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Osmolyte concentrations in *Atriplex halimus* L. and *Atriplex canescens* (Pursh) Nutt. adapted to salinity and low temperature (Chenopodiaceae)

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Resumen

Concentración de osmolitos en Atriplex halimus *L. y* Atriplex canescens *(Pursh) Nutt. adaptados a salinidad y bajas temperaturas (Chenopodiaceae)*

En este trabajo hemos investigado los efectos de la tolerancia a la congelación de *Atriplex halimus* y *Atriplex canescens* de diferentes poblaciones de Argelia. La tolerancia al frío se determinó mediante ensayos de pérdida de electrolitos en hojas de plantas que crecieron durante tres meses en maceta más un mes de aclimatación al frío. Para *A. halimus* se determinó el efecto que producía el incremento de salinidad en el suelo sobre la tolerancia al frío, comparando los niveles de cationes y osmolitos orgánicos en hojas de plantas que crecieron en soluciones salinas, con los de plantas que únicamente habían sido aclimatadas al frío. Se encontró una relación significativa entre la tolerancia al frío y la concentración de Na y Na + K en tejidos. Se relacionan los cambios en la concentración de osmolitos bajo condiciones de salinidad (cultivo hidropónico) con los cambios producidos mediante la interación de aclimatación al frío más salinidad del suelo.

Palabras clave: *Atriplex halimus*, *Atriplex canescens*, Cationes, Halofitos, Salinidad, Solutos compatibles, Tolerancia al frío.

Abstract

We investigated the effects of cold (freezing) tolerance for *Atriplex halimus* and *Atriplex canescens* (Chenopodiaceae) from different locations in Algeria. Plants were grown in pots of soil for three months, after a one-month acclimation period, the cold tolerance was determined, in leaf electrolyte leakage assays. For *A. halimus*, the effect of increased soil salinity (addition of NaCl) on tolerance was determined and the cold-acclimated plants were compared with those grown in saline nutrient solution, in relation to leaf levels of cations and organic osmolytes. There was significant correlation between the cold tolerance and the leaf tissue water concentrations of Na and Na+K. Here we relate changes in osmolyte concentration under cold acclimation/soil salinisation.

Key words: *Atriplex halimus*, *Atriplex canescens*, cations, cold tolerance, compatible solutes, halophytes, salinity.

Introduction

Atriplex halimus L. (Chenopodiaceae) (Saltbush) is a perennial C_4 shrub which grows throughout the Mediterranean basin and is used widely to provide forage, due to its drought and salt tolerance and its high protein content (Le Houérou, 1992). *Atriplex canescens* (Pursh) Nutt. (fourwing saltbush) originates from North America and possesses numerous ploidy levels, from diploid (2n = 2x = 18) to dodecaploid (2n = 12x = 108), which contribute to its adaptation to diverse environmental conditions (Sanderson et al. 1989). In Algeria, *A. halimus* is autochthonous whereas *A. canescens* was introduced in 1987, as a source of fodder in plantations. Here, these two species are grown over a wide range of soil salinity and minimum winter temperatures, from coastal areas to mountainous areas at more than 1100 m altitude.

Although *Atriplex* species are relatively cold-tolerant C_4 plants (Caldwell et al. 1977), their distribution and biomass production are restricted by sub-zero temperatures. Freezing damage arises mainly from the formation of ice in intercellular spaces, and the consequent cellular dehydration (Xin & Browse 2000). The physiological factors related to plant cold (freezing) tolerance include raised tissue concentrations of amino acids, proline, soluble sugars and quaternary ammonium compounds (QACs), such as betaine (Chen & Li 1977, Thomas & James 1993, Allard et al. 1998, Sakamoto et al. 2000, Xin & Browse 2000). Organic osmotica contribute to osmotic adjustment (OA) and also protect the structural integrity of cell membranes and proteins (Xin & Browse 2000). In halophytes, such as *A. halimus*, OA in response to salinity or drought, which also cause cellular dehydration, involves vacuolar accumulation of inorganic ions; simultaneously, compatible organic osmotica accumulate in the cytoplasm to maintain an osmotic equilibrium across the tonoplast (Matoh et al. 1987, Bajji et al. 1998, Martínez et al. 2003, 2004).

The aim of this work was to characterise cold tolerance in *A. halimus* and *A. canescens* populations from Algeria and, for two populations of *A. halimus*, determine the effect of soil salinity on cold tolerance. We also compared plants exposed to salinity in nutrient solution, and cold-acclimated plants, subjected to decreasing temperature and day length, with respect to tissue levels of cations and organic osmotica. The results will be used for selection of species and populations for use under particular conditions of soil salinity and winter temperatures.

Material and methods

Area of study

The Algerian locations of the plants from which fruits were obtained are shown in table 1. Analyses of the soils from these sites (the electrical conductivity (EC), pH and cation concentrations of the vacuum-filtered saturated paste) were performed as described in Walker et al. (2007).

