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REVIEW: PART OF A SPECIAL ISSUE ON HALOPHYTES AND SALINE ADAPTATIONS

Sodium chloride toxicity and the cellular basis of salt tolerance in halophytes

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• **Background** Halophytes are the flora of saline soils. They adjust osmotically to soil salinity by accumulating ions and sequestering the vast majority of these (generally Na^+ and Cl^-) in vacuoles, while in the cytoplasm organic solutes are accumulated to prevent adverse effects on metabolism. At high salinities, however, growth is inhibited. Possible causes are: toxicity to metabolism of Na^+ and/or Cl^- in the cytoplasm; insufficient osmotic adjustment resulting in reduced net photosynthesis because of stomatal closure; reduced turgor for expansion growth; adverse cellular water relations if ions build up in the apoplast (cell walls) of leaves; diversion of energy needed to maintain solute homeostasis; sub-optimal levels of K^+ (or other mineral nutrients) required for maintaining enzyme activities; possible damage from reactive oxygen species; or changes in hormonal concentrations.
• **Scope** This review discusses the evidence for Na^+ and Cl^- toxicity and the concept of tissue tolerance in relation to halophytes.
• **Conclusions** The data reviewed here suggest that halophytes tolerate cytoplasmic Na^+ and Cl^- concentrations of 100–200 mM, but whether these ions ever reach toxic concentrations that inhibit metabolism in the cytoplasm or cause death is unknown. Measurements of ion concentrations in the cytosol of various cell types for contrasting species and growth conditions are needed. Future work should also focus on the properties of the tonoplast that enable ion accumulation and prevent ion leakage, such as the special properties of ion transporters and of the lipids that determine membrane permeability.

Key words: Halophyte, salinity stress, sodium chloride toxicity, Na^+, Cl^-, saline soil, environmental stress, osmotic adjustment, cytoplasmic ion concentration.

INTRODUCTION

Halophytes are the flora of saline soils. Although arbitrary, halophytes can be defined as plants that complete their life cycle in a concentration of salt (most commonly dominated by NaCl) of ≥200 mM (see Flowers and Colmer, 2008); all less tolerant species are 'glycophytes'. However, there is a continuum in salt tolerance among plant species, from very sensitive species such as chickpea (*Cicer arietinum* L.) and rice (*Oryza sativa* L.) to the most tolerant halophytes (Greenway and Munns, 1980). Halophytes are characterized by their ability to tolerate high Na^+ and Cl^- concentrations in their shoots, concentrations that would prove damaging or even lethal in non-halophytes. Monocotyledonous halophytes tend to contain lower Na^+ concentrations than dicotyledonous halophytes, with a higher selectivity for K^+ uptake (Albert, 1975; Flowers and Colmer, 2008). The capacity of cells and tissues to continue to function without major lesions while containing high internal Na^+ and Cl^- concentrations can be described by the term 'tissue tolerance'. The basis of tissue tolerance is the major theme discussed in this review, within the context of the potential toxicity to metabolism of high Na^+ and Cl^- in shoot tissues of halophytes.

OSMOTIC ADJUSTMENT

For plants to grow in saline soil they need to adjust osmotically to maintain a positive turgor pressure. Consequently, cells must contain a total solute concentration greater than that of the external solution: just how much greater is not known for most halophytes as turgor pressures have rarely been measured directly, but where data are available turgor is generally lower (0·05–0·3 MPa, Table 1) than values in glycophytes with access to adequate water (around 0·5 MPa; Boyer, 2009). Maintaining a turgor pressure of 0·3 MPa in the cells of a plant growing in seawater with its osmotic potential of −2·3 MPa requires solutes that generate a cellular osmotic potential of −2·6 MPa, equivalent to about 530 mM NaCl. Halophytes commonly do contain Na^+ and Cl^- concentrations >500 mM (Flowers, 1985) and some up to almost 2 M Na^+ on a tissue water basis (e.g. *Tecticornia* species at extreme salinity; English and Colmer, 2013).

In dicotyledonous halophytes, Na^+ and Cl^- are the major components of the cellular osmotic potential. Accommodating high concentrations of Na^+ and Cl^- in tissues is generally thought to be achieved by intracellular compartmentation and the synthesis of compatible solutes: the bulk of the ions

TABLE 1. *Turgor pressures in the cells of some halophytes measured using a cell pressure probe*

Turgor pressure (MPa)	Species	Comment	Reference
0·20	*Suaeda maritima*	The maximum turgor pressure in cells of the youngest leaves of plants growing in 400 mM NaCl. The minimum turgor in old leaves was 0·050 MPa.	Clipson *et al*. (1985)
0·16	*Mesembryanthemum crystallinum*	Maximum turgor pressure in root cortical cells (layer 3, 25 mm from the tip) of plants growing in 400 mM NaCl.	Rygol and Zimmermann (1990)
0·10	*Aster tripolium*	Rhizodermis of plants growing in 300 mM NaCl. Maximum turgor pressure in root cortical cells (layer 7, 20 mm from the tip) was 0·6 MPa.	Zimmermann *et al*. (1992)
0·33	*Sarcobatus vermiculatus*	Pre-dawn in mature leaves of plants growing in 300 mM NaCl.	James *et al*. (2006)
0·22	*Sarcobatus vermiculatus*	Mid-day in mature leaves of plants growing in 300 mM NaCl.	James *et al*. (2006)

are compartmentalized within vacuoles, and organic solutes adjust the osmotic potential of the cytoplasm (see Flowers and Colmer, 2008). Successful Na^+ and Cl^- sequestration into vacuoles requires tonoplast-located ion exchangers and the H^+ pumps that generate the electrochemical difference of H^+ across the tonoplast to drive them; the H^+ pumps also contribute to the membrane potential, which in turn influences channel transport activity (Flowers and Colmer, 2008; Munns and Tester, 2008; Hasegawa, 2013; Shabala, 2013). Charge differences mean that sequestration of Na^+ is likely to be more expensive energetically than sequestration of Cl^-, as the potential inside the vacuole is positive relative to the cytoplasm (Rea and Poole, 1993), but the energetics would also depend upon the concentration differences and type/mechanism of the transporters involved (the concentration differences, membrane potentials and mechanisms of the transport of Na^+ across the tonoplast have been reviewed by Shabala, 2013). Apart from these transport mechanisms, efficient retention of ions in the vacuoles is also required (i.e. very low membrane permeability to leakage of Na^+ and Cl^- back to the cytoplasm) if significant amounts of ATP are not to be utilized (Yeo, 1981, 1983; Greenway and Munns, 1983) in reloading of Na^+ (and Cl^-) at the tonoplast into the vacuoles (similar to the suggestion for the plasma membrane by Britto and Kronzucker, 2006). How a low permeability of the tonoplast is achieved is unclear, but may be through control of Na^+-permeable SV (slow vacuolar) and FV (fast vacuolar) channels (Shabala, 2013). Certainly, there are many SV channels in the leaves of the halophyte *Suaeda maritima* but, under physiological conditions, the gating frequency is low so that these largely remain closed, thus preventing the ions from leaking to the cytoplasm (Maathuis *et al*., 1992). A similar situation appears to occur in old leaves of *Chenopodium quinoa* where there is a decrease in SV channel activity compared with young leaves (Bonales-Alatorre *et al*., 2013). An additional feature of the tonoplast of at least one halophyte that is likely to contribute to low leakage of Na^+ is the lipid composition; the tonoplast from *S. maritima* has a high phospholipid:protein ratio (1·1:1·0) with highly saturated fatty acids and a significant (30 %) contribution of cholesterol to the membrane sterols (Leach *et al*., 1990*a*, *b*).

