RESEARCH PAPER

NaCl alleviates polyethylene glycol-induced water stress in the halophyte species Atriplex halimus L.

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Abstract

Atriplex halimus L. is a C4 xero-halophyte species well adapted to salt and drought conditions. To collect information on the physiological impact of low salt levels on their water-stress resistance, seedlings were exposed for 6 d to nutrient solution containing either 0% or 15% polyethylene glycol 10 000 (PEG), in the presence or in the absence of 50 mM NaCl. Similar experiments were performed with one PEG-resistant and one PEG-sensitive selected cell line exposed for 50 d to 0% or 15% PEG on standard Linsmaier and Skoog (LS) medium, on LS medium supplemented with 50 mM NaCl, or on Na+ -free medium. NaCl mitigated the deleterious impact of PEG on growth of both whole plants and PEG-sensitive cell lines and improved the ability of stressed tissues to perform osmotic adjustment (OA). Water stress reduced CO2 net assimilation rates quantified in the presence of high CO2 and low O2 levels (A), stomatal conductance and transpiration, but NaCl improved water use efficiency of PEG-treated plants through its positive effect on A values, especially in young leaves. PEG increased the internal Na+ concentration. The resistant cell line accumulated higher concentration of Na+ than the PEG-sensitive one. The complete absence of Na+ in the medium endangered the survival of both cell lines exposed to PEG. Although Na+ by itself contributed only for a small part to OA, NaCl induced an increase in proline concentration and stimulated the synthesis of glycinebetaine in response to PEG in photosynthetic tissues. Soluble sugars were the main contributors to OA and increased when tissues were simultaneously exposed to PEG and NaCl compared with PEG alone, suggesting that Na+ may influence sugar synthesis and/or translocation.

Key words: Atriplex halimus, halophyte, NaCl, osmotic adjustment, osmotic stress salinity, sodium, water stress.

Introduction

In semi-arid climatic conditions, desertification is becoming a serious problem, with a progressive reduction of the vegetation cover coupled with rapid soil erosion. Drought resistance is a complex trait involving several interacting properties and there is increasing interest in studying the physiological behaviour of xero-halophyte species in order to identify and understand drought-resistance mechanisms. Several species belonging to the genus Atriplex are well adapted to harsh environmental conditions and therefore constitute a useful material for the identification of physiological mechanisms and genes involved in abiotic stress resistance (Shen et al., 2003; Cabello-Hurtado and Ramos, 2004; Wang and Showalter, 2004). Atriplex halimus is a Mediterranean xero-halophyte saltbush species highly resistant to drought (Le Houe`rou et al., 2000), salinity (Bajji et al., 1998), and heavy-metal stress (Lutts et al., 2004).

One of the main adaptive mechanisms contributing to water-stress resistance in plants is osmotic adjustment (OA) which involves the net accumulation of solutes in cells in response to a fall in the water potential of the environment (Zhang et al., 1999). In Chenopodiaceae species, such as those belonging to the genus Atriplex, OA is thought to be mainly performed by the accumulation of glycinebetaine (GB) which may also assume positive functions in relation to the maintenance of membrane integrity and stability of other cellular structures under water-stress conditions (Shen et al., 2002; Wang and Showalter, 2004). GB accumulation mainly occurs in chloroplasts under light conditions...
habitats could involve peculiar abilities to accumulate K⁺ through a Na+/H⁺ symport (Ohnishi, 1987) to control pyruvate translocation across membranes (Brownell and Bielig, 1996). According to Qiu et al., help in the maintenance of chloroplast structural integrity of Na⁺ to OA is difficult to quantify precisely at the whole plant level, since a considerable part of Na⁺ accumulates in both the vacuoles and cytosol of numerous plant species (Pitman, 1981).

The specialization of some Atriplex species for saline habitats could involve peculiar abilities to accumulate K⁺ and Na⁺ ions. Under well-irrigated conditions, low NaCl doses were shown to stimulate plant growth in A. gmelini (Matoh et al., 1986), A. hortensis (Jeschke and Stelter, 1983), and A. rhabdoioides (syn. A. amnicola) (Mahmood and Malik, 1987), but had no positive impact on the growth of A. griffithii (Khan et al., 2000) and A. nummularia (Ramos et al., 2004). Data concerning the impact of Na⁺ on the Atriplex response to soil drying under non-saline conditions are scarce. In A. halimus, however, it has been recently hypothesized that Na⁺ may assume a positive function in response to water stress, since the plants specifically increased Na⁺ absorption when submitted to drought on a non-saline substrate (Martínez et al., 2003, 2004). The underlying reasons for such specific increase remain unknown.

Most C₄ species such as A. halimus require Na⁺, although in small amounts, to convert pyruvate to phosphoenolpyruvate in light conditions (Murata et al., 1992), to control pyruvate translocation across membranes through a Na⁺/H⁺ symport (Ohnishi et al., 1990), and to help in the maintenance of chloroplast structural integrity (Brownell and Bielig, 1996). According to Qiu et al. (2003), the beneficial impact of Na⁺ in stressed plants of A. centralasiatica may also be related to an increase in zeaxanthan concentration, which suggests that the xanthophyll cycle plays an important role in protecting the photosynthetic apparatus from stress-induced damage. Na⁺ was also reported to stimulate GB synthesis in redbeet (Subbarao et al., 2001) and to hasten stomatal closure in some halophyte species (Kerstiens et al., 2002). All these putative functions of Na⁺ in water-stress resistance directly depend on the integrity of the whole plant system and the presence of light. According to Glenn and Brown (1998), tolerance to water and salt stresses in A. canescens is linked through a common mechanism of Na⁺ uptake which is directly used for OA. In A. halimus, the real contribution of Na⁺ to OA is difficult to quantify precisely at the whole plant level, since a considerable part of Na⁺ accumulates in trichomes covering the leaf surface (Mozafar and Goodin, 1970). Moreover, Martínez et al. (2003, 2004) estimated that despite a significant stress-induced stimulation of Na⁺ absorption by the plant, the direct contribution of this element to OA is negligible from a quantitative point of view. The use of undifferentiated proliferating cell lines devoid of trichomes and maintained on an appropriate in vitro culture medium may therefore help to gain valuable information about the precise impact of Na⁺ on stressed tissues.