Effects of salinity on *Atriplex halimus*

In a growth room (day/night temperatures of 27/20 ºC, 14-h day, photosynthetically-active radiation (PAR) of 400 µmol m^{-2} s^{-1}, relative humidity 60%), fruits of *A. halimus* populations (El Biodh, El Kasdir and Maamoura) were sown in trays of vermiculite and watered with tap water until they

Species	Population	Latitude (N)	Longitude	Altitude (msl)	Mean temperature coldest month	Soil parameters			
						pH (saturated paste)	EC (dS m^{-1})	Soluble Na meq kg^{-1}	Soluble K meq kg^{-1}
A. halimus	El Biodh	33º54´27´´	00º20´43´´ W	989	-8 ºC	7.61	4.69	3.40	0.63
	El Kasdir	33º42´52´´	01º23´31´´ W	981	-6 ºC	7.66	3.18	1.34	0.39
A. canescens	El Kheiter	34º08´29´´	00º04´15´´ E	989	-8 ºC	7.80	32.80	92.7	9.27
	Maamoura	34º37´29´´	00º33´00´´ E	1106	-6 ºC	7.89	1.30	2.08	0.11
	Oran	35º38´23´´	00º36´46´´ W	92	-5 ºC	7.29	25.10	86.7	2.08
	Ain El Ha I	34º45´31´´	00º07´08´´ E	1008	-8 ºC	8.09	1.84	2.69	1.53
	Ain El Ha II	34º45´42´´	00º08´50´´ E	1036	-10 ºC	8.08	0.93	1.96	0.05
	Maamoura	34º36´34´´	00º33´03´´ E	1106	-6 ºC	7.93	1.61	2.37	0.06

Tabla 1. Localización de las poblaciones de origen de *A. halimus* y *A. canescens*.

Table 1. Description of the original locations of the populations of *A. halimus* and *A. canescens*

were 26 days-old. They were then transplanted to pots (5 plants/pot) containing 1.5 litres of aerated-Hoagland nutrient solution (Hoagland & Arnon 1938), adjusted to pH 6.5 with 0.7 mM NaOH. Two days later, addition of NaCl started. In addition to the control plants, there were two salinity treatments consisting of 100 mM NaCl (in 2 days) and 400 mM NaCl (in 5 days), with three repetitions for each species-treatment combination. The nutrient solution was changed every 4-8 days and the plants were harvested when 49 days-old. The roots of the intact plants cultured with control solution, 100 or 400 mM NaCl were incubated for eight minutes with 10, 120 or 400 mM sorbitol, respectively (plus 1 mM $Ca(NO_3)_2$). The plants were then rinsed with deionised water and the roots and shoots were separated, fresh weighed, uncivilised and re-weighed.

Parameter	Value
Water-holding capacity (%)	34.9
Texture:	Sandy-loam
sand (> 0.02 mm) (%)	62.9
silt (0.002-0.02 mm) (%)	22.3
clay (< 0.002 mm) (%)	14.8
CEC (cmol kg^{-1})	8.45
pH (saturated paste, with water)	7.46
EC (saturated paste, with water) (dS m^{-1})	1.72
$CaCO_3$ (%)	23.7
Available (bicarbonate-extractable) P (µg g^{-1})	6.76
Soluble K (meq kg^{-1})	0.74
Soluble Na (meq kg^{-1})	0.38
Soluble Ca (meq kg^{-1})	3.45
Soluble Mg (meq kg^{-1})	2.43
Total N (µg g^{-1})	1.85
Organic C (µg g^{-1})	14.1

Tabla 2. Características del suelo usado en los ensayos. Los valores son para suelo desecado a 105 ºC.

Table 2. Characteristics of the soil used in the pot experiment, values for soil dried at 105 ºC

Cold tolerance assays

Fruits were sown in trays of vermiculite, in a growth chamber (set to day/night temperatures of 25/21 ºC, 14-h day, PAR of 350 µmol m^{-2} s^{-1}, relative humidity 60%), and watered alternately with tap water (electrical conductivity, EC,=1.36 dS m^{-1}) and Hoagland nutrient solution (Hoagland & Arnon, 1938) for four weeks, before being transplanted to pots containing 3.5 kg of air-dry soil, under the same conditions. The principal characteristics of this soil, determined according to Walker et al. (2007), are given in Table 2. The plants were watered as necessary with tap water. For two populations of *A. halimus*, El Biodh and El Kasdir, 350 mL of 0.6 M NaCl were added 8 weeks after transplanting to half the pots; samples of soil were taken from these pots, one on each assay day, to measure the EC and cation concentrations of the vacuum-filtered saturated paste. For each population-treatment combination, there were six pots, each containing 12 plants.

