

Assessment of the Relevance of Osmolyte Biosynthesis for Salt Tolerance of Halophytes under Natural Conditions

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ABSTRACT

The study of the mechanisms of response to salt stress is one of the most active research topics in plant biology, not only due to its unquestionable academic interest but also because of its economic implications, since high soil salinity is – together with drought – one of the major causes of reduction of crop yields worldwide. These studies have shown a series of basic mechanisms of response to abiotic stress, which include, among others, the synthesis and accumulation in the cytoplasm of ‘compatible solutes’ or osmolytes, used for osmotic balance and as ‘osmoprotectants’. Today, there is overwhelming evidence that osmolyte, and especially proline accumulation, represents a general and reliable biochemical marker for salt stress. However, and despite the large amount of data available regarding this response mechanism, our knowledge of the importance of osmolyte biosynthesis for salt tolerance of any given species under natural conditions, is still very limited. This is partly due, in our opinion, to the approaches commonly used in these studies, which rely on experiments performed with salt-sensitive plants (glycophytes) – instead of halophytes, plants naturally adapted to high soil salinities – under artificial laboratory or greenhouse conditions. In this review, we describe and comment on data supporting these ideas, and point out that extreme caution should be taken when assessing the biological relevance of laboratory results.

Keywords: plant stress responses, salt stress, salt tolerance, halophytes, osmolytes, proline, stress markers

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INTRODUCTION

Drought and soil salinity are the major environmental factors affecting crop productivity and plant distribution (Boyer 1982; Owens 2001; Bartels and Sunkar 2005; Munns and Tester 2008). Excessive salinity in marginal soils results from natural processes, whereas in arable land it is mostly anthropically generated, due to the progressive accumulation of the ions dissolved in irrigation water (Neumann 1997). This ‘secondary salinisation’ represents an increasing problem for agriculture, especially in the most productive zones of the world, those cultivated under irrigation in arid and semi-arid regions: about half of their surface is already affected by salt stress, to a higher or lower degree, and more than 10 million ha of agricultural land are lost every year due to salinisation (Owens 2001; Munns 2002; Munns and Tester 2008).

The need to improve the salt tolerance of crops – apart from the academic interest of this topic – has stimulated the study of the mechanisms of plant responses to salt stress, at

the biochemical, cellular and physiological levels. As a result of the work carried out over the last four decades, today these basic mechanisms are relatively well known, mainly involving: i) the control of ion homeostasis and osmotic balance; ii) the synthesis of ‘protective’ metabolites and proteins; and iii) the activation of chemical and enzymatic antioxidant systems; these general responses are triggered not only by high salinity but also under other environmental stress conditions causing cellular dehydration (osmotic stress), such as drought and too high or too low temperatures (Zhu 2001; Munns 2002; Wang *et al.* 2003; Vinocur and Altman 2005; Hussain *et al.* 2008).

It is generally assumed that plants rely on the above-mentioned mechanisms to tolerate salt stress, although, paradoxically, most of these studies have been carried out using glycophytes, i.e., salt-sensitive plants, either crops (e.g. tomato, tobacco, maize, or rice) or *Arabidopsis thaliana*, the model species in plant molecular biology, instead of plants naturally tolerant to high salinity – the halophytes – which *a priori* would appeared to be more suitable for this

kind of research.

The definition of halophytes – or halophilic ('salt-loving') plants – is not as simple as it would seem at first sight. Even the terminology used to describe the relationships between plants and salinity is very heterogeneous and often confusing (for extensive comments see: Grigore 2008a, 2008b; Grigore and Toma 2010a, 2010b). For example, halophytes have been defined as plants living in natural saline environments, where they are able to complete their life cycles in the presence of salt concentrations of at least 200 mM NaCl. Although there exists a continuous variation in the degree of plant salt tolerance, from very salt-sensitive species to extreme halophytes, this operational definition seems appropriated, excluding about 99% of all angiosperm species, which will not survive under those conditions (Flowers *et al.* 1986; Flowers and Colmer 2008).

The basic mechanisms of salt tolerance in halophytes seem to be mostly dependent on their capacity to sequester toxic ions (Na^+ , Cl^-) in the vacuoles and to accumulate compatible osmotica in the cytoplasm (Flowers *et al.* 1977; Wyn Jones *et al.* 1977; Glenn *et al.* 1999). Exclusion of ions by the roots, synthesis of specific proteins or activation of antioxidant systems most likely also contribute to the overall salt tolerance of a given species, but the relative importance of these responses is largely unknown. Besides, many halophytes possess a large diversity of additional, often constitutive, mechanisms of defence against high salinity in the soil: salt glands or salt bladders (Grigore and Toma 2010a), succulent tissues and specific photosynthetic adaptations such as Kranz anatomy structure (Grigore and Toma 2010b).