Organic solutes (these include sucrose, sugar alcohols, proline and glycinebetaine and are listed in Flowers and Colmer, 2008; Gil *et al*., 2013) are accumulated by halophytes and most probably contribute to osmotic adjustment in the cytoplasmic compartments of vacuolated cells (Flowers *et al*., 1977; Wyn Jones *et al*., 1977; Greenway and Munns, 1980) rather than the whole cell. From an energetic point of view, cellular osmotic adjustment is more efficiently achieved by the use of ions than organic solutes (Greenway and Munns, 1983; Yeo, 1983), the synthesis of which would divert C and N from the supply of assimilates for growth processes (e.g. for N in *Spartina alterniflora*; Colmer *et al*., 1996). As large amounts of energy would be needed to synthesize these organic compounds in sufficient quantities to adjust the whole volume of cells, it follows that evolutionary pressure has resulted in plants using Na^+ and Cl^- as osmotica in vacuoles while preventing these ions rising to toxic concentrations in the cytoplasm (Flowers *et al*., 2010). Entry of these ions into some critical cells and tissues, such as meristems and very young growing cells, might also be restricted to keep concentrations relatively low (Greenway and Munns, 1980; Flowers *et al*., 2010).

ION TOXICITY

The concept of toxicity of various mineral elements in plants (including essential nutrients) is long standing in the study of plant mineral nutrition (e.g. Marschner, 1995). Toxicity can be described, in an agronomic context, as the soil (i.e. external) concentration at which the additional supply of a particular element causes a decline in plant growth (e.g. see fig. 11·1 in Römheld, 2012). As discussed by Römheld (2012), for a critical toxic concentration of a mineral element in soil to be established, the criterion used to define a growth reduction also needs to be set (e.g. 90 % of maximal growth). The discipline of plant nutrition has also frequently related internal (i.e. leaf tissue) concentrations to plant growth/yield responses, to establish 'tissue tests' as a diagnostic tool to determine the deficiency, sufficiency or toxicity status of crops (Reuter and Robinson, 1986). Setting such critical concentrations for crops (which can then be used as a diagnostic guide) might be based on economic yield, but, for a wild species (especially for perennials), where survival is the functional criterion, this is difficult to do, especially where there may be competition from other plants and a variable environment.

Limits to salt tolerance in natural habitats are not established for halophytes, owing to the complexities of assessing plant performance under natural conditions, and have rarely been assessed even in controlled conditions. Soil salinity is spatially heterogeneous even within the root zone of individual plants

and, together with temporal changes, both influence the physiology of halophytes (Bazihizina *et al*., 2012*a*, *b*). Even for crops, the concentration in the soil at which an element restricts growth can be influenced by interacting factors. For halophytes and NaCl, the various interacting factors that influence growth responses to salinity include the supply of other elements (e.g. silicon, Mateos-Naranjo *et al*., 2013; nitrogen source, Al Daini *et al*., 2013), humidity (e.g. Devinar *et al*., 2013) and other abiotic stresses (e.g. drought, Yue *et al*., 2012; waterlogging, Colmer and Flowers, 2008). An additional complexity for understanding toxicity effects generally is that the length of exposure of the organism influences the outcome, from various degrees of injury up to death (e.g. Miller *et al*., 2000). We do know, however, that even the most salt-tolerant halophytes are killed by high concentrations of NaCl in the root zone – of around 2 M (English and Colmer, 2013). Moreover, growth of halophytes is inhibited at external salinities well before death occurs (Greenway and Munns, 1980; Flowers and Colmer, 2008). Possible causes of these growth reductions and eventually death of halophytes as soil salinity becomes very high, are: (1) insufficient osmotic adjustment resulting in reduced net photosynthesis because of stomatal closure and reduced turgor for expansion growth, due to insufficient ion uptake or adverse cellular water relations if ions build up in the apoplast (cell walls) of leaves; (2) diversion of energy needed to regulate ion transport and compartmentation and for the synthesis of organic 'compatible' solutes; (3) toxicity to metabolism of Na^+ and/or Cl^- in the cytoplasm; and/or (4) damage from reactive oxygen species (ROS). The focus of the present review is on how leaf tissues tolerate high Na^+ and Cl^- as well as the potential toxicity to metabolism of high Na^+ and Cl^- in shoot tissues of halophytes; the other factors listed above are briefly considered.

The discipline of toxicology has traditionally evaluated toxicity (both chronic and acute) as doses required to be lethal to 50 % of a population (LD_{50}); the LD_{50} provides a convenient quantitative summary for a particular agent on particular organisms/populations under specific conditions. For ion toxicity in plants, as discussed below, toxicity can be defined as the concentrations of Na^+ or Cl^- within a tissue above which metabolism (and growth) is inhibited; although concentrations within a specific compartment(s) such as the cytoplasm probably determine the response. Observations by Yeo and Flowers (1983) that genotypes of rice showed differences in the severity of chlorosis that were not simply related to differences in the concentrations of Na^+ in these tissues, resulted in their concept of 'tissue tolerance' – which they quantified as the concentration of Na^+ in the leaf at which the chlorophyll concentration was reduced by 50 % (Yeo and Flowers, 1983). The assumption was that Na^+ toxicity resulted in the observed tissue damage, and the dysfunction was reflected by the degree of chlorosis; in addition, these authors were seeking to establish a quantitative screening method to determine quantitative trait loci (QTLs) for selection of this trait in breeding for more salinity-resistant rice. For halophytes, there are not the thousands of varieties that are available for rice, varieties that differ at least to some degree in tolerance but have relatively similar leaf morphologies and physiologies. So, comparisons of changes in a metabolic function with leaf ion concentrations in diverse species of halophytes are more complicated to interpret. Furthermore, halophytes function with vacuolar Na^+ and Cl^- concentrations of $\geq$500 mM, so it is not possible simply to relate cytoplasmic ion concentrations to whole-tissue concentrations – as the cytoplasm is such a small proportion of the tissue: cytoplasmic ion concentrations could double when tissue concentrations only change by about 10 % (a doubling of concentration in a tissue with 3 % cytoplasm and 80 % vacuole). Consequently, without a detailed study of the effect of increases in external salt concentration on cytoplasmic Na^+ and Cl^- concentrations, it is impossible to evaluate whether these ions reach toxic concentrations in halophytes or whether other factors are responsible for decreases in growth and eventually death as the external salt concentration rises.