The plants were used for cold tolerance assays and tissue analyses when four months-old. During the month before the assays, the day/night temperatures and day length were decreased gradually to 10/2ºC and eight hours, respectively, and maintained thus for one week, to achieve cold acclimation. Three separate assays were carried within a one-week period. On each day, several plants were removed from two pots per population-treatment combination and their leaves used for the cold tolerance assay. The dry mass (DM) content was determined, for each day of assay, by weighing samples of fresh and lyophilised leaves. One of the two pots, containing six-eight intact plants, was also used in the assay. Cold tolerance was determined by measuring the freezing-induced electrolyte leakage from detached, whole leaves (Warren et al., 1996), at -8, -14 and -22 ºC. Leaves were rinsed with deionised water, dried, weighed (approximately 2 g), rinsed again (excess water was removed) and placed in glass tubes. The temperature in the freezing-assay cabinet (model CET.25/480; Dycometal, Barcelona, Spain) was lowered to -1.5 ºC, maintained at this temperature for one hour, lowered at 2.5 ºC h^{-1} to the final temperature, maintained at this temperature for 30 min and then raised at 2.5 ºC h^{-1} to 5 ºC. The tubes were then removed from the cabinet, deionised water (10 mL) was added to each tube and the tubes were shaken, at 20 ºC, for four hours. The EC of the "extract" was measured and the tubes were then heated to 95 ºC for 30 min, before cooling and re-measurement of EC. The freezing damage (%) was calculated as: 100 x (EC after freezing/EC after 95 ºC), after removal of the percentage value for control leaves, prepared in the same way but left in the growth room during the assay. The temperature at which 50% of electrolytes were released (LT_{50}) was calculated from best-fit curves fitted to the data (Warren et al. 1996). At the end of each assay, the pots containing intact plants were returned to the growth room and visual symptoms of freezing damage (leaf and meristem death) were assessed weekly after the assays.

Analysis of plant material

The milled, lyophilised tissue from the assays (digested at 210 ºC in a 2:1 nitric acid:perchloric acid mixture) was used for determination of cations by inductively-coupled plasma-optical emission spectrometry (Varian Vista-MPX, Varian Ltd., Mugrave, Australia). This tissue was also used for determining organic osmotica. Proline was extracted by incubating 100 mg of tissue in 10 mL of deionised water for 1 hour at 100 ºC (Tiefert 1988) and determined according to Bates et al. (1973). QACs were extracted by shaking 50 mg of tissue in 10 mL of deionised water for 24 hours at 25ºC and determined according to Grieve & Grattan (1983). Soluble sugars and amino acids were extracted by incubating 40 mg of tissue twice in 5 mL of 60% ethanol, for 30 minutes each time, at 35 ºC. Each extract was centrifuged at 3500 x g for 10 minutes, at 20 ºC, and the two supernatants combined. Chloroform (5 ml) was added and the mixture shaken before centrifugation at 2700 x g for 10 minutes, at 20 ºC. The upper, colourless layer (20% ethanol) was used for determination of amino acids according to Lee & Takahashi (1966). Another sample was diluted 4-fold with absolute ethanol to produce an extract in 80% ethanol for measurement of soluble sugars according to Buysse & Merckx (1993). The residual material from the extraction with 60% ethanol was hydrolysed with 3% HCl, for 3 hours at 125 ºC, and the soluble sugars released measured as an estimate of the starch content.

Statistical analyses

ANOVA was performed, using SPSS version 11.5 software, for the plant parameters, to determine if there were significant ($P<0.05$) effects of treatment, species (for the cold-tolerance experiment) and population. For the two populations of *A. halimus*, El Biodh and El Kasdir, grown with and without soil salinisation, the SPSS General Linear Model was used to determine the effects of population and salinisation and their interaction. Where such effects existed, and there were more than two levels of the factor, the Student Neuman Keuls (SNK) test was used to compare means. Where necessary, the data were log-transformed to achieve normality. Percentage values were arc-sin-transformed before analysis. Pearson correlation coefficients were determined for the relationships between LT_{50} and the plant parameters.

Results

Salinity assay

The figure 1 shows the similarity of the growth (dry matter, DM) responses of the three tested populations of *A. halimus* with respect to increasing salinity; root growth was inhibited significantly, relative to the control ($P<0.05$, SNK test), at 400 mM NaCl for all populations (by an average of 66%) but at 100 mM only for Maamoura (by 32%). Shoot growth was only reduced significantly at 400 mM NaCl (by an average of 49%). As root Na concentration increased with external salinity (Fig. 2D), tissue Ca, Mg and K levels showed no significant changes for any population ($P>0.05$) (Figs. 2A-C). In the shoot (Fig. 3), as the Na level increased with external salinity, the Ca, Mg and K levels declined similarly for the three

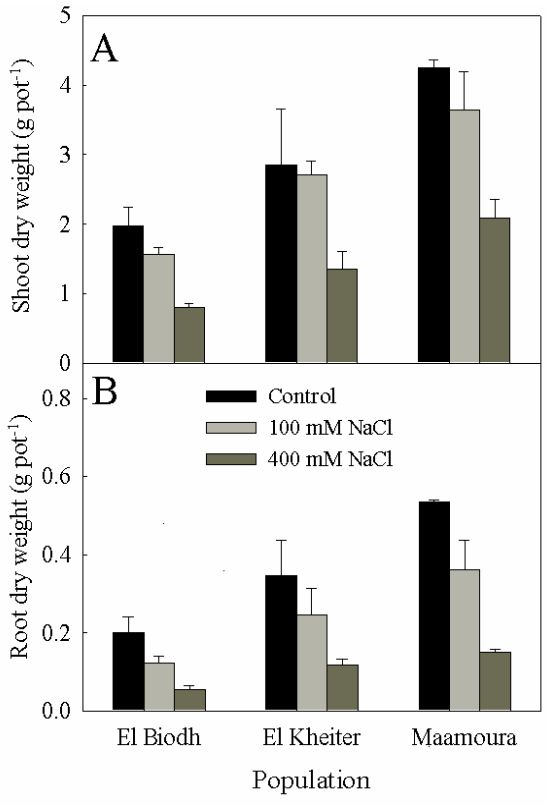

Figura 1. Crecimiento de raíces y tallos de las poblaciones de *A. halimus* de El Biodh, El Kasdir y Mammoura durante 23 días en solución hidropónica, con la presencia de NaCl a 0, 100 y 400 mM respectivamente.