In this study, the behaviour of whole plants of A. halimus submitted in controlled conditions to a transient water stress induced by PEG in the presence or in the absence of a low NaCl dose was analysed in relation to water status, ion nutrition, and organic solute accumulation. Results were compared with those obtained with selected PEG-sensitive and PEG-resistant proliferating cell lines maintained under dark conditions in the presence of PEG and/or NaCl. Results suggest that Na⁺ may assume positive functions in stressed tissues, probably through the increase in total soluble sugar accumulation and the resulting improvement of OA.

Materials and methods

Whole plant material and growth conditions

Fruits (seeds with enclosing bracts) collected on Atriplex halimus L. plants growing wild in the region of Kairouan (Tunisia) were used in this study. More than 400 seeds were surface-sterilized and germinated as previously described (Martínez et al., 2004). Uniformly sized 7-d-old seedlings were transferred and acclimated in a greenhouse at 28/20 °C (day/night) under a photoperiod of 16 h consisting of natural daylight supplemented with Philips mercuric lamps (HPLN 400 W) to reach a minimum photon flux density of about 250 μmol m⁻² s⁻¹; daytime relative humidity was about 70%. Twenty-six days after sowing, the young seedlings were transferred to plugged holes (plant-to-plant distance: 6 cm) in polystyrene plates (20×12 cm) floated over 2.5 l plastic tanks containing a modified Hoagland nutritive solution containing (in mM) 5 KNO₃, 1 NH₄H₂PO₄, 0.5 MgSO₄, 5.5 Ca(NO₃)₂ and (in μM) 25 KCl, 1 H₃BO₃, 1 MnSO₄, 1 ZnSO₄, 0.25 CuSO₄, 10 Na₂MoO₄ and 50 mg l⁻¹ FeEDTA. Solutions were renewed each week. After a 2-week acclimation period, plants were divided into four batches (16 tanks per batch in a complete randomized block design; 4 plants per tank) which were subjected or not to a PEG water stress (15% PEG 10 000) for 6 d, in the presence or in the absence of 50 mM NaCl. Thus, there were ultimately four different treatments: (i) in the absence of PEG water stress: solution at 0% PEG containing 0 or 50 mM NaCl (osmotic potentials (Ψₛ) of −0.12 and −0.42 MPa, respectively) and (ii) in the presence of PEG water stress: solution at 15% PEG containing 0 or 50 mM NaCl (osmotic potentials (Ψₛ) of −0.40 and −0.87 MPa, respectively). PEG was obtained from Sigma-Aldrich (Belgium) as Biochemika Ultra. The absence of Na⁺ and heavy metal traces in the used PEG was checked beforehand after acid digestion of a small sample and analysis by flame atomic absorption spectrophotometry (Varian spectra-300).

Solutions were continuously agitated and aerated with a stream of air and kept at a constant O₂ concentration of 7.5 mg l⁻¹ (87%) measured with a dissolved oxygen probe (CellOx 325, WTW) and a precision instrument (ProfiLine Dissolved Oxygen Meter Oxi 197-S, WTW). For each treatment (PEG×NaCl), 24 randomly chosen seedlings were harvested just prior to the application of the PEG treatment, and then, after 6 d of stress, were used for the estimation of growth, water status, and ion concentrations. Roots, stems, young (level A) and old (level B) leaves of the main stem were analysed separately. Levels A and B contained the same number of leaves (when the main stem bore an uneven number of leaves, the median leaf was attributed to level A).
Selection of PEG-sensitive and PEG-resistant cell lines

After removal of the bracts, 800 seeds were surface-sterilized as described above. The seeds were then rinsed three times with sterile deionized water and transferred into the dark at 28 °C on two layers of Whatman No. 41 filter paper moistened with sterile deionized water for 48 h. Two hundred hypocotyls were excised under sterile airflow and used as explant sources for callus cultures in Petri dishes. The basal medium consisted of Linsmaier and Skoog (1965) (LS) supplemented with 3% (w/v) sucrose, 0.5 mg l⁻¹ 2,4-D, 1 mg l⁻¹ BAP, 1 mg l⁻¹ NAA, 100 mg l⁻¹ myo-inositol, and 0.1 mg l⁻¹ thiamine-HCl. The pH of the medium was adjusted to 5.7 with 1 N KOH prior to the addition of 2.5 g l⁻¹ Gelrite (Phytagel, Sigma) and autoclaving for 20 min at 120 °C and 150 kPa. Five explants were used per Petri dish on 25 ml LS medium.

After 2 months, the calli obtained were divided into small fragments (c. 50 mg fresh weight) which were cultivated in separate Petri dishes on media containing either 0 or 20% (w/v) PEG for 8 months. Fragments issued from a given callus were distributed among the two media. The selection criterion for the sensitive lines was early necrosis and death within 2 months on the medium supplemented with 20% PEG. The identified sensitive lines were then derived from calli proliferating on the PEG-free control medium, but issued from the same mother-calli as those which failed to develop in stress conditions. They were cultivated on a PEG-free multiplication medium for 16 months. The resistant lines were derived from calli that survived and grew on the medium containing 20% (w/v) PEG, and the non-necrotic calli were transferred to the multiplication medium with 20% PEG for 16 months. Calli were divided for multiplication before transfer to fresh medium every 10 d and kept in the dark at 28 °C. Two callus lines, one sensitive and one resistant, were used for further physiological characterization.