Plants accumulate Na^+ and Cl^- in their shoots to very different concentrations: glycophytes generally die when their leaves contain concentrations that halophytes tolerate. For example, the Na^+ concentration in the leaves was 500–600 mM in the halophyte *S. maritima* growing optimally [external salt concentration of 340 mM (Yeo and Flowers, 1980); based on a water content of 10 g g^{-1} dry weight (Flowers and Yeo, 1986)]. Some halophytes can even continue to grow when shoot tissues contain 1·5 M Na^+ on a tissue water basis (e.g. *Tecticornia* species; English and Colmer, 2013). Rice (*Oryza sativa*), on the other hand, cannot tolerate (for long) tissue concentrations of Na^+ (based on tissue water) in the leaves much above 100 mM (Ul Haq *et al*., 2013). Since the majority of Na^+ and Cl^- accumulated in leaves is likely to be in the vacuoles, as the vacuoles make up the major part of leaf volume (55 % in mature sunflower, Fagerberg, 1984; 72·5 % in mesophyll cells of *S. maritima* growing in 340 mM NaCl, Hajibagheri *et al*., 1984*a*), then tissue concentrations will largely reflect vacuolar concentrations and differences in tissue tolerance presumably reflect differences in abilities to accumulate Na^+ and Cl^- within vacuoles. An important implication of this conclusion is that Na^+ and Cl^- are toxic if not compartmentalized, i.e. if not in a compartment surrounded by a membrane that can take up but not leak out these ions (i.e. in the vacuoles). However, it is important that, as Cheeseman (2013) has pointed out, this potential toxicity is not associated with a particular target, but with a sensitivity of many enzymes or cellular functions such as signalling systems to elevated concentrations of Na^+ and/or Cl^-, and that the tolerable concentrations in cytoplasm are poorly defined.

NA^+ AND CL^- TOXICITY AND TISSUE TOLERANCE AS REVEALED BY *IN VITRO* STUDIES ON ENZYMES AND ORGANELLES

Enzymes

The study of the activities of enzymes *in vitro* formed the basis of much of our understanding of biochemistry (Dixon and Webb, 1964), and during the late 1960s and early 1970s a number of enzymes, but by no means all, were shown to be inhibited by the NaCl concentrations found in healthy plant tissues (Johnson *et al*., 1968; Flowers, 1972*a*, *b*; Greenway and Osmond, 1972; Osmond and Greenway, 1972). The argument was made that tissue concentrations of Na^+ and Cl^- could not be uniform across the cell, and ions must be compartmentalized with higher concentrations in vacuoles than in the cytoplasm (Flowers *et al*., 1977), a view supported by the presence in tissues of organic solutes (mentioned above) that are neutral with

respect to enzyme activity (Wyn Jones *et al*., 1977; Gibson *et al*., 1984) or even protective (Pollard and Wyn Jones, 1979; Manetas, 1990). These organic solutes can, then, balance what would otherwise be osmotic imbalances across the tonoplast when ions accumulate in vacuoles. However, while the arguments in favour of compartmentation hold today, it was clear even by the mid-1970s that apparently salt-sensitive enzymes might not be as sensitive to Na^+ and/or Cl^- as originally reported: the response to NaCl added *in vitro* could be altered by the substrate concentration (Osmond and Greenway, 1972; Flowers *et al*., 1976*a*, *b*) and the preparative procedures used to extract enzymes from tissues (Blackwood and Miflin, 1976; Flowers *et al*., 1976*a*, *b*) and the protein concentration (Manetas, 1990). It is likely that enzymes thought to be salt sensitive, such as malate dehydrogenase (Flowers, 1972*a*; Greenway and Osmond, 1972), can tolerate, *in vitro*, NaCl concentrations of around 200 mM without significant loss of activity (Flowers *et al*., 1976*b*; Kalir and Flowers, 1982). Inhibition of the activity of phosphoenolpyruvate carboxylase by NaCl (a salt-sentitive enzyme that has been extracted from *Salsola soda*, concentrated by ammonium sulphate precipitation and then desalted) decreased by 60 % as the protein concentration was increased 10-fold to 400 $\mu g\,ml^{-1}$ (Manetas, 1990). These findings are, however, all consistent with a view that the functionality of enzyme pathways in the cytosol depends on stabilizing forces requiring ionic strengths of >100 mM (see Cheeseman, 2013).

It is difficult to know the extent to which the response of enzymes *in vitro* mirrors that *in vivo*, as there is a clear difference between the protein concentrations *in vivo* and that used in assays carried out *in vitro* (e.g. Greenway and Osmond, 1972; Manetas, 1990). Cheeseman (2013) has pointed out that enzymes are likely to exist as complexes, and the behaviour of such complexes in the cytoplasm may be very different from that of enzymes in a dilute solution *in vitro*. The effects of NaCl on the activity of such complexes have not, to our knowledge, been examined. However, where a complex of enzymes such as the ribosomal system that catalyses protein synthesis has been investigated, the stability of polysomes from both halophytes and glycophytes decreased as the concentration of KCl increased above 125 mM; polysomes were less stable in Na^+ than in K^+ and also less stable in Cl^- than in acetate (Brady *et al*., 1984). Cl^- at concentrations >80 mM became inhibitory to the translation of mRNA from *Triticum aestivum*, *Pisum sativum*, *Beta vulgaris*, *Chenopodium album* and *Hordeum vulgare* and the halophyte *Atriplex nummularia* by wheat germ ribosomes (Gibson *et al*., 1984) or those of *S. maritima* (Flowers and Dalmond, 1992). Incorporation of [^{35}S]methionine into protein was more tolerant to substitution of K^+ by Na^+ during elongation of message in preparations from the halophytes *Atriplex isatidea*, *Inula crithmoides* and *S. maritima* than the glycophytes *P. sativum*, *O. sativa* or *T. aestivum* (Flowers and Dalmond, 1992) suggesting some adaptation of halophytes to a low cytosolic K^+/Na^+ ratio. These studies on ribosomes suggest that an Na^+ concentration of 100 mM would be tolerable (in the presence of 100 mM K^+) and that Cl^- is more inhibitory than Na^+, at least to this aspect of metabolism. While recent work on the enzymes of halophytes has concentrated on changes in expression patterns (see, for example, Koyro *et al*., 2013), it would be timely to look again at the effects of monovalent ions on the activities of particularly salt-sensitive proteins and some multiprotein complexes (cf. Cheeseman, 2013). The response of organelles such as mitochondria and chloroplasts that have been extracted and exposed to different salt concentrations *in vitro* could also throw some light on the response of enzyme complexes to changes in salt concentration: these organelles presumably contain proteins at a similar concentration *in vivo* and *in vitro*.