Figure 1: Root and shoot growth of Atriplex halimus populations El Biodh, El Kasdir and Maamoura grown for 23 days in hydroponic nutrient solution, in the presence of 0, 100 or 400 mM NaCl.

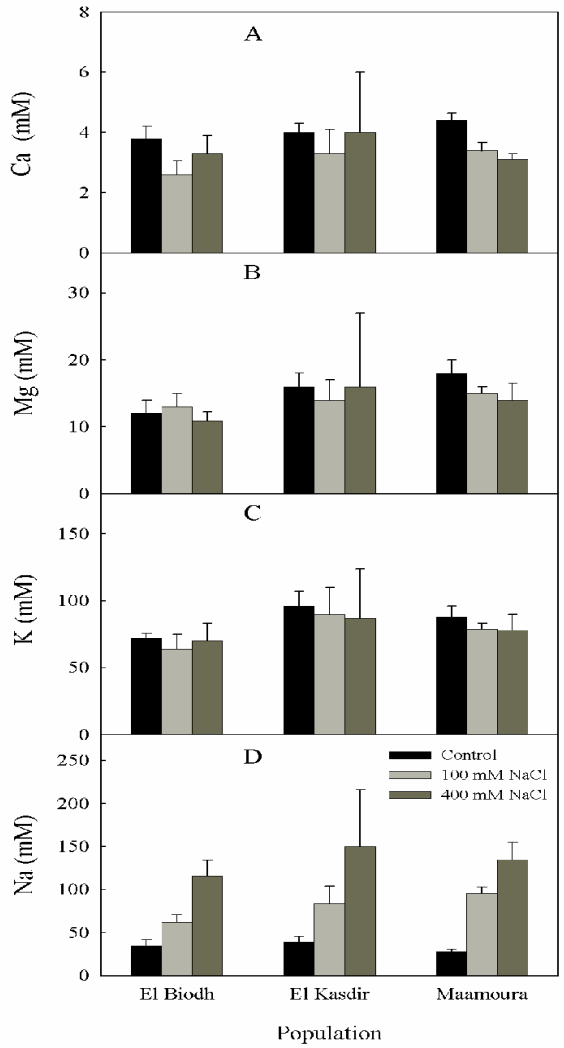

Figura 2. Concentraciones de (A) Ca, (B) Mg, (C) K y (D) Na en las raíces de individuos pertenecientes a las poblaciones de *Atriplex halimus* de El Biodh, El Kasdir y Maamoura que crecieron durante 23 días en solución hidropónica, con la presencia de NaCl a 0, 100 y 400 mM respectivamente.

Fig. 2: Root tissue water concentrations of (A) Ca, (B) Mg, (C) K and (D) Na, for *Atriplex halimus* populations El Biodh, El Kasdir and Maamoura grown for 23 days in hydroponic nutrient solution, in the presence of 0, 100 or 400 mM NaCl.

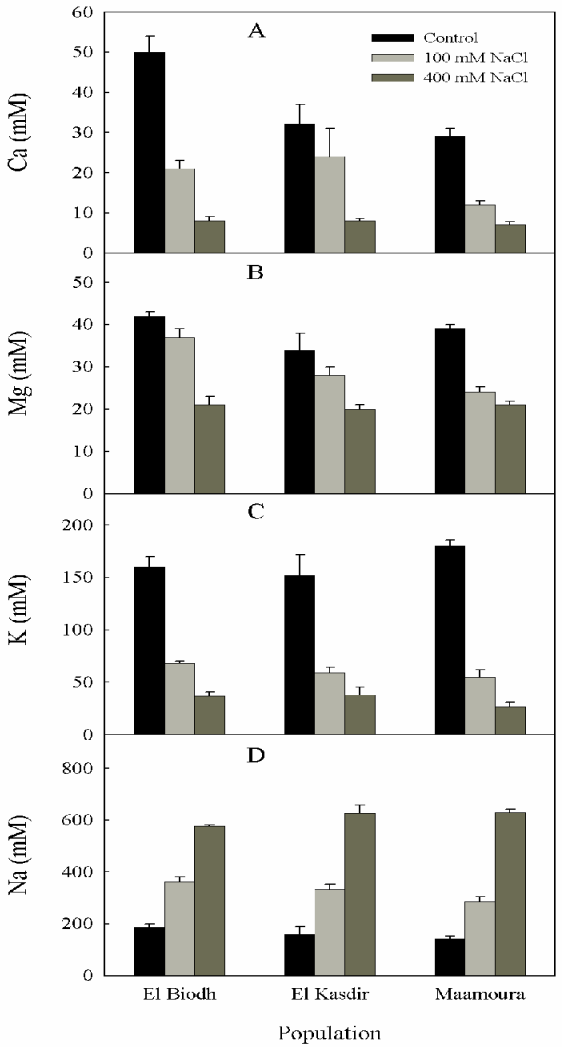

Figura 3. Concentraciones de (A) Ca, (B) Mg, (C) K y (D) Na en los tallos de individuos pertenecientes a las poblaciones de *Atriplex halimus* de El Biodh, El Kasdir y Maamoura que crecieron durante 23 días en solución hidropónica, con la presencia de NaCl a 0, 100 y 400 mM respectivamente.