As a very active field in plant biology research, hundreds, if not thousands of reports on the responses of plants to high salinity have been published during the last years – using mostly salt-sensitive species. We can also include here experiments performed to assess the possible increase in salt tolerance of transgenic plants expressing putative halotolerance genes. A few examples of these studies are summarized in **Table 1**.

It is important to note that practically all studies carried out to investigate the mechanisms of salt stress responses use, basically, the same experimental approach. Tissue culture material, germinating seeds, or whole plants at different developmental stages are subjected to increasing salt concentrations (NaCl, generally) for different times, under otherwise optimal conditions – regarding substrate, water and nutrient availability, light, temperature or relative humidity – in the controlled environment of the laboratory or the greenhouse. The 'response' of the plants is then assessed by determining changes in different morphological, physiological or biochemical parameters – which could include from simple growth measurements to the expression of specific genes – in salt-treated plant material, as compared to non-treated samples, which are considered as 'controls'. This artificial situation is different from the 'real life' of plants in their natural habitats, where they are simultaneously subjected to different, uncontrollable environmental stress conditions (Grigore and Toma 2008), which may frequently change, qualitatively and quantitatively, in unpredictable ways, and where the 'non stress' situation simply does not exist. Molecular, biochemical or physiological data on plant responses to salt stress under natural conditions are very scarce, due to the obvious difficulties of performing this kind of research. Therefore, despite the large amount of data obtained in the laboratory and the attempts to generalise them, our knowledge of the mechanisms of salt tolerance operating in halophytes in their natural habitats is still very limited.

The responses of plants to high soil salinity and the possible mechanisms of salt tolerance – and/or tolerance to other abiotic stresses – have been summarized and discussed, with emphasis on different aspects, in many reviews published during the last decade (in addition to references cited above, see for example: Hasegawa *et al.* 2000; Marcum 2002; O'Leary 2002; Sen *et al.* 2002; Tester and Davenport 2003; Sairam and Tyagi 2004; Munns 2005; Par-

vaiz and Satyawati 2008; Ruan *et al.* 2010; Ruan and Teixeira da Silva 2011). In this mini-review our aim is to reconsider the possible biological relevance for salt tolerance mechanisms of some biochemical markers of specific stress response pathways according to the ideas outlined in the previous paragraphs. We will focus on osmolytes, and especially on proline (Pro) biosynthesis. We will mention some of the numerous data supporting the notion that Pro could be considered a reliable stress marker, since it indeed accumulates in many plants subjected to salt – or drought – stress; but some contradictory results will also be described, suggesting that Pro accumulation may be relevant for salt tolerance in some species, but not in others. Finally, we will propose some possible future lines of research, complementary to the most common approaches use today, which may contribute to improving our knowledge of the mechanisms of salt tolerance of halophytes in their natural habitats.

GENERAL RESPONSES OF PLANTS TO SALT STRESS

Theoretically, both halophytes and glycophytes face the same problems induced by high salinity of the soil solution: i) lowering of the water potential, leading to cellular dehydration (osmotic stress); ii) toxicity of absorbed Na^+ and Cl^- ions, due to inhibition of many enzymatic activities and different cellular processes (protein synthesis, pre-mRNA processing, direct inactivation of proteins and macromolecular structures, or generation of reactive oxygen species (ROS) (oxidative stress); and iii) interference with the uptake of essential nutrients, such as K^+ and Ca^{2+} (Zhu 2001; Forment *et al.* 2002; Munns 2002; Wang *et al.* 2003; Flowers and Flowers 2005; Vinocur and Altman 2005).

There is much evidence that all plants, salt-sensitive as well as tolerant, use the same basic mechanisms of response to salt stress mentioned above, involving control of ion homeostasis, synthesis of compatible solutes and protective proteins, and oxidative stress management (Hussain *et al.* 2008). However, the very fact that halophytes can tolerate NaCl concentrations which will kill any glycophyte (including most wild species and all crops) clearly indicates that all those responses of plants to salt stress generally do not lead to salt tolerance. Therefore, the mechanisms that halophytes use to cope with high soil salinity – apart from anatomic or other adaptations which may be important for particular species – must be much more efficient than those operating in glycophytes, although they may share the same molecular basis in both types of plants. This also highlights the drawbacks and limitations of using salt sensitive models to investigate salt tolerance mechanisms.