Mitochondria

For mitochondria, the outer membrane is freely permeable to solutes up to a size of approx. 5 kDa due to the presence of a porin (a voltage-dependent anion channel): it is the inner membrane that has differential permeability to solutes (see Philippar and Soll, 2007) and mediates the volume changes reported in various studies of swelling and contraction (e.g. Hanson and Miller, 1969; Malone *et al*., 1974). Mitochondrial respiration is most commonly lower in mitochondria isolated from salt-stressed plants than in those from control plants, although the response of overall respiration is very variable (Jacoby *et al*., 2011). Once isolated from their respective cells, mitochondrial respiration of the halophyte *S. maritima* and the glycophyte *P. sativum* was inhibited by 50 % when NaCl was increased to 300 mM (Flowers, 1974), with the cytochrome pathway being more sensitive than that to the alternative oxidase, most probably because cytochrome *c* is located in the intermembrane space, whereas the alternative oxidase is protected by the inner membrane (Jacoby *et al*., 2011). Mitochondrial malate dehydrogenase in halophytes is also salt sensitive (even in *Borrichia frutescens* where the cytosolic malate dehydrogenase appears salt resistant; Cavalieri and Huang, 1977). Mitochondrial respiration from glycophytes, in addition to *P. sativum* described above, also showed similar inhibition by NaCl (Flowers and Hanson, 1969; Campbell *et al*., 1976). Presumably, Na^+ and H^+ might compete for translocation into the mitochondrion so that, with high cytosolic Na^+ concentrations, mitochondria might be less likely to generate ATP, and more likely to absorb Na^+. However, if this were the case, K^+ might also compete with H^+ (especially in glycophytes) and so whether there is a net increase in competition for ATP production under saline conditions might depend on the concentration of $(Na^+ + K^+)$ rather than Na^+ or K^+ alone. The internal Na^+ and Cl^- concentrations within the isolated mitochondria exposed to these NaCl treatments are unknown, so this limits conclusions regarding the NaCl concentration that inhibits functioning of enzyme complexes.

Chloroplasts

In preparations of spinach chloroplasts (that were judged 75 % intact and again where the protein concentration was presumably similar to that *in vivo*), light-saturated, uncoupled rates of electron transport from water to oxaloacetate were stimulated by additions of 150 mM KCl (Marsho *et al*., 1980). A similar concentration of NaCl in a medium that contained 2·5 mM oxaloacetate and 30 mM methylamine was 85 % as effective as KCl (Marsho *et al*., 1980). For chloroplasts isolated from *S. maritima*, a salt concentration of 300 mM NaCl was optimal

TABLE 2. *Na^+ concentrations in some algae from freshwater (FW), euryhaline (Eh) or seawater (SW) habitats*

Alga	Habitat	External NaCl (mM)	Cytoplasmic Na (units)	Notes	Reference
Dunaliella parva	Eh/SW	400	37 (mol m^{-3} of analysed volume)	X-ray microanalysis. Vacuole contained 349 mol m^{-3} analysed volume.	Hajibagheri *et al*. (1986)
Chlorella emersonii	FW	335	21 (mM)	Atomic absorption spectrophotometry of cells that contain only small vacuoles.	Greenway and Setter (1979)
Acetabularia acetabulum	SW	460	4–295 (mM)	Na-sensitive microelectrode. Large variation but mean of about 60 mM.	Amtmann and Gradmann (1994)
Ulva lactuca	Eh/SW	468	8 (mmol kg^{-1})	Efflux analysis.	Ritchie (1988)

Euryhaline means that the organism is found in habitats of a wide range of salinities.

for oxygen evolution using ferricyanide in the Hill reaction (Hajibagheri *et al*., 1984*a*). Oxygen evolution from isolated thylakoids from *Sarcocornia quinqueflora* was close to maximal at an external Cl^- concentration of 200 mM at pH 7·8 (Preston and Critchley, 1986), but more sensitive to KCl than NaCl, a similar response to that of thylakoids isolated from *Avicennia marina* (Preston and Critchley, 1987). These data for isolated chloroplasts (where, in contrast to the situation for mitochondria, there are estimates for the concentrations of Cl^- in the chloroplast; see Table 4 below), as well as the data above for isolated mitochondria, are all consistent with the cells of halophytes being able to cope with significant (e.g. $\geq$100 mM) Na^+ and Cl^- in the cytoplasm.

WHAT NA^+, K^+ AND CL^- CONCENTRATIONS ARE IN THE CYTOPLASM OF HALOPHYTES?

Cytoplasm

We define the cytoplasm as all the cellular structures contained within the space between the plasma membrane and the tonoplast, excluding the nucleus. The cytoplasm is made up of the cytosol and the membrane-bound organelles (mitochondria, chloroplasts, microbodies and endoplasmic reticulum). In this section, we comment on ion concentrations within the cytosol, chloroplasts and mitochondria.

There have been a number of measurements made on algae, initially because some have very large cells (up to 1 cm in length) where measurements of the cytoplasmic and vacuolar ion concentrations could be more easily made than in most vascular plant cells – although the complex nature of the cytoplasm of the Cladophorales may confound some of these data (Shepherd *et al*., 2004). In general, freshwater algae growing in the absence of external salt contain between 1 and 50 mM Na^+ (Raven, 1976; Cameron *et al*., 1986; Okihara and Kiyosawa, 1988; Whittington and Bisson, 1994). Early studies on salt-tolerant algae tabulated by Raven (1976) suggest a similar range of cytoplasmic Na^+ (20–60 mM) for algae growing in external concentrations of 470–500 mM Na^+, and these earlier observations are confirmed by more recent data shown in Table 2 (cytoplasmic Na^+ is generally <40 mM).