Fig. 3: Shoot tissue water concentrations of (A) Ca, (B) Mg, (C) K and (D) Na, for *Atriplex halimus* populations El Biodh, El Kasdir and Maamoura grown for 23 days in hydroponic nutrient solution, in the presence of 0, 100 or 400 mM NaCl.

populations. For all three populations, the shoot QAC and proline levels, with respect to the control, were only increased significantly at 400 mM NaCl (Figs. 4A, B). Averaging across treatments, the level of total amino acids was significantly higher in population El Biodh (91 µmol g^{-1}) than in El Kasdir (68 µmol g^{-1}) or Maamoura (56 µmol g^{-1}) and in Maamoura declined with external salinity (Fig. 4C). There was no significant difference in shoot DM soluble sugar concentration among the populations or treatments, but the concentration was higher in control plants for Maamoura and at 400 mM NaCl for the other populations (Fig. 4D). Overall, the starch concentration (Fig. 4E) was much lower in population Maamoura, for which it declined with increasing salinity, whereas the opposite occurred for El Biodh.

Cold tolerance assays

The salinisation of the soil for the *A. halimus* populations El Biodh and El Kasdir increased greatly the soil EC and soluble Na, doubled the soluble Ca and Mg and increased soluble K (Table 3).

The LT_{50} values derived from the electrolyte leakage assays with leaves (Table 4) show a good deal of variation in cold tolerance among popula-

tions of *A. halimus*; some were more tolerant than the chosen populations of *A. canescens* and others less tolerant. For populations from Maamoura, *A. halimus* showed greater tolerance than *A. canescens*. Salinisation of the soil decreased the LT_{50} values for *A. halimus* populations El Biodh, by 7.5 ºC, and El Kasdir, by 5.0 ºC. All plants frozen at -14 or -22 ºC died within 12 days, but for plants subjected to -8 ºC, the order of damage was: all *A. canescens* populations and *A. halimus* population Oran (75-95% of leaves and meristems dead) > *A. halimus* El Kheiter, Maamoura and El Biodh (±NaCl) (30-50%)> *A. halimus* El Kasdir (±NaCl) (<25% damage). This order is more or less in agreement with that obtained in the electrolyte leakage assays.

Tables 5 and 6 show, respectively, the mean values of leaf tissue water cation concentrations for each population and the statistical significance of the species, population and soil salinisation on leaf cation concentrations. Although soil salinisation increased leaf K when El Biodh and El Kasdir were considered together, its only significant effect when the two populations were considered separately was an increase in leaf Na. Excluding the plants grown in salinised soils, the leaf Ca concentration was significantly higher in *A. halimus* (mean of 4.57 mM) than in *A. canescens* (3.67 mM). The leaf K level was higher in A. canescens (267 mM) than in *A. halimus* (138 mM) and differed significantly among populations of both species. The leaf Mg level differed significantly among populations of *A. halimus*. The leaf Na concentration was nearly 4-fold higher for *A. halimus* (455 mM) than for *A. canescens* (117 mM), with significant differences among the populations of both species. There was no relationship between tissue cation levels and the soil EC or soluble cation concentrations at the original sites at which the studied populations grow (Table 1). Regarding the leaf concentrations of organic osmolytes (Table 7), there were no significant differences (P≥0.104) between species or populations, and no effect of soil salinity, although for *A. halimus* population El Biodh, increased soil salinity virtually doubled the shoot sugar content.