For each line, calli were exposed for 50 d to either 0% or 15% PEG on classical LS medium in the concomitant absence or presence of 50 mM NaCl. Another set of experiments was performed in order to determine the impact on callus behaviour of the complete absence of Na⁺. Calli were transferred to Na⁺-free medium (hereafter designed as LS-Na⁺ medium), which was obtained by substituting (NH₄)₂MoO₄ for Na₂MoO₄ (present in the classical LS medium). All chemicals were purchased from Sigma as ‘SigmaUltra’ with a minimal purity level of 99%. The absence of Na⁺ in LS-Na⁺ medium was checked using an inductively coupled argon plasma emission spectrophotometer: sodium was never present in detectable amounts. Calli from the above-mentioned cell lines were then exposed to either 0% or 15% PEG in the absence of Na⁺ as described previously.

Growth, water and osmotic potentials

Mean relative growth rates (RGR) of shoots, roots, and calli were calculated as: \( \text{RGR} = \frac{\ln W_f - \ln W_i}{\delta t} \), where \( W_i \) and \( W_f \) are final and initial dry weights, respectively, and \( \delta t \) is the time elapsed (d) between the two measurements.

Shoot water potential \( \Psi_s \), leaf relative water content \( \Psi_{wr} \), leaf osmotic potential \( \Psi_s \) and osmotic potential at full turgor \( \Psi_{100} \) were determined between 12.00 h and 14.00 h. \( \Psi_s \) was evaluated immediately after sampling using the pressure chamber method. Fully expanded leaves of five plants (two leaves for each level per plant) were harvested on the main stem. RWC was calculated as: \( \text{RWC} = \frac{(W_F - W_D)/(W_T - W_D)}{100} \times 100 \), where \( W_F \) is the fresh weight, \( W_T \) is the turgid weight measured after 24 h of saturation (when leaf weight reached a plateau) on deionized water at 4 °C in the dark, and \( W_D \) is the dry weight determined after 48 h in an oven at 70 °C.

\( \Psi_s \) was quantified on extracted tissue sap (Martínez et al., 2003, 2004) using a vapour pressure osmometer (Wescor 5500). For the measurement of \( \Psi_{100} \), tissues were rehydrated on filter paper moistened with deionized water for 24 h at 4 °C in the dark. Osmotic adjustment (OA) was calculated as the difference of \( \Psi_{100} \) at full turgor between unstressed \( \Psi_{100}^{ss} \) and PEG-stressed \( \Psi_{100}^{0} \) treatments (Zhang et al., 1999):

\[
\text{OA (MPa)} = \Psi_{100}^{ss} - \Psi_{100}^{0} \]

All data were estimated on 12 different individuals (plants or calli) per treatment.

Ion concentration

For K⁺ and Na⁺ quantification, tissues harvested from five plants (or calli) per treatment were oven-dried at 70 °C for 48 h and 50 mg dry weight (DW) were digested in 35% (v/v) HNO₃. Analyses were conducted using an inductively coupled argon plasma emission spectrophotometer (Jobin-Yvon JY 48). Chloride was estimated on fresh material using the feric ammonium sulphate and mercuric thiocyanate colorimetric method according to Guerrier and Patolia (1989).

Gas exchange parameters

Instantaneous CO₂ assimilation in saturating conditions and transpiration rates were measured at the end of the stress treatment using a CO₂ and water vapour analyser (LCA 2 8.7, ADC, Hertshire, UK) and an air supply unit (ASU 10.87, ADC, Hertfordshire, UK), mounted in series in an open system. Gas exchange was first measured using a PLC (N) Parkinson leaf cuvette on intact leaves for 1 min (20 records min⁻¹), with an air flow of 300 ml min⁻¹. Leaves were illuminated with 4 Philips mercury-vapour lamps (HPLN 400 W). The photosynthetic photon flux density (PPFD) at the leaf surface was set to 500 μmol m⁻² s⁻¹. Temperature and relative humidity were set to 25 ± 2 °C and 70 ± 5%, respectively. In order to quantify the impact of water stress on the chemical processes of photosynthesis independent of its effect on stomatal closure, a CO₂ molar ratio was used that was high enough to saturate the carbon-salinity: air jets of 1000 μmol mol⁻¹ CO₂ and 2% O₂ in N₂ were directed to both surfaces and the recorded instantaneous CO₂ assimilation in saturating conditions \( \text{(A)} \) and the instantaneous transpiration rates \( \text{(E)} \) readings were corrected for water vapour, temperature, and atmospheric pressure (von Caemmerer and Farquhar, 1981). Leaf stomatal conductance \( (g_s) \) was measured on the abaxial surface of leaves exposed to a PPFD of 500 μmol m⁻² s⁻¹ with an automatic porometer (MK III, Delta-T Devices, UK). For each treatment, six different plants were analysed.

Glycinobetaine, proline and total soluble sugars

Tissues belonging to six plants per treatment were pooled prior to organic compounds analysis and quickly frozen in liquid nitrogen. GB was extracted and quantified according to Bessieres et al. (1999) after HPLC separation on a Spherisorb 5 ODS2 column (250 x 4.6 mm) and detection by a UV detector (Bio-Rad 1801 UV monitor). For free proline quantification, 1 g of tissue was extracted with 5 ml of salicylic acid 5%; after centrifugation at 5000 g, free proline was specifically quantified according to Bates et al. (1973). Total soluble sugars (TSS) were extracted in 80% ethanol from 1 g of leaf fresh tissue and quantified by the classical anthrone method (Yemm and Willis, 1954) using a spectrophotometer (Beckman DU® 640, USA). A standard curve was established using glucose, and the results were therefore expressed in μmol equivalent glucose g⁻¹ FW.