Cytosol

Direct measurement of the Na^+ concentrations in the cytosol of vacuolated plant cells is difficult as the cytosol is a thin layer of 1–2 μm width around a large central vacuole. Of the various methods available (see Flowers and Läuchli, 1983), the most direct is to insert ion-selective microelectrodes into a cell, although an additional barrel in the electrode (in addition to the Na^+-selective electrode and one to measure membrane potential) that reports either H^+ or Ca^{2+} concentration is required (i.e. triple-barrelled) so that the position of the electrode tip, which is not visible to the electrophysiologist, can be inferred (Carden *et al*., 2003). Unfortunately, there are, as far as we are aware, no such measurements on the leaves of halophytes, so we have collected data obtained from less direct methods.

The thin layer of the cytosol means that elemental analyses on vacuolated cells cannot normally be carried out by scanning X-ray microanalysis, but requires the resolution of transmission analytical electron microscopy (TAEM), with the attendant problems of tissue dehydration and sectioning. Studies carried out during the 1980s and 1990s, where tissue was frozen rapidly and rigorous precautions taken to avoid the presence of liquid water by dehydration at low temperatures (a technique known as freeze-substitution; see Hajibagheri and Flowers, 1993) together with dry sectioning, before analysis using a transmission electron microscope, provided estimates of cytosolic ion concentrations (see Table 3). In the mesophyll cells of leaves of *S. maritima* growing at optimal external salinity, Na and Cl concentrations were reported as 116 and 60 mol m^{-3} of analysed volume (Harvey *et al*., 1981). However, in order to provide data on a basis that can be compared with *in vitro* measurements, estimates of the cytosolic water content and activity coefficients are required. Calculations suggest cytosolic concentrations of 166 and 86 mM, for Na^+ and Cl^-, respectively (see Flowers and Yeo, 1988): these would equate to activities of 120 and 67 mM, respectively. While assumptions are made in generating these numbers (that preparative procedures are effective in preventing ion migration during sample preparation, the water content of the cytosol and that the activities are those of the ions in a simple aqueous solution), they are consistent with the ion concentrations estimated from experiments carried out on *in vitro* systems to be tolerable by metabolism.

TABLE 3. *Data providing estimates of cytoplasmic ion concentrations in cells of halophytes and in a salt-adapted cell culture of tobacco*

Species	Tissue	External NaCl (mM)	Estimated cytoplasmic ion concentration (mM)			Na/K ratio	Original units	Method of analysis	Comment	Reference
			Na	K	Cl					
Suaeda maritima	Mature leaf mesophyll cytoplasm-cell wall	340	101–209	0–40	30–191	5·2–8·8	mol m^{-3} analysed volume	X-ray microanalysis using transmission microscopy following freeze-substitution; cytosol	Calibrated X-ray microscopy. The values are calculated from mol m^{-3} analysed volume assuming a water content of 70 %. The area analysed included some cell wall.	Harvey *et al.* (1981)
Suaeda maritima	Leaf cells	340	165	–	–	–	Counts per unit tissue	Flux analysis using ^{22}Na	Cell and cytoplasmic volumes estimated from electron micrographs.	Yeo (1981)
Zostera marina	Leaf mesophyll	Sea water	ND	ND	ND	0·11	Counts s^{-1}	X-ray microanalysis after freeze drying and dry sectioning	Data not calibrated, but vacuolar counts for Na in the leaf mesophyll were 4·7 times those in the cytoplasm. Leaf Na^+ concentrations ranged from 245 to 317 mM based on plant water concentration.	Ye and Zhao (2003)
Atriplex spongiosa	Mature leaf	600	See comments		–	0·29	Counts s^{-1}	X-ray microanalysis of frozen hydrated specimens using scanning microscopy	The authors calculated Na + K to be 107 mM, a much lower value than the tissue concentration of about 800 mM (270 mM Na^+ + 128 mM K^+).	Storey *et al.* (1983*a*)
Atriplex spongiosa	Bundle sheath mature leaf	200 400 600	See comments			0·53 0·44 0·47	Counts s^{-1}	Cells with little vacuolation X-ray microanalysis of frozen hydrated specimens using scanning microscopy	The authors calculated (K + Na) in the cytoplasm to be 30, 20 and 14 % of that in the vacuole in 200, 400 and 200 mM NaCl, respectively.	Storey *et al.* (1983*a*)
Suaeda maritima	Root mature cortical cells 10–20 mm behind tip	200	168	78	128	2·1	mol m^{-3} analysed volume	X-ray microanalysis using transmission microscopy following freeze-substitution	The tabulated values are calculated from mol m^{-3} analysed volume assuming a water content of 70 %. Standards were used for calibration.	Hajibagheri and Flowers (1989)
Suaeda maritima	Root cells	340	150	–	–	–	Counts per unit tissue	Flux analysis using ^{22}Na	Cell and cytoplasmic volumes estimated from electron micrographs.	Yeo (1981)
Atriplex spongiosa	Root meristematic cells	200 400 600	 80	 120	 70	0·20 0·38 0·79	Counts s^{-1}	Cells with little vacuolation. X-ray microanalysis of frozen hydrated specimens using scanning microscopy	Calculated from counts using a value of 8·2 μmol g^{-1} water). Na/K ratios are for counts data. Uncalibrated X-ray data.	Storey *et al.* (1983*b*)
Suaeda maritima	Root tip 60-day-old plants	340	105	227	–	0·49	mol m^{-3} tissue volume	Cells with little vacuolation. Atomic absorption spectroscopy	Concentrations are for apical mm as quoted in table 4 of the paper.	Hajibagheri *et al.* (1985)

(Continued)