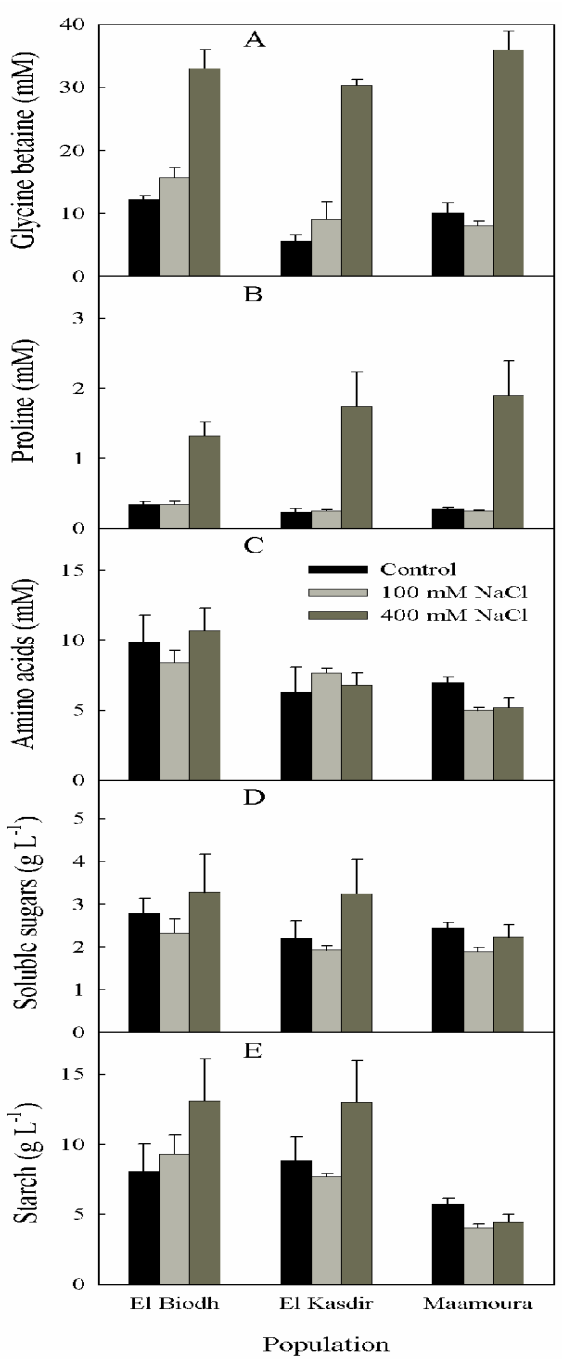

Figura 4. Concentraciones de (A) QACs, (B) prolina, (C) aminoácidos totales, (D) azúcares solubles y (E) almidón en los tallos de individuos pertenecientes a las poblaciones de *Atriplex halimus* de El Biodh, El Kasdir y Maamoura que crecieron durante 23 días en solución hidropónica, con la presencia de NaCl a 0, 100 y 400 mM respectivamente.

Figure 4: Shoot tissue water concentrations of (A) QACs, (B) proline, (C) total amino acids, (D) soluble sugars and (E) starch, for *Atriplex halimus* populations El Biodh, El Kasdir and Maamoura grown for 23 days in hydroponic nutrient solution, in the presence of 0, 100 or 400 mM NaCl.

Discussion

Salinity assay

Although growth declined with increasing extern-

Population	Soil ± NaCl	EC (dS m^{-1})	Cation concentration in saturated extract (µg g^{-1} soil)			
			Ca	Mg	K	Na
El Biodh	-	5.76b	231b	88a	40a	148b
	+	27.83a	453a	177a	62a	1345a
El Kasdir	-	5.30b	213b	73a	37a	143b
	+	26.78a	486a	188a	57a	1318a

Tabla 3. Valores medios de conductividad eléctrica y concentración de cationes de extractos de pasta saturada de suelo, usados para el desarrollo de *A. halimus* de las poblaciones de El Biodh y El Kasdir. (+) indica que se han añadido 350 mL de NaCl 0.6 M al suelo cuando las plantas tenían 8 semanas, (-) indica que no se ha añadido nada. En todos los casos la salinización del suelo fue significativa ($P<0.01$) pero no se observaron diferencias significativas entre poblaciones, ni entre poblaciones con interacción de la salinización.

Table 3. Mean (n = 3) electrical conductivity (EC) values and cation concentrations for saturated paste extracts of the soil used to grow *A. halimus* populations El Biodh and El Kasdir grown without (-) and with (+) the addition of 350 mL of 0.6 M NaCl to the soil when plants were 8 weeks old. In all cases, the effect of soil salinisation was significant ($P<0.01$) but there was no significant population effect or population x salinisation interaction ($P>0.05$)

Species	Population	Soil ± NaCl	LT_{50} (ºC)
A. halimus	El Biodh	-	-16.0
	El Kasdir	+	-23.5
	Maamoura	-	-15.6
	El Kheiter	+	-20.6
	Oran	-	-15.0
		-	-9.5
		-	-8.0
A. canescens	Maamoura	-	-9.9
	Saida I	-	-9.5
	Saida II	-	-11.4

Tabla 4. Valores de LT_{50} (ºC), obtenidos mediante ensayos de fuga de electrolitos para *A. halimus* y *A. canescens*.

Table 4. Values of LT_{50} (ºC), determined by electrolyte leakage assays for *A. halimus* and *A. canescens*.

Species	Population	Soil ± NaCl	Cation concentration (mM)			
			Ca	Mg	K	Na
A. halimus	El Biodh	-	3.57	56.9ab	139cdB	485abB
		+	5.62	64.7	164AB	773 A
	El Kasdir	-	4.50	64.5a	162c AB	502a B
		+	4.62	55.3	182 A	696 A
	Maamoura	-	3.37	47.9b	127d	471ab
	El Kheiter	-	3.78	57.4ab	121d	400c
	Oran	-	3.12	45.2b	139cd	418bc
A. canescens	Maamoura	-	4.60	54.4ab	221b	163d
	Saida I	-	4.30	51.2ab	295a	113de
	Saida II	-	4.82	53.5ab	286a	76e

Tabla 5. Concentración media de cationes en tejido de hojas (n=6) de individuos de *Atriplex halimus* y *A. canescens*, que crecieron durante 4 meses antes de la realización de los ensayos de tolerancia al frío. Las diferencias significativas observadas ($P<0.05$, SNK test) se señalan con letras minúsculas cuando se consideran todos los valores obtenidos, y con mayúsculas cuando se consideran únicamente las poblaciones de *A. halimus* de El Biodh y El Kasdir que crecieron con y sin la adición de 350 mL de NaCl 0.6 M al suelo cuando las plantas contaban con 8 semanas de edad.