BIOSYNTHESIS OF COMPATIBLE SOLUTES

General remarks

As mentioned before, the stress caused by salinity has two major components: osmotic (water) stress causing cellular dehydration, and salt (ion) toxicity (Schulze *et al.* 2005). Water deficits have been shown to induce a lowering of the osmotic potential in plants as a means of maintaining cellular turgor (Jones *et al.* 1981). This reduction in the osmotic potential as a response to water deficit can be achieved by solute accumulation within the plant cell or by a decrease in the cell volume, leading to an increased concentration of osmotic solutes as water leaves from the vacuole (Heuer 1999). These phenomena are described as osmoregulation and osmotic adjustment. Osmotic adjustment is a very important mechanism involved in drought and salt tolerance, because it enables maintenance of turgor, cell expansion, stomatal and photosynthetic adjustments and plant growth.

Since sodium (and chloride) ions are toxic at relatively low concentrations, they cannot accumulate in the cytoplasm and must be sequestered in the vacuole; in fact, salt tolerance depends to a great extent on the cellular compart-

Table 1 Examples of experiments performed to reveal the responses of plants to salt stress.

Species	Plant material	Registered parameters	References	Comments/Remarks
<i>Arabidopsis thaliana</i> (transgenic)	¹ Adult plant		¹ Nanjo <i>et al.</i> 1999a	¹ Alteration of Pro metabolism by inhibition of Pro dehydrogenase (antisense approach)
	² Adult plant		² Nanjo <i>et al.</i> 1999b	² Alteration of Pro metabolism by inhibition of Δ - pyrroline-5-carboxylate synthetase (antisense approach)
	³ Adult plant	³ Growth	³ Forment <i>et al.</i> 2002	³ Overexpression of splicing factors
	⁴ Germinating seeds; adult plants	⁴ Germination frequency, growth	⁴ Naranjo <i>et al.</i> 2006	⁴ Overexpression of a GDSL-lipase
<i>Nicotiana tabacum</i> (transgenic)	¹ Adult plant		¹ Kishor <i>et al.</i> 1995	¹ Overexpression of Δ - pyrroline-5-carboxylate synthetase
	² Tissue culture material (cells adapted to NaCl)		² La Rosa <i>et al.</i> 1991	Role of Δ - pyrroline-5-carboxylate synthetase in the regulation of Pro biosynthesis
<i>Lycopersicon esculentum</i> *, <i>L. pennellii</i>	¹ Excised leaf discs	¹ Accumulation of Pro and Pro precursors (asparagine, ornithine, glutamate)	¹ Guerrier 1998	¹ Higher accumulation of Pro in salt-sensitive <i>L. esculentum</i>
	² Adult plants	² Growth, ionic relations and Pro accumulation	^{2*} Inal 2002	² Salt treatments reduced biomass and increased Pro accumulation
	³ Young and old plants	³ Growth, Pro accumulation	^{3*} Claussen 2005	³ The highest concentration of Pro was found in growing leaves
<i>Oryza sativa</i>	¹ Seedling stage of salt-resistant and salt-sensitive cultivars	¹ Ions and Pro accumulation	¹ Lutts <i>et al.</i> 1996	¹ The salt-resistant cultivar accumulated less Pro than the salt-sensitive
	^{2,3} Seedling stage of different genotypes	² Seed germination, early seedling growth, water content, Pro accumulation ³ Seed germination, seedlings growth, Pro content	² Momayezi <i>et al.</i> 2009 a ³ Momayezi <i>et al.</i> 2009b	^{2,3} The amount of Pro within seedlings was reduced by high salt levels
	⁴ Callus	⁴ Cell growth, water content, Pro accumulation, Na ⁺ , K ⁺ , Ca ²⁺ determination	⁴ Summart <i>et al.</i> 2010	⁴ Pro content was 2.0-2.5 -fold higher in salt-stressed cells than in the control cells
	¹ Excised leaves	¹ Na ⁺ and Pro content, water potential, osmotic potential	¹ Buhl and Stewart 1983	¹ Pro accumulation under salt stress
<i>Hordeum vulgare</i>	² Excised leaves	² Pro, Na ⁺ , K accumulation	² Voetberg and Stewart 1984	² Pro increased for about 16 h of salt exposure, to remain at the same level afterwards
	³ Young plants	³ Relative water content, Pro accumulation	³ Kocheva and Georgiev 2008	³ Pro levels showed a significant increase in ornithine- and Pro-pretreated plants
	⁴ Adult plants of salt-sensitive and salt-tolerant cultivars	⁴ Plant growth, ions determination, compatible solutes	⁴ Chen <i>et al.</i> 2007	⁴ Salt treatments increased leaf Pro contents, but more in salt-sensitive cultivars
	⁵ Adults plants	⁵ Growth, ions, organic solutes determinations	⁵ Garthwaite <i>et al.</i> 2005	⁵ Increase in Pro was much larger in the expanding leaf blade
	¹ Calli of bean cultivars	Pro accumulation	Cardenas-Avila <i>et al.</i> 2006	Higher Pro content in tolerant varieties
<i>Pennisetum glaucum</i>	Ten-day-old seedlings of lines differing in salt sensitivity	Pro accumulation, ion accumulation and antioxidative enzyme activities	Mukhopadhyay <i>et al.</i> 2007	Relatively higher increase of Pro levels in tolerant lines
<i>Triticum aestivum</i>	Adult plants	Pro, protein contents and peroxidase activity	Goudarzi and Paknyat 2009	Higher accumulation of Pro in stress tolerant cultivars
<i>Saccharum officinarum</i>	Plantlets	¹ Growth, photosynthetic parameters, Pro accumulation	¹ Cha-Um and Kirdmanee 2008	¹ Pro content was maximum in the plantlets treated with 300 mM mannitol
	Plantlets	² Growth, Pro and ion accumulation	² Errabii <i>et al.</i> 2006	² Accumulation of Pro occurred in both cultivars
<i>Linum usitatissimum</i>	Adult plants	Growth, leaf relative water content, enzyme activities, net photosynthetic rate, chlorophyll content, Pro accumulation	Khan <i>et al.</i> 2007	Pro accumulation was registered in three of the four investigated genotypes
<i>Olea europaea</i>	Adult plants	Photosynthetic capacity and Pro content	Ben-Rouina <i>et al.</i> 2006	Increase in Pro levels in leaves and roots of stressed plants
<i>Phoenix dactylifera</i>	Callus	Growth, ions and Pro accumulation	Al-Khayri 2002	Pro accumulation was correlated to callus growth inhibition
<i>Sorghum bicolor</i>	Adult plants	Growth, ions and Pro accumulation	Weimberg <i>et al.</i> 1984	Pro increased 60 and 18 fold in Chloride - and Sulphate-plants