TABLE 3. *Continued*

Species	Tissue	External NaCl (mM)	Estimated cytoplasmic ion concentration (mM)			Na/K ratio	Original units	Method of analysis	Comment	Reference
			Na	K	Cl					
Atriplex amnicola	Root tip (12 mm)	200	66	225	–	0·27	mM	Cells with little vacuolation. Atomic absorption spectroscopy and coulometric titration	Based on protoplast water for apical 12 mm sections of roots.	Jeschke *et al.* (1986)
		400	161	225	181	0·72				
Atriplex nummularia	Roots	50	198	143	–	1·4	Counts per unit tissue	Flux analysis using ^{22}Na and ^{86}Rb	Using sections, to estimate volumes; cytoplasmic volume of 5 %.	Mills *et al.* (1985)
Triglochin maritimum	Root cells	100	104		–		Counts per unit tissue	Flux analysis using ^{24}Na or ^{42}K as NaCl and KCl	Cell sizes estimated from sections and light microscopy.	Jefferies (1973)
		500		71		2·1				
		(8 K^+)	148							
Eleocharis uniglumis	Root cells	74	192	–	–		Counts / unit tissue	Flux analysis using ^{22}Na	The concentration was estimated by dividing the quantity in the compartment by its estimated volume determined from sections.	Shepherd and Bowling (1979)
Puccinellia peisonis	Root rhizodermis	40	70	2	–	0·02	Counts	X-ray microanalysis of frozen hydrated specimens using scanning microscopy	Used aqueous standards containing BSA that slightly underestimated concentrations.	Koyro and Stelzer (1988)
Spartina townsendii	Root rhizodermis	40	5	32	–	0·16	Counts	X-ray microanalysis of frozen hydrated specimens using scanning microscopy	Used aqueous standards containing BSA that slightly underestimated concentrations.	Koyro and Stelzer (1988)
Nicotiana tabaccum cell culture	Adapted cells in early stationary phase	428	96	–	96	–	Counts (plus morphometry and whole cell concentration data)	Cells with little vacuolation. X-ray microanalysis	Cytoplasm was 45 % of cell volume. Vacuolar concentration was 780 mM Na^+ and 624 mM Cl^-.	Binzel *et al.* (1988)

BSA, bovine serum albumin; ND, not determined.

TABLE 4. *Ion concentrations in isolated chloroplasts vs. whole-leaf tissue for three halophytes grown at 340–400 mM NaCl*

Species	Chloroplast or whole leaf	Na^+ (mM)	K^+ (mM)	Cl^-(mM)	Method and reference
Mesembryanthemum cristallinum (400 mM NaCl)	Chloroplast	156–234	35–54	42–64	Isolated chloroplasts, Demming and Winter (1986)
	Leaf	498	19	374	
Suaeda australis (350 mM NaCl)	Chloroplast	104	73	98	Isolated chloroplasts, Robinson and Downton (1985)
	Leaf	683	67	372	
Suaeda maritima (340 mM maCl)	Chloroplast*	133	28	126	X-ray microanalysis of whole leaf, Harvey *et al*. (1981)
	Leaf	494	39	351	
Suaeda maritima (340 mM NaCl)	Chloroplast*†‡	108	29	110	X-ray microanalysis of whole leaf, Hajibagheri *et al*. (1984*b*)

*Adjusted for 78 % solute available space.
†Data for 90 % of chloroplasts; 10 % had values of Na 329, K 46 and Cl 272 mM.
‡Leaf concentrations would have been similar to the values in the row above.

An alternative approach to estimating cytosolic ion concentrations has been to use flux analysis. Flux analysis involves loading cells with an isotope and interpreting efflux over time in terms of ion loss from three compartments: cell wall, cytoplasm (cytosol and organelles) and vacuole (e.g. see section 2.2.6 in Flowers, 2007). The analysis provides data on half-times and quantities in the three compartments (mitochondria and chloroplasts are not generally considered separately from the cytosol and efflux data do generally fit a three-compartment model), provided each reaches the specific activity of the loading solution. The use of $^{22}Na^+$ or $^{36}Cl^-$ with suitable data on cell and compartmental volumes provides estimates of compartmental Na^+ and Cl^- concentrations; it is technically more difficult to obtain estimates for K^+ as its radioactive isotopes are short lived and so $^{86}Rb^+$ has often been used as a tracer for K^+, but there is evidence that it is not a perfect tracer at least in *S. maritima* (Yeo, 1974). In spite of the assumptions required, the flux analysis data in Table 3 suggest that the cytosolic Na^+ concentration in halophytes is in the range 150–200 mM, a similar range to those from X-ray microanalysis.

Cells with little vacuolar volume

Measurements of tissues or organs containing, predominantly, cells with small vacuoles, such as shoot and root apices, have also been used to approximate likely cytoplasmic ion concentrations. As an alternative to X-ray microanalysis, Jeschke and Stelter (1976) conceived the idea of using conventional atomic absorption spectrometry to measure the ion contents of longitudinal profiles of roots, where the meristematic cells of the apex were almost non-vacuolated and so tissue ion concentrations reflected those in the cytoplasm. Examples shown in Table 3 again suggest cytoplasmic Na^+ concentrations in the range 80–200 mM. Cells of tobacco, which, although it is not a halophyte, can be adapted to grow at high NaCl concentrations in tissue culture (Binzel *et al*., 1985), had a cytoplasmic Na^+ concentration close to 100 mM.

Chloroplasts

Ion concentrations have been estimated for the chloroplasts from three species of halophyte, both in isolated organelles and by X-ray microanalysis (Table 4). It appears that chloroplasts contain in excess of 100 mM Na^+ and a little lower Cl^-, and that these concentrations are regulated in as much as they are different from the concentrations of the same ions in the leaf sap (on average, chloroplastic Na^+, K^+ and Cl^- were 26, 122 and 27 %, respectively, of the values in the leaf sap; calculated from data in Table 4).

Mitochondria

We have been unable to find estimates of the concentrations of Na^+ and Cl^- in plant mitochondria. In heart cells of rats, Na^+ in mitochondria estimated from fluorescence measurements appears to be about 10 mM, about half that in the cytoplasm of these cells (Donoso *et al*., 1992).

Summing up on the topic of cytoplasmic ion concentrations

In looking at the data available, which are limited to a few species, but analysed by a number of different techniques (albeit without the most direct one of using ion-specific microelectrodes), we are drawn to the conclusion that in halophytes the cytoplasmic concentration of Na^+ is 100–200 mM (Table 3), somewhat higher than the concentration reported for algae (Table 2 and above). Overall, there seems a consensus that in young tissues Na^+/ K^+ ratios typically are around 0·5–1 (Table 3). Techniques as different as flux analysis, measurements of slightly vacuolated tissues by atomic absorption spectroscopy and, for cells, by X-ray microanalysis all tell the same story (a conclusion borne out for Na^+ and K^+, but not for Cl^-, in a comparison of techniques using a non-halophyte, maize; Hajibagheri *et al*., 1988). However, there remains uncertainty in the distribution of ions within the cytoplasm; for example, the cytoplasm of the coenocytic algae is not homogeneous (Shepherd *et al*., 2004). Moreover, there has long been discussion of vesicular transport of ions in higher plants (see Lazof and Cheeseman, 1986, and references therein; Shabala and Mackay, 2011). Enclosing ions in (pinocytotic) vesicles (e.g. Field *et al*., 1980; Balnokin *et al*., 2007) is a way in which metabolic processes could be 'protected' from potentially damaging concentrations of monovalent cations and anions and a means by which the essential symplastic transport of Na^+ and Cl^- through the cytoplasm is accomplished. Overall, the evidence (metabolic and analytical) is in favour of substantial

(100–200 mM) concentrations of Na^+ and Cl^- (with activities of 76–140 mM) in the cytoplasm of halophytes, although we have no evidence of their homogeneity.