For each type of compound, extraction was performed on three distinct subsamples and all measurements for a given subsample were performed in triplicate. In order to eliminate the effect of water loss on the possible changes in organic solute concentrations (when leaf RWC was significantly affected by PEG-induced water stress), solute concentrations were adjusted to the RWC of unstressed tissues according to: \( X \times Y/Z \), where \( X \) is the organic solute concentration and \( Y \) and \( Z \) are the leaf RWC of the stressed and unstressed tissues, respectively.
Statistical analysis

For the whole plant treatment, two independent experiments were performed with results exhibiting similar tendencies. Data were analysed using a three-way analysis of variance (ANOVA) at a significance level of $P < 0.05$ (*) or $P < 0.01$ (**). The model is defined on the basis of fixed effects and hierarchical classification criteria. The main effects were considered to be PEG, NaCl, and leaf age (except for $RGR$ and $\Psi_W$) as well as their interactions. A similar three-way analysis of variance (ANOVA) was performed for data obtained with calli, considering callus line, PEG, and NaCl as the main factors. When the ANOVA was significant at $P < 0.05$, Duncan’s Multiple Range Test was used for means comparisons. All data were analysed by a MSTATC statistical package.

Results

NaCl effects on growth of PEG-treated plants and calli

In the absence of PEG, root $RGR$ was not significantly affected by the presence of 50 mM NaCl while shoot $RGR$ was slightly increased ($P < 0.05$; Fig. 1). In the presence of 15% PEG and the absence of NaCl, a significant reduction in both shoot and root $RGR$ was observed ($P < 0.01$). The presence of 50 mM NaCl in the nutrient solution mitigated to some extent the deleterious impact of PEG on both root and shoot growth.

In the absence of PEG, PEG-sensitive and PEG-resistant calli exhibited similar $RGR$ (Fig. 2). The growth of the PEG-sensitive cell line on standard LS medium was clearly inhibited by PEG while the growth of the resistant cell line was not affected. The addition of 50 mM NaCl to PEG-containing culture medium clearly improved the $RGR$ of the PEG-sensitive cell line, but had no impact on that of PEG-resistant calli. When cell lines were maintained on Na+-free medium (LS-Na+) in the absence of PEG, $RGR$ were only marginally affected. By contrast, when PEG was added to Na+-free medium, both cell lines were strongly affected and no difference was recorded between PEG-sensitive and resistant calli.

Shoot water potential, relative water content, osmotic potential, and osmotic adjustment

Shoot $\Psi_w$ decreased in response to the PEG stress ($\Psi_w= -0.54$ MPa in controls and $-1.48$ MPa in PEG-treated shoots; $P < 0.01$). NaCl had no effect on this parameter, whatever the PEG concentration (detailed data not shown). Leaf $RWC$ markedly decreased in response to the PEG stress ($P < 0.01$) and $RWC$ values of leaves were higher in the presence than in the absence of NaCl ($P < 0.05$) (Table 1). At the callus level, PEG added to standard LS medium reduced the $RWC$ of both sensitive and resistant calli. The addition of 50 mM NaCl to LS medium had no significant impact on the $RWC$ of calli maintained in the absence of PEG, but improved the $RWC$ of both PEG-treated lines (Table 1). By contrast, the absence of Na$^+$ in the medium clearly decreased $RWC$ of both lines exposed to PEG since $RWC$ was lower than 70% for resistant as well as for sensitive calli (detailed data not shown).

In the absence of PEG, NaCl induced a slight, although significant decrease in $\Psi_s$ in roots (not shown) and leaves (Table 1). When the plants were grown at 15% PEG, $\Psi_s$ values were strongly reduced ($P < 0.01$), the reduction being more marked in the presence of NaCl ($P < 0.01$) and in the young leaves ($P < 0.05$) (Table 1). At the callus level, PEG also induced a significant decrease in $\Psi_s$ ($P < 0.01$) and differences between sensitive and resistant calli were significant for the PEG treatment only. While NaCl had no impact in the absence of PEG, it clearly improved the ability of the PEG-treated cell lines to reduce $\Psi_s$ and no significant difference was recorded between the two cell lines considered in this respect (Table 1). By contrast, the complete absence of Na$^+$ in the medium compromised

Fig. 1. Relative growth rate ($RGR$) of roots and shoots of Atriplex halimus L. Plants were exposed for 6 d to nutrient solution containing 0% or 15% PEG in the absence (open columns) or presence (filled columns) of 50 mM NaCl ($n=12$; vertical bars are SE). Values sharing a common letter are not significantly different at $P < 0.05$.

Fig. 2. Relative growth rate ($RGR$) of PEG-sensitive and PEG-resistant cell lines of Atriplex halimus L. maintained for 50 d on LS-standard medium supplemented with 0 (LS, open columns) or 50 mM NaCl (LS+50 mM NaCl, filled columns) and on sodium-free medium (LS-Na+, hatched columns) ($n=12$; vertical bars are SE). Values sharing a common letter are not significantly different at $P < 0.05$. 

$\Psi_s$ Photosynthesis, $\Psi_W$ Water potential, $RWC$ Relative water content.
the ability for OA in the presence of PEG in both sensitive and resistant calli (Ψₛ = −0.53 and −0.61 MPa, respectively). In response to PEG, OA values were more than double in 50 mM NaCl compared with those in 0 mM NaCl, regardless of the tissues examined (P < 0.01) (Table 1).