superscript numbers indicate different reports on the same species

mentalisation of toxic ions. Osmotic adjustment under stress then requires the synthesis and accumulation in the cytoplasm of 'compatible solutes' (osmolytes), very soluble organic compounds which do not interfere with normal metabolism, even at high concentrations; their chemical nature is quite diverse, including mostly polyols (glycerol, sorbitol, mannitol), sugars (trehalose, sucrose) and some amino acids and derivatives (Pro, glycine betaine) (e.g., Sairam and Tyagi 2004; Bartels and Sunkar 2005; Flowers and Colmer 2008). Osmolytes are synthesized by many species, not only halophytes, in response to all environmental conditions leading to water stress; in fact, osmolyte accumulation represents a striking case of convergent evolution in solving osmotic problems by all organisms, ranging from microorganisms to plants (Yancey *et al.* 1982; Burg *et al.* 1996).

Functions of osmolytes in stress response mechanisms

It was initially presumed that osmolytes played their main biological role in osmotic adjustment, but they appear to have additional functions during the stress response, acting as 'osmoprotectants', by directly stabilizing proteins and membrane structures under dehydration conditions, and by protecting the cell against oxidative stress as scavengers of ROS; can also function as molecules for the cellular storage under stress conditions of C and/or N (and energy), nutrients which will be reutilised by the plant during recovery from stress (Shen *et al.* 1997; Hong *et al.* 2000; Akashi *et al.* 2001; Zhu 2001; Chen and Murata 2002; Ashraf and Foolad 2007; Szabados and Savaouré 2010). Thus, it has been shown that, *in vitro*, Pro reduces the denaturation of enzymes caused by elevated NaCl concentrations; the presence of increased concentrations of Pro, and/or glycine betaine, provides protection against the biologically unfavourable consequences of dehydration-induced thermodynamic perturbations (Hamilton and Heckathorn 2001). Pro may also function as a protein-compatible hydrotrope (Srinivas and Balasubramanian 1995), alleviating cytoplasmic acidosis, and maintaining appropriate NADP⁺/NADPH ratios compatible with metabolism (Hare and Cress 1997). Other additional functions, which could be essential for plant recovery from stress, have been attributed to Pro, for example acting as a signalling molecule to affect cell proliferation, cell death, or to activate the expression of specific genes (Szabados and Savaouré 2010, and references therein).