POSSIBLE FACTORS IN ADDITION TO NA^+ AND CL^- TOXICITY THAT COULD REDUCE GROWTH AND POTENTIALLY CONTRIBUTE TO DEATH OF HALOPHYTES AT HIGH SALINITY

Although the focus of research has often been on Na^+ toxicity, other factors could contribute to the reductions in growth and ultimately death of halophytes when NaCl is progressively increased. These include (1) low cytoplasmic K^+ (see Shabala and Cuin, 2008); (2) Cl^- toxicity *per se* (Gibson *et al*., 1984; Flowers and Dalmond, 1992); (3) effects on plant water relations resulting in dehydration (see below); (4) possible deficiency of Ca^{2+} (as suggested for glycophytes by Greenway and Munns, 1980) or, in the case of halophytes, Mg^{2+} (as this is a significant component of seawater and many experiments are carried out in 'conventional nutrient solutions' that contain lower Ca^{2+} and Mg^{2+} than in seawater and/or by changing the concentration of NaCl and not all the salts present in seawater); (5) changes to other mineral nutrients (see below); (6) adverse effects on water relations and decreased stomatal conductance limiting net photosynthesis (e.g. Flowers and Colmer, 2008; Bazihizina *et al*., 2012*b*); (7) possible hormonal imbalances (e.g. Ben Hassine and Lutts, 2010); (8) increased damage from ROS (e.g. Ozgur *et al*., 2013); or (9) the energetic requirements of either ion transport or the synthesis of compatible solutes, or both (cf. Yeo, 1981, 1983).

Potassium has long been established as an essential element within the cytoplasm, where its concentration in glycophytes is around 100 mM (Leigh and Wyn Jones, 1984). A significant proportion of leaf K^+ is in the vacuoles, where it functions in turgor generation: this role can be replaced by Na^+ (Leigh and Wyn Jones, 1986; Kronzucker *et al*., 2013). Salinity commonly reduces the K^+ concentrations in plant tissues (Greenway and Munns, 1980), but not necessarily the concentration in the cytoplasm. High salinity, high enough to reduce growth, may reduce cytoplasmic K^+ concentrations, and Shabala (2013) has argued that in roots this is as a consequence of depolarization of the plasma membrane and ROS-induced activation of channels allowing K^+ efflux.

Regarding Cl^- toxicity, the uptake and transport of Cl^- within plants is tightly regulated (White and Broadley, 2001). However, as far as we are aware, nothing is known of Cl^- transporters in halophytes and so future investigations into the role of Cl^- channels (CLC proteins; see Barbier-Brygoo *et al*., 2011; Wege *et al*., 2014) would be very worthwhile. The situation for Cl^- in halophytes was summarized by Flowers and Colmer (2008) as having received far less attention than for Na^+, but that ($Na^+ + K^+$) exceeds Cl^- by about 35 % in dicotyledonous species and by at least double in halophytic grasses. Interestingly, Cl^- is as, or more, inhibitory to protein synthesis than Na^+ (Flowers and Dalmond, 1992), and Flowers *et al*. (1986) concluded that regulation of cytoplasmic Cl^- as well as of Na^+ is of importance for salt tolerance. Research on glycophytes has used different types and/or combinations of salts in attempts to evaluate separately the effects of Na^+ vs. Cl^- (e.g. barley; Tavakkoli *et al*., 2011), but the high treatment concentrations required for such experiments on halophytes means that the concentration of any accompanying counterion complicates interpretations.

There is too little information available on halophytes to be able to evaluate whether supra-optimal concentrations of NaCl affect their mineral nutrition to such a degree that it is eventually lethal. Experimentally, it is difficult to diagnose mineral deficiencies or excesses as, just as for Na^+ and Cl^-, stores in the vacuoles confound the estimation of cytoplasmic concentrations. Perhaps the only effective method will be to investigate the effects of supplementation in enhancing survival, although the absence of an effect provides little or no information. For glycophytes, the importance of Ca^{2+} in mitigating the effects of salinity has long been known (see Greenway and Munns, 1980) and for some halophytes there is evidence that additional Ca^{2+} and Mg^{2+} can counteract detrimental effects of NaCl alone (e.g. Nedjimi *et al*., 2009; Ben Amor *et al*., 2010; Grigore *et al*., 2012; English and Colmer, 2013). The results of such experiments support the view that seawater (or an ion composition that resembles that of the habitat) should be the basis of any hydroponic culture of halophytes.

In addition to potential direct or indirect effects of changes in mineral nutrition, other processes could also work in concert to damage halophytes or at least result in lower resilience to stress. In brief, apart from the normal maintenance processes that occur in all plant cells, halophytes require energy for ion transport, combatting oxidative stress (the presence of ROS), and the synthesis of compatible solutes, particularly under conditions where photosynthesis is reduced. Taking an energetic perspective for one of these processes, sequestration of Na^+ in vacuoles has a need for ATP or pyrophosphate to generate the H^+ electrochemical difference across the tonoplast required to energize tonoplastic Na^+/H^+ antiporters; this activity has been suggested to place an additional demand on the ATP pool and on the respiratory activity of halophytes at high salinity (see Shabala, 2013). ROS might also be a factor, although this is difficult to assess as it depends on the site of generation and the defence proteins and antioxidants at that site (Jacoby *et al*., 2011). One report found that it took 1 week or more before effects of salt concentrations of 300 mM or higher had any effect on lipid peroxidation in the more tolerant halophytes (Ozgur *et al*., 2013); such lipid peroxidation could damage membranes and affect the retention of ions within membrane-bound compartments. Other consequences of ROS accumulation are damaged proteins, unfolded proteins and the release of transition metals (Jacoby *et al*., 2011). Finally, the situation is similar for the consequences of salinity on hormone concentrations in halophytes. Halophytes respond to salinity with changes in abscisic acid and ethylene concentrations (Clipson *et al*., 1988; Ben Hassine and Lutts, 2010), and such changes have been shown to affect the metabolism (e.g. Chu *et al*., 1990; Ueno, 1998; Woodward and Bennett, 2005; Ben Hassine and Lutts, 2010). Changes in signals from the roots could alter shoot growth rates, but, in our view, are very unlikely to bring about the death of a plant.