**Ion concentrations**

Whatever the PEG and NaCl concentrations present in the nutrient solution, Na⁺ concentrations were always higher in leaves than in roots: more than 65% of the total plant Na⁺ content was present in the aerial parts and this proportion increased in response to PEG (Fig. 3). Even in standard solutions containing a small Na⁺ concentration (20 μM), the addition of 15% PEG induced an increase in the Na⁺ concentration in both young and old leaves. As expected, the endogenous Na⁺ concentration was higher in the presence than in the absence of salt, but exposure to PEG of salt-treated plants clearly increased Na⁺ accumulation in all parts of the plant. Such an increase was especially marked in the oldest leaves: statistical analysis showed a significant PEG×NaCl×organ interaction (P < 0.05).

On standard LS medium, Na⁺ concentration was higher in the PEG-resistant cell line than in the sensitive one, even in the absence of PEG (Fig. 4). Although the addition of PEG increased Na⁺ concentrations in both lines, the resistant line accumulated higher Na⁺ concentrations than the sensitive one. When callus lines were exposed to 50 mM NaCl, PEG-induced increase in Na⁺ was still recorded, but there was no significant difference between cell lines. When calli were exposed to PEG on Na⁺-free medium, the low endogenous concentration of Na⁺ was related to the previous period of callus maintenance on standard medium before treatment; in this case, PEG did not induce any significant modification in Na⁺ concentration, except a slight increase which may be linked to the growth inhibition of PEG-treated calli compared with calli maintained in the absence of PEG.

**Table 1. Relative water content (RWC, %), osmotic potential (Ψₛ, MPa) and osmotic adjustment (OA, MPa) of young leaves, old leaves, PEG-sensitive and PEG-resistant calli of Atriplex halimus L. exposed to PEG in the presence or absence of NaCl**

<table>
<thead>
<tr>
<th>Organ</th>
<th>PEG (%)</th>
<th>RWC (%)</th>
<th>Ψₛ (MPa)</th>
<th>OA (MPa)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 mM NaCl</td>
<td>50 mM NaCl</td>
<td>0 mM NaCl</td>
<td>50 mM NaCl</td>
</tr>
<tr>
<td>Young leaves</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>75.1 ± 1.5 a</td>
<td>83.4 ± 0.7 b</td>
<td>−1.78 ± 0.11 a</td>
<td>−2.01 ± 0.17 b</td>
</tr>
<tr>
<td>15</td>
<td>65.3 ± 1.6 c</td>
<td>79.5 ± 0.6 ab</td>
<td>−3.17 ± 0.21 d</td>
<td>−4.88 ± 0.09 f</td>
</tr>
<tr>
<td>Old leaves</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>85.4 ± 1.2 b</td>
<td>88.3 ± 1.0 d</td>
<td>−1.98 ± 0.12 ab</td>
<td>−2.27 ± 0.10 c</td>
</tr>
<tr>
<td>15</td>
<td>68.1 ± 0.4 c</td>
<td>77.5 ± 0.3 a</td>
<td>−2.83 ± 0.07 d</td>
<td>−4.01 ± 0.21 e</td>
</tr>
<tr>
<td>PEG-sensitive calli</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>95.4 ± 0.1 de</td>
<td>94.8 ± 0.3 e</td>
<td>−0.32 ± 0.04 g</td>
<td>−0.31 ± 0.01 g</td>
</tr>
<tr>
<td>15</td>
<td>83.3 ± 0.2 b</td>
<td>87.2 ± 0.2 d</td>
<td>−0.70 ± 0.05 h</td>
<td>−1.79 ± 0.05 j</td>
</tr>
<tr>
<td>PEG-resistant calli</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>96.3 ± 0.1 e</td>
<td>95.1 ± 0.2 e</td>
<td>−0.38 ± 0.02 g</td>
<td>−0.41 ± 0.04 g</td>
</tr>
<tr>
<td>15</td>
<td>85.2 ± 0.4 b</td>
<td>90.3 ± 0.4 d</td>
<td>−0.82 ± 0.09 i</td>
<td>−1.74 ± 0.14 j</td>
</tr>
</tbody>
</table>

Exposure of whole plants to PEG slightly increased K⁺ concentration (P < 0.01) while NaCl decreased it in both young and old leaves (Table 2). When both 15% PEG and 50 mM NaCl were present, the K⁺ concentration was similar to that recorded in the complete absence of both. By contrast, at the callus level, PEG induced a decrease in K⁺ concentration of both the resistant and the sensitive cell lines independent of the absence or presence of NaCl. Salinity clearly induced an increase in Cl⁻ concentration in...
all tissues, but PEG had no impact on this parameter and no significant difference was recorded in this respect between resistant and sensitive cell lines (data not shown).

**Instantaneous CO₂ assimilation and transpiration rates quantified in the presence of high CO₂ and low O₂ levels and stomatal conductance**

PEG induced a decrease in stomatal conductance ($g_s$, estimated by porometry); $g_s$ was slightly decreased by NaCl in PEG-exposed plants, especially in the youngest leaves ($P < 0.01$). PEG induced a decrease in $A$ (quantified in the presence of high CO₂ and low O₂ levels) (Table 3). NaCl had no impact on $A$ values in the absence of PEG, but it stimulated CO₂ assimilation in PEG-stressed young leaves. NaCl also decreased the transpiration rate estimated in both young and old leaves of PEG-treated plants and drastically increased the instantaneous efficiency of photosynthesis in PEG-treated plants by 62% in young leaves and by 51% in old ones.

