* plants subjected to 250 mM NaCl treatment for 4 days (Figure 4). This result also indicates that the salt treatment was sufficient to generate an important but still reversible oxidative damage in *L. japonicus* leaves.

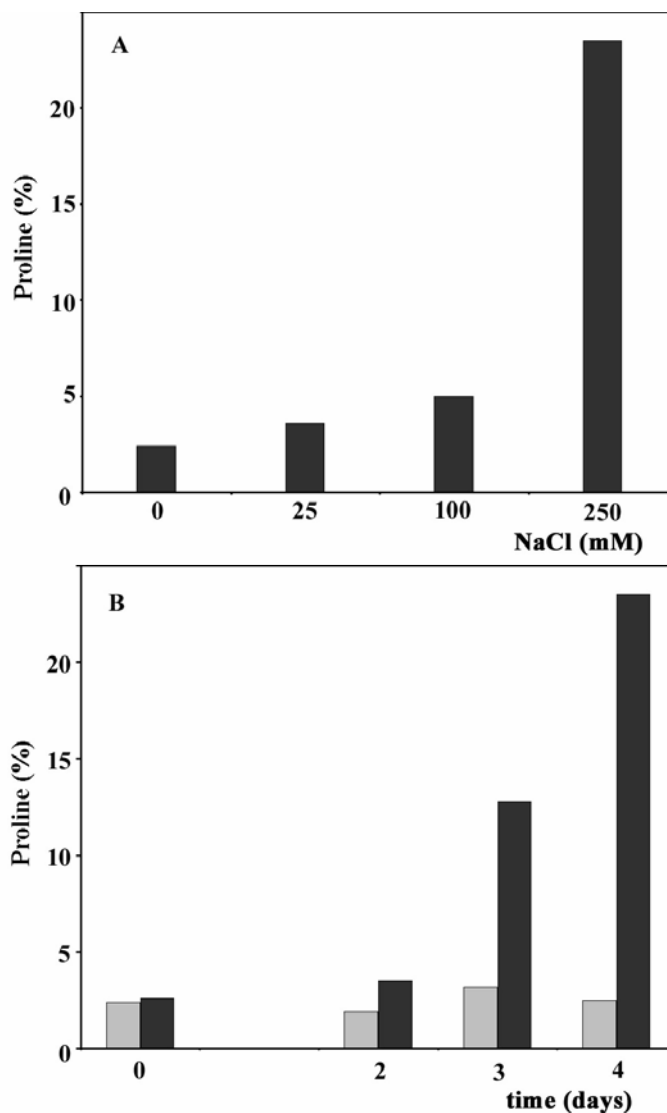


Figure 5. Proline accumulation during salt stress in *L. japonicus* leaves. A: Effect of different NaCl concentrations for 4 days of salt treatment. B: Proline accumulation at different times of treatment with 250 mM NaCl (black bars) and control without NaCl (grey bars). Other details as described in previous figures and in the text.

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