

Freezing tolerance in Algerian populations of *Atriplex halimus* and *Atriplex canescens*

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Abstract

The freezing tolerance of populations of the fodder species *Atriplex halimus* L. and *Atriplex canescens* (Pursh.) Nutt (Chenopodiaceae) from different locations in Algeria was investigated, in relation to plant provenance and leaf cation concentrations. For two populations of *A. halimus*, the effect of increased soil salinity (addition of NaCl) on tolerance was determined. Tolerance was determined in leaf electrolyte leakage assays and by assessment of visual damage, after exposure to temperatures between -5 and -25°C. There was significant correlation ($P < 0.005$) between freezing tolerance and the leaf sap concentrations of Na and Na+K, tolerance being improved markedly by soil salinisation, but no relationship between tolerance and the soil salinity or minimum winter temperatures of the original sites of the populations. It is concluded that, for these halophytic species, the soil salinity (supply of ionic osmolytes) at cold-winter sites will be of great importance regarding the likely freezing damage.

Additional key words: cations, cold, flow cytometry, halophytes, salinity

Resumen

Tolerancia a la congelación en poblaciones argelinas de *Atriplex halimus* y *Atriplex canescens*

Se ha investigado la tolerancia a la congelación de distintas poblaciones de las especies forrajeras *Atriplex halimus* L. y *Atriplex canescens* (Pursh.) Nutt (Chenopodiaceae) de diversos lugares en Argelia, en relación con las concentraciones de cationes en la hoja y con la distinta procedencia de la plantas. Se determinó el efecto de la salinidad del suelo (adición de NaCl) sobre la tolerancia al frío para dos poblaciones de *A. halimus*. La tolerancia a la congelación fue determinada por análisis del contenido de electrólitos en las hojas y por el gravamen del daño visual, tras la exposición a temperaturas entre -5 y -25°C. Existió una correlación significativa ($P < 0,005$) entre la tolerancia a la congelación y las concentraciones de Na y Na+K en la savia de las hojas, siendo la tolerancia mejorada de forma acusada mediante la salinización del suelo, pero no existió ninguna relación entre la tolerancia a la congelación y las temperaturas mínimas del invierno o la salinidad del suelo de los lugares originales de las poblaciones. Se concluye, por tanto, que, para estas especies halófitas, la salinidad del suelo (suministro de osmolitos iónicos) en sitios de invierno frío será determinante del riesgo de daño por congelación.

Palabras clave adicionales: cationes, citometría de flujo, frío, halófitas, salinidad.

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Introduction

Atriplex halimus L. (Chenopodiaceae) (Mediterranean saltbush) is a perennial 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). *A. halimus* exists as both diploid ($2n=2x=18$) and tetraploid ($2n=4x=36$) populations, the former occurring in Spain and France and the latter in North Africa and Eastern parts of the Mediterranean basin (Walker *et al.*, 2005). *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 and Stutz, 2001). In Algeria, *A. halimus* is native whereas *A. canescens* was introduced from the USA, in 1987, as a source of fodder in plantations. Here, these two species grow 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 injury arises mainly from cellular dehydration, due to movement of intracellular water to ice in the extracellular space, and damage to cell membranes (Xin and Browse, 2000; Uemura *et al.*, 2003). It has been shown recently (Walker *et al.*, 2008) that, under field conditions, freezing tolerance of *A. halimus* populations seemed to be related to leaf concentrations of Na and K and to ploidy, diploid populations being more tol-

erant than tetraploid. Interestingly, in this work, freezing tolerance measured in laboratory assays with detached leaves, was similar for plants grown in two contrasting sites, mild-winter/more-saline soil and cold-winter/less-saline soil, although the former plants had never been exposed to sub-zero temperatures in the field.

The aim of the current work was to extend previous freezing tolerance studies in order to compare *A. halimus* and *A. canescens* from Algeria, in relation to the location and soil characteristics of the populations. For two populations of *A. halimus*, it was intended to determine the effect of soil salinity on freezing tolerance, for plants grown under controlled conditions – thus eliminating variables such as climate and soil type. The results would be used to select species and populations appropriate for providing fodder and soil stabilisation in cold-winter and/or saline-soil sites in Algeria.

Material and methods

The populations studied and their original sites

In Algeria, fruits were obtained at the locations of the principal plantations of *A. halimus* and *A. canescens* (var. *occidentalis*, tetraploid, $2n=4x=36$; Sanderson and Stutz, 2001) in this country, including high-altitude (low and high soil salinity) and low-altitude (saline) sites (Table 1). Analyses of the soils from these sites (electrical conductivity [EC], pH and cation concentrations of the vacuum-filtered saturated paste) were performed as described in Walker *et al.* (2007).