**Organic solutes accumulation**

GB concentration (Fig. 5) increased in response to PEG and exogenous NaCl reinforced the accumulation of this compound, the effect being higher in young ($P < 0.01$) than in old leaves. By contrast, NaCl had no obvious impact on GB concentration in the absence of PEG. Only very low levels of GB were detected in roots (less than 0.1 µmol g⁻¹ FW) and neither PEG nor NaCl had any impact on the root GB concentration (data not shown). Similarly, GB was not detected in calli, whatever the cell line considered or the composition of the exogenous medium.

PEG had no impact on the proline concentration at the whole plant level, whatever the organ (Fig. 6A), but NaCl increased this compound, mainly in roots and old leaves. In plants exposed to PEG, TSS increased mainly in the leaves (Fig. 6B). When NaCl was added on its own to the standard solution, it had no impact on TSS concentration. However, when NaCl was added to the PEG-containing nutritive solution, it drastically increased the sugar concentration in all organs, including roots (Fig. 6B).

At the callus level, both PEG and NaCl induced a significant increase in the proline concentration (Table 4). While PEG and NaCl had no additive effects on the proline concentration of the resistant cell line, the proline concentration of the sensitive line reached its maximal
value in the simultaneous presence of PEG and NaCl. Calli
maintained on Na+-free medium were still able to accumu-
late proline in response to PEG. When calli were exposed to
PEG in the absence of NaCl, the PEG-resistant cell line
contained higher amounts of TSS than the sensitive one.
When NaCl was added to this PEG-containing LS medium,
it induced a strong increase in the sugar concentration of
the sensitive line and, to a lower extent, of the resistant
one (Table 4). From a relative point of view, NaCl impact
was clearly higher for sugars than for proline. TSS con-
centrations in calli were clearly reduced when maintained
on Na+-free medium and in this case, calli were unable
to accumulate sugars in response to the addition of PEG.

Discussion

Xero-halophyte species such as salt bushes are often
thought to be adapted to a doubly harsh environment in
halomorphic arid soils. *Atriplex halimus* may be found in
both the intertidal zone of coastal deserts and dry-saline
inland habitats. Surprisingly, few studies until now have
considered the combined effects of water and salt stresses
on plants (Glenn and Brown, 1998).

*Sodium improves water-stress resistance in
*Atriplex halimus*

This research shows that, even in the absence of PEG, a low
NaCl dose may improve growth in *A. halimus*. Similar

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**Table 3.** Instantaneous CO₂ assimilation rate (A, µmol CO₂ m⁻² s⁻¹), stomatal conductance (gₛ, cm s⁻¹), and instantaneous transpiration rates (E, mmol H₂O m⁻² s⁻¹) in young and old leaves of *Atriplex halimus* L.

Both A and E were estimated in saturating conditions under high CO₂ and low O₂ levels. Plants were exposed to nutritive solution containing either 0% or 15% PEG in the presence of 0 mM or 50 mM NaCl. Measurements were made after 6 d of treatment. Stomatal conductance was quantified by porometry independently of A measurements. Each value represents mean ± SE (n=12). Values sharing a common letter in each column (parameter) are not significantly different at *P* <0.05.

<table>
<thead>
<tr>
<th>Organ</th>
<th>PEG (%)</th>
<th>NaCl (mM)</th>
<th>A (µmol CO₂ m⁻² s⁻¹)</th>
<th>gₛ (cm s⁻¹)</th>
<th>E (mmol H₂O m⁻² s⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Young leaves</td>
<td>0</td>
<td>0</td>
<td>38.3±2.1 a</td>
<td>0.27±0.04 a</td>
<td>6.11±0.72 a</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>50</td>
<td>40.8±2.9 a</td>
<td>0.25±0.01 a</td>
<td>5.88±0.53 a</td>
</tr>
<tr>
<td>Old leaves</td>
<td>0</td>
<td>0</td>
<td>20.7±1.8 c</td>
<td>0.22±0.02 b</td>
<td>5.13±0.25 c</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>50</td>
<td>27.0±2.4 b</td>
<td>0.11±0.00 c</td>
<td>4.12±0.07 d</td>
</tr>
</tbody>
</table>

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**Fig. 5.** Effects of PEG-induced water stress on GB concentration in young and old leaves of *Atriplex halimus* L. Plants were exposed for 6 d to nutrient solution containing 0% or 15% PEG in the absence (open columns) or presence (filled columns) of 50 mM NaCl (n=3; vertical bars are SE). Values sharing a common letter are not significantly different at *P* <0.05.

**Fig. 6.** Effects of PEG-induced water stress on (A) proline and (B) total soluble sugars in roots, young and old leaves of *Atriplex halimus* L. Plants were exposed for 6 d to nutrient solution containing 0% or 15% PEG in the absence (open columns) or presence (filled columns) of 50 mM NaCl (n=3; vertical bars are SE). Values sharing a common letter are not significantly different at *P* <0.05.
results have already been reported in this species (Bajji et al., 1998) and in other species of the genus Atriplex (Jeschke and Stelter, 1983; Matoh et al., 1986; Mahmood and Malik, 1987). This work, however, mainly demonstrates that low salinity levels may also improve the plant’s ability to cope with PEG-induced water stress. Indeed, growth (Fig. 1), OA (Table 1), and A (Table 3) were clearly higher in PEG-treated plants simultaneously exposed to 50 mM NaCl compared with plants exposed to PEG in the absence of salt. Conversely, gs and E (Table 3) clearly decreased in PEG-treated plants exposed to NaCl.