HOW RELEVANT IS OSMOLYTE ACCUMULATION FOR SALT TOLERANCE?

There is a general agreement in considering osmolyte accumulation as a conserved response of plants to abiotic stress conditions that include an osmotic component, as is the case with salt stress. A different question is the relative contribution of osmolyte biosynthesis to the stress tolerance of a particular species, which could be minor, as compared to other stress response mechanisms.

There is some confusion regarding the concepts of *stress responses* and *stress tolerance*. As mentioned before, all plants appear to use the same basic mechanisms of *response* to different abiotic stress conditions, and any model could be used, in principle, to investigate those mechanisms. However, only in a limited number of species those responses seem to be efficient enough to confer *tolerance* to a particular stress. Thus, in absolute terms and by definition, only halophytes should be considered as 'salt-tolerant'; therefore, in our opinion, studies to elucidate salt tolerance mechanisms should better focus on halophytic species. Of course, the concept of salt tolerance or salt resistance can also be applied to glycophytes, but in relative terms – obviously, there are differences in the degree of resistance between different species, or between different cultivars of the same species. Comparative studies trying to correlate osmolyte contents with the relative tolerance levels of related

taxa could also give useful information on salt tolerance mechanisms.

Correlation between salinity and proline accumulation in halophytes

A large number of published reports support the notion that the concentration of Pro – or other osmolytes – in plants increases in parallel with an increase of the external salinity level. Most available data refer to experiments with glycophytes, either *Arabidopsis* or some crop species, but there are also examples in halophytes; in both cases, practically all those experiments were performed under artificial laboratory conditions. There are also reports on the absolute Pro contents in halophytes collected from the field. However, studies to determine possible *changes* in Pro levels of halophytes in their natural habitats, in response to changes in soil salinity – for example, sampling the plant material under different climatic conditions or in different seasons – are much scarcer.

Perhaps some of the earliest data regarding accumulation of Pro in halophytes are those recorded by Stewart and Lee (1974). These authors found that, under non-saline conditions, Pro levels in *Triglochin maritima* were low, but increased as the salinity was raised, so that at high external salt concentrations free Pro could reach values representing 10-20% of the shoot dry weight. Cavalieri and Huang (1979) showed that when some salt marsh halophytes were exposed to increasing salinities, they accumulated Pro once a threshold salinity had been reached. Three general patterns were reported. *Limonium carolinianum* and *Juncus roemerianus* began to accumulate Pro at 0.25% NaCl (*ca.* 40 mM) reaching levels up to 63.6 µmoles/g fresh weight at higher salinities. In the same work, it was found that C₄ grasses (*Spartina alterniflora*, *Spartina patens* and *Distichlis spicata*) had threshold salinity levels around 0.5 M NaCl and accumulated Pro to 27.4 µmoles/g fresh weight. Succulent species, *Salicornia bigelovii*, *S. virginica* and *Borrichia frutescens*, did not accumulate Pro until very high salinities (0.7 M) were reached.

Youssef (2009) reported the analysis of several compounds involved in the response to salt stress, in five succulent halophytes – *Halocnemum strobilaceum*, *Arthrocnemum macrostachyum*, *Halopeplis perfoliata*, *Suaeda vermiculata* and *Seidlitzia rosmarinus* – collected from two natural habitats with different salinity levels (as determined by electrical conductivity and ions content measurements). For all these species, Pro levels were higher in the plants from the sampling site with higher soil salinity.

It has been suggested that there exists a correlation between the type of compatible solute(s) used by different species and their taxonomic classification. Several attempts have been made to determine which particular osmolyte accumulates in specific families and/or genera (Gorham *et al.* 1980; Gorham *et al.* 1981; Vernon and Bohnert, 1992; Rhodes and Hanson, 1993; Hanson *et al.* 1994; Popp and Albert, 1995; Ishitani *et al.* 1996; Stoop *et al.* 1996; Naidu *et al.* 2000; Noiraud *et al.* 2001; Murakeozy *et al.* 2003; Arndt *et al.* 2004; Koyro 2006; Tipirdamaz *et al.* 2006).