In addition to possible toxicity of Na^+ and Cl^- within the symplast of leaf cells, Oertli (1968) pointed out that if ions transported to the leaves were not quickly taken up by the cell and transported into vacuoles, these ions would accumulate in

the cell walls and cause cells to lose water. Because the cell wall is only about 3 % of the cell volume (see Flowers and Yeo, 1986), ion concentrations rise much faster than in the cell as a whole (see fig. 1 in Flowers and Yeo, 1986) and so apparently low whole-tissue ion concentrations could have disastrous effects on the cells if a proportion of these ions remain in the apoplast (i.e. external to the symplast of the leaf tissue). It has proven difficult to obtain evidence for or against this hypothesis because of the difficulty of measuring intra- and intercellular ion concentrations. Flowers *et al*. (1991) analysed the apoplastic ion concentrations in two genotypes of rice grown for 6·5 d in 50 mM NaCl using X-ray microanalysis of thin sections prepared by freeze-substitution; the data showed ion concentrations in the cell walls to exceed greatly those in the cytoplasm and the vacuoles, providing evidence in favour of the Oertli hypothesis. Using a technique in which leaf segments were first vacuum infiltrated with sorbitol and then centrifuged, Speer and Kaiser (1991) estimated higher apoplastic ion concentrations in *P. sativum* than in *Spinacia oleracea*; as *S. oleracea* is more tolerant of salinity than *P. sativum*, these data were interpreted as offering some support for the Oertli hypothesis. Mühling and Läuchli (2002*a*), using a similar 'infiltration' technique, also showed ions in washings from the leaf apoplast of maize and cotton, but not in sufficiently high concentrations to result in cell damage, a result supported with more recent data for maize (Shahzad *et al*., 2012) and with cotton using fluorescence ratio imaging (Mühling and Läuchli, 2002*b*). Conversely, a rise in Na^+ concentration estimated in the apoplast of the salt-sensitive *Vicia faba* on salinization (Shahzad *et al*., 2013) was interpreted as more evidence in favour of the Oertli hypothesis, as the rise was reduced by adding silicon to the growth medium, which also enhanced salt tolerance.

All the results described in the preceding paragraph were obtained with glycophytes, and there is little information on halophytes. In 60 % of mesophyll cells of *S. maritima*, the cell wall contained a quarter of the Na^+ and one-tenth of the Cl^- concentration (per unit of volume analysed by X-ray microanalysis) of the vacuole; only in 30 % of cells were cell wall and vacuolar concentrations similar (Harvey *et al*., 1981). The apoplast of root cortical cells of the same halophyte had similar concentrations to the cytoplasm and lower than in vacuoles (Hajibagheri and Flowers, 1988), suggesting that *S. maritima* is (as expected) able to regulate the uptake and compartmentation of ions. In the halophytic shrub *Sarcobatus vermiculatus*, high apoplastic ion concentrations have been seen as a means to regulate low water potentials recorded before dawn (James *et al*., 2006), again suggesting that this species is able to regulate ion compartmentation. The low turgor pressures in mature cells of *S. maritima* (Clipson *et al*., 1985) can be interpreted as a consequence of ions accumulating in the apoplast (see Flowers and Yeo, 1986).

It has been hard to draw an unequivocal conclusion on the Oertli hypothesis – that excessive ion concentrations in the leaf apoplast can be the cause of cellular damage – as the techniques available have potential flaws. Ion movement could occur during the vacuum infiltration process (see Flowers and Yeo, 1986), and, in both this technique and X-ray microanalysis, concentrations have to be estimated because cell wall water contents are not known (the vacuum infiltration technique depends on determining the water content of the apoplast by measuring dilution of a relatively large volume of sorbitol by a small volume of water from the apoplast, and the X-ray microanalysis data are obtained per unit volume of analysed material, which is embedded in resin). Furthermore, estimating cation activities in the cell walls is confounded by interactions with fixed negative charges. However, high concentrations of ions in the apoplast may occur and be a contributory factor in cell death in some species (Flowers *et al*., 1991; Shahzad *et al*., 2013), although it is still not possible to distinguish between dehydration or elevated ion concentrations in the cytoplasm as the cause.

CONCLUSIONS

The limited data available indicate that halophytes appear to function with cytoplasmic Na^+ and Cl^- concentrations of 100–200 mM in the face of substantially higher whole-tissue concentrations. Halophytes sequester Na^+ and Cl^- in vacuoles, with compatible solutes contributing to osmotic adjustment of the cytoplasm in addition to the K^+, Na^+ and Cl^- present. The cytoplasmic concentrations of Na^+ and Cl^-, like that of K^+ and those of other solutes and metabolites, are apparently well regulated so that adverse effects on metabolism are probably minimized. Tissue tolerance of Na^+ and Cl^- (i.e. tissue functioning despite high concentrations of these two ions) is evident in halophytes. However, confirming the activities of these two ions in cellular compartments remains problematic. Although high-pressure freezing of tissue and analysis by secondary ion mass spectrometry is feasible, quantification is difficult (Moore *et al*., 2012) and so priorities for future experiments should be direct measurements of ions in the cytoplasm of various cell and tissue types of halophytes using ion-selective microelectrodes, together with analyses of membrane properties and identification and assessment of the characteristics of transporter proteins, for both the tonoplast and plasma membrane. Contrasting species of halophytes (mono- and dicotyledonous species, with and without salt glands), when under a range of defined conditions (such as different external NaCl concentrations, Na^+/Mg^{2+} and Na^+/Ca^{2+} ratios), should be studied.

Growth of all halophytes does occur as soil salinity increases beyond the optimal range, but, as plant tolerance limits are approached, any or all of high cytosolic Na^+ or Cl^- concentrations, low cytoplasmic K^+, cellular dehydration, Ca^{2+}, Mg^{2+} or other mineral deficiencies, decreased stomatal conductance limiting net photosynthesis, hormonal imbalances, increased damage from ROS, the energetic requirements of either ion transport or the synthesis of compatible solutes or both could contribute to reduced growth and eventual death. The possible detrimental effects we list also depend on the duration of exposure and interacting effects of other stresses (e.g. drought, flooding); these aspects require further study, and the energy budgets (maintenance requirements, increased demands related to acclimation, as well as growth requirements) of halophytes at different NaCl concentrations should be of priority to assess. Salinity in the soil and the high Na^+ and Cl^- in the tissues have multiple effects that need to be distinguished if we are to make gains in the understanding of the mechanisms of salt tolerance in halophytes. Such knowledge is crucial in revealing the genetic and molecular basis of salt tolerance in plants, with potential applications in breeding of crops and pasture plants to

enhance utilization of saline soils to help sustain global food production.

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