Results obtained with selected cell lines suggest that the positive effect of NaCl may be due to Na+. On the LS-standard medium, PEG-resistant calli accumulated more Na+ than the PEG-sensitive ones (Fig. 4) while no differences were no longer recorded between cell lines and the ‘resistant’ calli were unable to grow (Fig. 2) and to perform OA efficiently (Table 1) in the presence of PEG. By contrast, when 50 mM NaCl were added to LS medium, the PEG-sensitive cell line was able to accumulate amounts of Na+ similar to those of the PEG-resistant one and both lines exhibited similar performances in terms of growth and OA. These data thus suggest that Na+ is involved in resistance to PEG-induced water stress and that the sensitive cell line was less efficient than the resistant one in taking up Na+ from an external medium containing low Na+ concentrations. The differences between cell lines disappeared when higher external Na+ concentrations were used.

At the whole plant level, additional experiments recently demonstrated that 50 mM KCl was unable to improve plant growth in the presence of 15% PEG, despite significant accumulation of Cl− (S Lutts, unpublished results), thus confirming that Na+ and not Cl− is involved in water-stress resistance and that K+ may not substitute for Na+ in this respect. Even in the absence of salt, PEG induced an increase in the internal Na+ concentration in both young and old leaves (Fig. 3), although the external concentration of this element was low. PEG also induced an increase in K+ concentration in leaves in the absence of salt (Table 2), but this increase was, from a relative point of view, far lower than the recorded increase in Na+. This indicates a high selectivity for the absorption of Na+ in relation to the putative involvement of this element in PEG-treated plant metabolism. This selectivity was confirmed by the analysis of the callus where PEG induced an increase in Na+ concentration on standard LS-medium (Fig. 4), but also a significant decrease in K+ (Table 2).

In other halophyte species, high selectivity for K+ absorption by K+ channels is a key component of salinity resistance as well as the efficiency of an endodermis barrier to Na+ movement through apoplasm (Peng et al., 2004). This does not appear to be valid for A. halimus which behaves as a typical includer. These results are therefore in accordance with those of Reimann and Breckle (1993), who observed that, in A. acuminata and A. rosea, more than 70% of the total Na+ content in the plant is located in the leaves.

### Low NaCl dose increased water use efficiency in PEG-treated plants

When the two osmotic agents were used alone, gs was significantly decreased by PEG but was not affected by NaCl (Table 3) although both agents induced the same external Ψw (−0.4 MPa). In this situation, high Na+ amounts were recorded in leaf tissues for NaCl compared to PEG (Fig. 3), suggesting that stomatal closure may not be directly linked to Na+ content in A. halimus in contrast to Aster tripolium (Kerstiens et al., 2002). In the presence of both agents, gs was more affected than in the presence of PEG alone. In this case, stomatal closure might be a reaction to the decrease in Ψw (−0.87 MPa) rather than to Na+ accumulation. It is noteworthy that, despite this enhancement of stomatal closure, instantaneous CO2 assimilation rate quantified in the presence of high CO2 and low O2 levels (A) was maintained in old leaves and was even surprisingly increased by NaCl in young leaves of PEG-treated plants. As a consequence, NaCl increased the instantaneous efficiency of transpiration in PEG-treated plants, which, in turn, would lead to an increase in the water use efficiency.

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**Table 4.** Proline (μmol g−1 FW) and total soluble sugar concentrations (μmol equivalent glucose g−1 FW) in PEG-sensitive and PEG-resistant cell lines of Atriplex halimus L.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>PEG (%)</th>
<th>Proline (μmol g−1 FW)</th>
<th>Total soluble sugars (μmol equivalent glucose g−1 FW)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>LS</td>
<td>LS+NaCl</td>
</tr>
<tr>
<td>PEG-sensitive</td>
<td>0</td>
<td>6.1±1.9 a</td>
<td>11.7±0.9 b</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>16.4±0.5 c</td>
<td>30.9±2.1 e</td>
</tr>
<tr>
<td>PEG-resistant</td>
<td>0</td>
<td>8.5±1.7 a</td>
<td>16.1±3.6 c</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>25.2±2.9 b</td>
<td>26.4±1.7 d</td>
</tr>
</tbody>
</table>
A positive impact of \( \text{Na}^+ \) on photosynthesis in \( \text{C}_4 \) plants has already been suggested: \( \text{Na}^+ \) was found to increase the conversion of pyruvate to phosphoenolpyruvate in light conditions (Murata et al., 1992) and to be involved in pyruvate translocation across membranes through a \( \text{Na}^+/\text{H}^+ \) symport (Oinishi et al., 1990). According to Johnston et al. (1988), a normal photosynthesis in the \( \text{C}_4 \) species \( \text{A. spongiosa} \) requires an exogenous \( \text{Na}^+ \) concentration of 0.1 mM. In \( \text{A. lentiformis} \), Zhu and Meinzer (1999), however, have demonstrated that the quantum yield for \( \text{CO}_2 \) uptake was maximal in plants grown at 50 mM NaCl. In \( \text{A. halimus} \), external NaCl had, once again, no impact on \( \text{CO}_2 \) assimilation in the absence of PEG, thus suggesting that the small amount of \( \text{Na}^+ \) present in the standard control condition is sufficient for pyruvate translocation. However, NaCl increased \( A \) values in young leaves of PEG-treated plants. One hypothesis is that in those tissues, \( \text{Na}^+ \) present within the cell is less available for chloroplast translocation from the cytosol because it accumulates in other cell compartments such as vacuoles to ensure OA (see below); thus, in this condition, the addition of 50 mM NaCl to the nutrient solution, with subsequent accumulation of \( \text{Na}^+ \), would help the plant to maintain a higher photosynthetic rate.