In a recent study, Tipirdamaz *et al.* (2006) analysed 51 halophytic species from an inland, non-submerged salt marsh in Central Anatolia and found that proline and glycine betaine 'behave as almost omnipresent solutes'. Their results confirm previous findings (Stewart *et al.* 1979, Briens and Larher 1982) that the osmolyte type permits discrimination between taxa, and underline that species that are Pro accumulators show low levels of glycine betaine, and *vice versa*. Tipirdamaz *et al.* (2006) also discuss the importance of nitrogen as limiting factor in the inland salt marshes and the fact that nitrogen requirements for accumulation of nitrogenous osmolytes may represent an adaptive trait of 'paramount importance'.

Proline contents have also been determined in man-grove species, with mixed results. Pro accumulation in response to salt stress has been reported in roots and shoots of

Aegialitis annulata (Popp and Polania 1989). On the other hand, Popp and Albert (1995) found that, out of twelve mangrove species investigated, only two – *Acanthus ilicifolius* and *Xylocarpus granatum* – contained relatively high levels of Pro; similarly, *Avicennia marina* also accumulates very low Pro in its shoot (Yasseen and Abu-Al-Basal 2008). These data suggest that mangrove species generally do not behave as Pro accumulators, which could be related to the complexity and diversity of adaptive mechanisms coping with salinity in coastal ecosystems (Walsh 1974; Tomlinson 1986; Adam 1990; Hogart 1999; Cronk and Siobhan Fennessy 2001; Lüttge 2002; Hogart 2007).

The level of Pro increased with the salinity of the habitat in three submerged aquatic halophytic species of *Ruppia* (Brock, 1981). In general, Pro content increased in young plants during the phase of active plant growth, decreasing later on during the life cycle. Since earlier developmental stages are more sensitive to stress than adult plants, higher Pro levels may provide increased protection to the more sensitive phase of vegetative growth.

Flowers and Hall (1978) reported a 20-fold increase of Pro levels in shoots of *Sueda maritima* plants grown in the presence of 340 mM NaCl, as compared to control plants grown in the absence of salt. The related taxon *Suaeda salsa* – previously considered as a subspecies of *S. maritima* – as well as *Limonium bicolor* also accumulated Pro in the shoots with increasing external NaCl concentrations; in this case, the shoots of *L. bicolor* contained more Pro than those of *S. salsa*, especially at higher salinity levels (Liu *et al.* 2008). Pro accumulation in response to salt stress has also been reported for another member of the Chenopodiaceae, *Atriplex farinosa* (Morsy 2008), or in leaves of *Lepidium crassifolium* from an inland saline area (Mile *et al.* 2002).

These and many other studies clearly indicate that synthesis and accumulation of Pro represents a general response to salt stress in many halophytes, at least qualitatively; the same function is probably fulfilled by glycine betaine or other osmolytes in those species that are not Pro accumulators. There are, however, significant differences among species regarding quantitative aspects of this response, such as the threshold salinity level which triggers osmolyte accumulation, the endogenous osmolyte concentration that can be reached or the kinetics of the reaction.

Is proline a physiological osmolyte in *Plantago crassifolia*?

As mentioned before, most studies on the mechanisms of response to high salinity are carried out under controlled experimental conditions, generally quite different from those to which plants are subjected in their natural habitats. Therefore, one should always be aware that the results obtained in the laboratory might not be relevant regarding the physiological mechanisms of salt tolerance of a given halophyte, as shown by our own work with *Plantago crassifolia*.