Table 5: Mean leaf tissue water cation concentrations (n = 6) for *Atriplex halimus* and *A. canescens* plants grown for four months in soil before cold tolerance assays. For each species considered separately, significant differences between mean values ($P<0.05$, SNK test) are denoted by differing lower-case letters when considering all values, and by differing upper-case ones when considering *A. halimus* populations El Biodh and El Kasdir grown without (-) and with (+) the addition of 350 mL of 0.6 M NaCl to the soil when plants were 8 weeks old.

Parameter	Species	Population (growth in non-salinised soil)		NaCl
		A. halimus	*A. canescens*	
Ca (mM)	6.433, 0.015	1.575, 0.212	0.189, 0.830	4.168, 0.055
Mg (mM)	0.237, 0.628	4.580, 0.007	0.415, 0.668	0.022, 0.884
K (mM)	198.4, < 0.001	4.044, 0.012	16.62, < 0.001	6.032, 0.023
Na (mM)	354.4, < 0.001	3.413, 0.023	15.33, < 0.001	25.26, < 0.001
QACs	0.764, 0.392	1.7775, 0.210	1.945, 0.223	0.055, 0.821
Proline	1.985, 0.173	2.390, 0.120	0.682, 0.541	0.664, 0.439
Amino acids (mM)	0.776, 0.388	1.459, 0.286	0.358, 0.713	0.792, 0.399
Sugars (g L^{-1})	0.431, 0.518	2.577, 0.102	2.931, 0.129	0.915, 0.367
Starch (g L^{-1})	2.973, 0.099	1.879, 0.191	5.746, 0.040	2.313, 0.167

Tabla 6. ANOVA de los factores que afectan a los parámetros de las plantas: NaCl únicamente para las poblaciones de El Biodh y El Kasdir que crecieron con salinización o sin ésta. En el resto de columnas se excluyen los valores para plantas que crecieron en suelos salinizados.

Table 6: ANOVA (F value, corresponding P value) for the factors affecting the plant parameters: "NaCl" refers only to the A. halimus populations El Biodh and El Kasdir, which were grown with and without soil salinisation, whereas the other columns exclude the values for plants grown in salinised soil.

Species	Population	Soil ± NaCl	QACs (mM)	Proline (mM)	Amino acids (mM)	Soluble sugars (g L^{-1})	Starch (g L^{-1})
A. halimus	El Biodh	-	32.6	0.261	4.5	3.9	9.2
		+	24.9	0.256	3.9	7.3	17.6
	El Kasdir	-	25.4	0.269	4.4	6.2	15.7
		+	29.7	0.200	3.7	7.5	15.6
	Maamoura	-	13.7	0.210	4.4	2.3	6.9
	El Kheiter	-	19.5	0.221	5.5	4.3	8.7
	Oran	-	15.7	0.160	3.0	6.4	14.8
A. canescens	Maamoura	-	31.3	0.210	4.5	5.0	19.5
	Saida I	-	19.3	0.159	4.7	3.1	10.0
	Saida II	-	24.9	0.201	5.4	4.2	15.4

Tabla 7. Concentración media (n=3) de los compuestos orgánicos en el tejido de las hojas de individuos de *Atriplex halimus* y *A. canescens* que crecieron durante 4 meses antes de los ensayos de tolerancia al frío. Las poblaciones de *A. halimus* de El Biodh y El Kasdir crecieron con y sin la adición de 350 mL de NaCl 0.6 M al suelo cuando las plantas contaban con 8 semanas de edad. No se diferencias significativas ($P<0.05$) ni en la salinización o no del suelo ni entre las poblaciones.

Table 7. Mean (n=3) leaf tissue water concentrations of organic compounds for *Atriplex halimus* and *A. canescens* plants grown for four months in soil before cold tolerance assays. *A. halimus* populations El Biodh and El Kasdir were grown without (-) and with (+) the addition of 350 mL of 0.6 M NaCl to the soil when plants were 8 weeks old. For no parameter was there a significant ($P < 0.05$) effect of population or soil salinisation.

al NaCl concentration, the decrease was only significant ($P<0.05$) at 400 mM NaCl, reflecting the halophytic nature of *A. halimus* (Le Houérou 1992, Bajji et al. 1998). As observed previously for *A. halimus* (Bajji et al. 1998), the plants adjusted to a saline root environment by accumulation of Na, whilst shoot Ca, Mg and K levels dropped. The levels of Ca, Mg and K decreased according to increasing salinity, but not significantly between different populations. We record a reduction rate between 8 and 22% for Ca, 5 and 26% for K and from 11 to 15% for Mg compared to control, knowing that the Na has been an increase between 19 and 41% always compared to the control. Regarding the compatible, organic solutes, which accumulate in the cytoplasm to counter, osmotically, the vacuolar accumulation of cations (Matoh et al. 1987, Martínez et al. 2004), accumulation of betaine and proline was only significant at high external NaCl, mirroring the findings of Bajji et al. (1998). The low levels of proline (<2 mM) indicate a non-osmotic role, possibly as an anti-oxidant (Smirnoff & Cumbes 1989).