Sodium may also be involved in the maintenance of the granal stacking which may provide a suitable environment for energy transfer between PSII and PSI (Brownell and Bieig, 1996; Qi et al., 2003). Although the stability of PSII was not analysed in this work, it is unlikely that this effect of \( \text{Na}^+ \) constitutes the key beneficial action in the tissue response to PEG; indeed, the beneficial impact of \( \text{Na}^+ \) in response to PEG was obvious at the callus level while both tested lines were maintained under dark conditions and did not differentiate any mature chloroplasts.

\( \text{NaCl} \) indirectly contributes to OA through an increase in organic solute accumulation

In recent years, considerable attention has been focused on the involvement of \( \text{Na}^+ \) in OA of halophyte species. The implication of \( \text{Na}^+ \) in OA processes may be considered, assuming that it is mainly present in the vacuoles and that these compartments occupy more or less about 90% of the total cell volume. It then appears that, in this material, the contribution of \( \text{Na}^+ \) to total OA never exceeded 15% in leaves. In fact, such a value should be regarded as maximal since a considerable part of the \( \text{Na}^+ \) absorbed accumulates in trichomes that cover the leaf surface in \( \text{A. halimus} \) (Mozafar and Goodin, 1970). \( \text{Na}^+ \), however, also contributed to OA (up to 27%) in calli devoid of trichomes. It is noteworthy that in both plants and calli, the relative contribution of \( \text{Na}^+ \) to \( \Psi_s \) was higher for tissues exposed to NaCl in the absence of PEG compared with those exposed to both PEG and NaCl. Since PEG induced an increase in \( \text{Na}^+ \) concentration and since NaCl strongly increased the ability of stressed tissues to perform OA in the presence of PEG, it is suggested that other compounds are involved in such adjustment and that \( \text{Na}^+ \) may directly or indirectly have a positive impact on their accumulation.

Apart from ions, organic compounds may also contribute to OA and are considered to accumulate in the cytosol and organelles. Members of the Chenopodiaceae are thought to accumulate high amounts of GB in response to salinity or drought (Rhodes and Hanson, 1993). Recent studies, however, suggest that some xero-halophyte species are able to accumulate several organic compounds for OA (Ramanjulu and Sudhakar, 2000; Di Martino et al., 2003) and this view is confirmed by these data since GB (Fig. 5), proline (Fig. 6A), and TSS (Fig. 6B) were all found to increase in stressed tissues of \( \text{A. halimus} \). However, two classes of compounds (GB and TSS) accumulated to the highest concentrations in the leaves when both PEG and NaCl were present in the solution. GB did not accumulate in response to NaCl alone, but 50 mM NaCl clearly improved the ability of \( \text{A. halimus} \) to accumulate this compound under water-stress conditions. Subbarao et al. (2001) also observed that \( \text{Na}^+ \) stimulated GB synthesis in redbeet, although the underlying biochemical explanation remains unknown. This study, however, is the first to show that an interaction may exist in this respect between \( \text{Na}^+ \) effect and water-stress resistance. From a quantitative point of view, however, GB contributed only a few per cent to OA, even if a chloroplastic sequestration is assumed. Its importance in stress resistance may, therefore, be linked to its protective properties for membranes and numerous endocellular structures, rather than to an osmotic function. The absence of GB in calli could be due to the absence of mature chloroplasts, which are considered to be the site of GB in light conditions.

At the whole plant level, assuming a cytoplasmic accumulation of TSS, these compounds accounted for more than 40% of OA. Once again, this should be regarded as a maximal theoretical value since some sugars, especially reducing hexoses, may accumulate in vacuoles. TSS accumulation can hardly be explained by a stress-induced modification in photosynthesis. An alternative explanation may be an activation of sucrose-phosphate-synthase activity by osmotic stress, as previously demonstrated in spinach (Toroser and Huber, 1997). TSS accumulation in roots suggests an increase in sugar translocation through the phloem in relation to an increase in sink strength. Their accumulation within the non-photosynthetic calli (Table 4) was sufficient to explain almost all OA. Such an accumulation occurring on LS+NaCl medium suggests that sugar translocation from the external medium to the proliferating cells might be increased by \( \text{Na}^+ \). This hypothesis is confirmed by the inability of both resistant and sensitive cell lines to accumulate sugar on LS–NaCl medium.

Proline accumulated to high concentrations in tissues of \( \text{A. halimus} \). While in most Chenopodiaceae, proline is considered to play a minor role in OA compared to GB or...
other quaternary ammonium compounds, these results unexpectedly showed the opposite trend: the contribution of proline to OA may be as high as 44% in old leaves exposed to NaCl while the contribution of GB remained lower than 5%. In this study, proline accumulated mainly in response to NaCl while PEG had no impact on such an accumulation (Fig. 6). Di Martino et al. (2003) demonstrated that, in spinach, proline accumulation occurs in response to the short-term imposition of salinity, while GB accumulated in the second phase, as a consequence of longer exposure to NaCl. This might be related to the fact that proline may be quickly recycled after removal of a temporary stress while GB is hardly catabolised in plants and thus accumulates in cases of permanent stress only. Since the plants were exposed to low NaCl dose for a short time only, it is possible that GB accumulates to a greater extent in the material studied after longer exposure to stress, as occurs in natural field conditions.

Although an efficient OA occurred in stressed leaves (Table 1), it had only a small impact on the whole shoot water potential, suggesting that a concomitant increase in turgor pressure could have contributed to the increased water uptake. Laboratory is now testing the impact of NaCl on the water potential, suggesting that a concomitant increase in GB could contribute to the increased water uptake. Long-term exposure to NaCl would be necessary to study this effect in more detail.

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