Comparative studies on the responses to salt stress in taxa with different levels of resistance to salt, but with similar genetic backgrounds (e.g. related species from the same genus), may help to establish which of those responses are essential for salt tolerance and which are not. The genus *Plantago* is a good choice for this experimental approach, since it includes species with considerable differences in their degree of tolerance, from salt-sensitive taxa, such as *P. media*, to typical halophytes like *P. maritima* (e.g. Erdei and Kuiper 1979). In our experiments, treatment of the halophyte *Plantago crassifolia* with 500 mM NaCl in the greenhouse resulted in a 20-fold increase in Pro levels in the leaves, as compared with control, non-treated plants, although most of this increment was observed at relatively high salt concentrations, over 300 mM NaCl (Vicente *et al.* 2004). These data, at first sight, seem to support the notion that Pro has indeed a physiological role in the responses to salinity in this species; however, they do not agree with pre-

vious studies showing that all *Plantago* species investigated – including salt-sensitive and salt-tolerant taxa – use sorbitol, not Pro, as osmolyte (e.g. Ahmad *et al.* 1979; Königshofer 1983). Since, to our knowledge, there are not known examples of plant species belonging to the same genus, which accumulate different compatible solutes in response to salt stress, the possibility that Pro could be a physiological osmolyte in *P. crassifolia* is extremely unlikely. In fact, *P. crassifolia* is never subjected to so high salinity levels in its natural habitats; the results obtained in the laboratory, although formally correct, have no ecological meaning: the observed accumulation of Pro in response to salt cannot be relevant for salt tolerance of this species under field conditions. In agreement with this statement, no significant differences in Pro content were found between *P. crassifolia* plants growing in two zones of a salt marsh with different soil salinity levels, as determined by electrical conductivity measurements and analysis of soil samples (M. Boscaiu *et al.*, unpublished data). This specific example highlights the necessity to analyse with extreme caution the results of laboratory experiments, to avoid erroneous conclusions and generalisations. This should be obvious, but it is common that reported salt treatments carried out in the lab include very high concentrations, higher than sea water and up to 1 M NaCl, which is extremely unlikely the plants can encounter in nature (see, for some examples: Khan and Weber 1986, Khan *et al.* 2000; Aghaleh *et al.* 2009).

CONCLUSIONS AND PERSPECTIVES

As a result of intensive research carried out over the last two decades, nowadays the deleterious effects for plants of salt stress, and the mechanisms used by them to respond to high soil salinity, are relatively well known. Comparatively, our knowledge of the mechanisms of salt tolerance, at the biochemical, cellular and physiological levels, is still very limited. Although there is no precise definition of ‘halophytes’, it is clear that most plants cannot survive even relatively mild conditions of salt stress. This indicates that all the responses triggered by stress in plants, which have been mentioned in the previous pages, do not generally lead to stress tolerance... except in halophytes. Still, it is not known which of those responses are important, and which provide only a minor contribution, for salt tolerance of a given salt-tolerant species.

It is not worth discussing here the obvious advantages of using model systems, such as *Arabidopsis thaliana*, for the study of the molecular mechanisms of response to stress; it is also logical to work on crops of economic interest. However, these species are all salt-sensitive; they have provided – and will continue to provide – valuable information on the biochemical and physiological responses of plants to high soil salinity... but probably are not the most appropriate to investigate salt tolerance mechanisms. In addition to current research – not as an alternative – we believe that increasing research on halophytes could substantially contribute to elucidate those mechanisms. Most likely, several halophytic ‘model species’ will have to be selected for these studies, as diverse salt-tolerance strategies are used by different halophytes (for a more extensive discussion on the value of models, see Flowers and Colmer 2008).

We also believe that more fieldwork will be necessary to define and understand biologically significant salt tolerance mechanisms in halophytes. Up to now, most data available on salt stress responses/tolerance have been obtained in laboratory set-ups, under artificial, highly controlled conditions, far different from the conditions the plants encounter in their natural habitats. This experimental approach is of course correct, and much easier than working in the field, but one should be aware of its limitations and drawbacks; the results of lab or greenhouse experiments should be analysed with caution, since they may have no ecological meaning.

A complementary approach could be based on the ana-

lysis of changes in the levels of different biochemical markers of stress responses (e.g., proline contents) in plant material collected in natural saline habitats and under different environmental conditions. These conditions (soil salinity, humidity, temperature, rainfall, etc.) cannot be controlled in nature, but they can be measured and correlated with the plant responses. These studies would then require the work of multidisciplinary research teams, including biochemists, botanists, ecologists and soil scientists. Also, comparative studies in taxa with different levels of salt tolerance but similar genetic backgrounds – for example, related species of the same genus – may help to establish which of the observed responses are essential for salt tolerance, and which are not.

Summarising, we propose to increase the research on the responses of halophytes to salt stress in their natural habitats, as a complement to the studies with salt-sensitive models in laboratory conditions. This strategy will most likely contribute significantly to improve our knowledge of salt tolerance mechanisms.

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