Cold tolerance assays

Atriplex species seem to be relatively cold-tolerant C_4 plants, their photosynthetic pathway remaining active at 4-10 ºC (Caldwell et al. 1977) but, in Mediterranean areas, their growth and survival are greatly affected by sub-zero temperatures. The idea of this work was to compare cold-acclimated plants of *A. halimus* with similar plants of *A. canescens* and with salinised plants. Non-acclimated plants were not used since the comparison of acclimated and de-acclimated plants is the subject of a separate study, with field-grown *A. halimus*.

Halophytic shrub species can achieve extremely negative ψs values, thus decreasing the freezing point of their cells (Newton and Goodin 1989) and minimising water movement from the cells to extracellular ice, the principal mechanism of cold (freezing) injury (Xin & Browse 2000). In drought or salt-exposed *A. halimus* plants, OA is achieved by (vacuolar) accumulation of Na and K and (cytoplasmic) accumulation of the QAC betaine, proline and soluble sugars (Bajji et al. 1998, Martínez et al. 2003, 2004).

When the *A. halimus* populations El Biodh, El Kasdir and Maamoura were grown in soil (± salinisation), their shoot Na concentrations were of the same order of magnitude as for the 400 mM NaCl-exposed plants of the salinity assay, but their K, Mg and proline levels were similar to the control plants. The low leaf Ca concentrations observed in the soil-grown plants probably represent lower vacuolar accumulation of Ca without effects on growth (Gilroy et al. 1987). In *A. halimus*, proline seems to accumulate in response to salinity but not water stress (Bajji et al. 1998, Martínez et al. 2004) so the lack of its accumulation in the soil-grown plants (± soil salinisation) indicates the absence of stress. However, the similarity of the QAC concentrations of the soil-grown plants to

those of the 400 mM NaCl-grown plants could indicate a degree of salt/water stress (Bajji et al. 1998, Martínez et al. 2004), possibly due to the increase in soil salinity (EC) (Tables 2, 4) resulting from the watering with tap water, although, for *A. halimus* populations El Biodh and El Kasdir, addition of NaCl did not raise tissue QAC levels. Betaine accumulation improves cold tolerance in other species (Allard et al. 1998, Sakamoto et al. 2000) and, in *A. halimus*, it may protect phosphoenolpyruvate carboxylase and pyruvate orthophosphate dikinase from cold inactivation (Salahas et al. 2002). Although during the cold acclimation of numerous species decreased and increased levels, respectively, of tissue starch and soluble sugars as well as increases in tissue proline and amino acid levels have been found (Chen & Li 1977, Thomas & James 1993, Xin & Browse 2000), in the current study there were no significant correlations between these factors and the LT_{50} values.

There was no correlation between the minimum temperatures at the original sites of the populations and the LT_{50} values, indicating that other factors influence cold tolerance more strongly. Considering both species together, the LT_{50} (ºC) was correlated significantly ($P<0.05$) only with leaf Na (mM) (Pearson correlation, $r=-0.817$, $P=0.004$) or Na + K (mM) ($r=-0.896$, $P<0.001$). Although Na seems to be required as a nutrient by C_4 species such as *A. halimus*, under stress conditions (salinity or drought) its role appears to be in OA, with its accumulation stimulating the synthesis of organic osmolytes (Bajji et al. 1998, Martínez et al. 2004). Increased tissue Na concentration is the obvious explanation for the increased cold tolerance of the salinised plants of *A. halimus* populations El Biodh and El Kasdir. Cytoplasmic K is regulated at 100-180 mM, to satisfy the metabolic requirements for K, with excess being stored in the vacuole (Walker et al. 1996), so the greater vacuolar accumulation of K in pot-grown *A. canescens* plants could have a role only in water relations. Glenn et al. (1996) found that populations of *A. canescens* originating from xeric and saline environments appeared to accumulate preferentially K or Na, respectively, in order to achieve OA. Martínez et al. (2004) found similar K and Na accumulations for two *A. halimus* populations (one from a saline site and one from a xeric site) during imposition of water stress. Our results for plants grown in non-salinised soil show that *A. halimus* accumulated relatively less K and more Na than *A. canescens*, but with no apparent relationship between the soluble Na and K levels in the original soils (Table 1) and the shoot accumulation of these cations in the non-salinised test soil (Table 6).

For species used as fodder, such as *A. halimus* and *A. canescens*, the tissue levels of inorganic and organic osmotica, as well as affecting stress tolerance, have repercussions for livestock nutrition. For example, ingestion of plant material with elevated salt content requires increased water intake and can depress appetite and betaine can affect fat deposition and carcass quality (Masters et al. 2001).

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