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

Species	Population	Latitude (N)	Longitude	Altitude (m asl)	Mean minimum temperature of coldest month (°C)	Soil parameters			
						pH (saturated paste)	EC (dS m ⁻¹)	Soluble Na (cmol kg ⁻¹)	Soluble K (cmol kg ⁻¹)
<i>A. halimus</i>	El Biodh	33°54'27''	00°20'43'' W	989	-8	7.61	4.69	0.34	0.06
	El Kasdir	33°42'52''	01°23'31'' W	981	-6	7.66	3.18	0.13	0.04
	El Kheiter	34°08'29''	00°04'15'' E	989	-8	7.80	32.80	9.27	0.93
	Maamoura	34°37'29''	00°33'00'' E	1106	-6	7.89	1.30	0.21	0.01
	Oran	35°38'23''	00°36'46'' W	92	-5	7.29	25.10	8.67	0.21
<i>A. canescens</i>	Ain El Ha I	34°45'31''	00°07'08'' E	1008	-8	8.09	1.84	0.27	0.15
	Ain El Ha II	34°45'42''	00°08'50'' E	1036	-10	8.08	0.93	0.20	0.01
	Maamoura	34°36'34''	00°33'03'' E	1106	-6	7.93	1.61	0.24	0.01

Freezing tolerance assays

Fruits were sown in trays of vermiculite, in a growth chamber (day/night temperatures of 27/21°C, 14-h day, photosynthetically-active radiation of 350 $\mu\text{mol m}^{-2} \text{s}^{-1}$, relative humidity 60%), and watered alternately with tap water (EC=1.36 dS m^{-1}) and Hoagland nutrient solution (Hoagland and Arnon, 1938) for one month, before being transplanted to pots containing 3.5 kg of air-dry soil, under the same conditions. The relevant characteristics of this soil (Walker *et al.*, 2007) are a sandy-loam texture, EC and pH (both saturated paste) of 1.72 dS m^{-1} and 7.46, respectively, and a cation exchange capacity of 8.45 cmol kg^{-1} . The plants were watered as necessary with tap water. For two populations of *A. halimus*, El Biodh and El Kasdir (chosen because they are the most important populations in terms of area grown), 350 mL of 0.6 M NaCl were added when the plant were three months-old, to half the pots; samples of soil were taken from three of 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 12 pots, each containing 12 plants. There were 12 blocks, with one pot per population-treatment (\pm NaCl) combination distributed randomly within each block.

The plants were used for freezing 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 10/2°C and eight hours, respectively, and maintained thus for one week, to achieve cold acclimation (Hou and Romo, 1998). Tolerance was determined by measuring the freezing-induced electrolyte leakage from detached leaves (Warren *et al.*, 1996). Two separate assays were carried out in a 3-day period. On each day, leaves were removed from each plant within each pot and mixed to provide 12 samples: two were weighed fresh and then freeze-dried for analysis and 10 used in the freezing assay. Leaves were soaked in deionised water for 5 min to remove salts released from burst vesiculated hairs (Bajji *et al.*, 1998), dried, weighed (2 g), moistened and placed in glass tubes. The soaking was the same for both control plants and those grown in salinised soil, although we did not verify whether the amounts of ion excreted are the same in both cases. Pots containing intact plants were also used (one at each assay temperature, on each day). For each population-treatment combination, there were two leaf samples for each temperature: -5, -10, -15, -20 and -25°C.

The temperature in the freezing-assay cabinet (model CET.25/480; Dycometal, Barcelona, Spain) was low-

ered to -1.5°C, maintained for one hour and then lowered at 2.5°C h^{-1} . At each of the selected temperatures the chamber was maintained for 30 min. Pots and tubes were removed from the cabinet and kept at 4°C overnight. Deionised water (10 mL) was added to each tube and they were shaken at 20°C for four hours. The EC of the “extract” was measured and the tubes were then heated to 95°C for 30 minutes before cooling and re-measurement of EC. The freezing damage (%) was calculated as $100 \times (\text{EC after freezing} / \text{EC after } 95^\circ\text{C})$, after removal of the value for leaves ($n=4$) prepared in the same way but kept at 20°C during the assay. The temperature at which 50% of electrolytes were released (LT₅₀) was calculated from non-linear curves fitted to the data (Warren *et al.*, 1996), using SPSS version 11.5 software, and the mean LT₅₀ for each population ($n=4$, two values per day) calculated using these values. The pots containing intact plants were returned to the growth chamber and visual symptoms of freezing damage to leaves were assessed weekly.

Analysis of plant material

Freeze-dried leaves were milled and acid-digested for determination of cations by inductively-coupled plasma-optical emission spectrometry (Varian Vista-MPX, Varian Ltd., Murgave, Australia).

Determination of nuclear DNA content and ploidy

Using tomato (*Solanum lycopersicum* L.) as the internal standard and propidium iodide as the stain, the nuclear DNA contents of *A. halimus* and *A. canescens* were determined by flow cytometry as described in Walker *et al.* (2005), using six leaves per population (one from each of six plants). The ploidy of the Algerian populations of *A. halimus* was determined by comparison with the nuclear DNA contents, determined simultaneously, of populations of *A. halimus* (Cala Tari-da and Butera; diploid and tetraploid, respectively) (Walker *et al.*, 2005).

Statistical analyses

ANOVA was performed, to determine if there were significant ($P<0.05$) effects of species and population

on LT_{50} and the plant cation concentrations and nuclear DNA content; when necessary, data were \log_{10} -transformed to achieve normality. For the two populations of *A. halimus*, El Biodh and El Kasdir, grown with and without soil salinisation, a General Linear Model was used to determine the effects of population and salinisation and their interaction. The least significant difference (LSD) test was used to compare means. Pearson correlation coefficients between LT_{50} and the plant cation concentrations were determined. All analyses were performed using SPSS version 11.5 software.

Results

The LT_{50} values derived from the electrolyte leakage assays with leaves (Table 2) show that *A. halimus* populations El Biodh, El Kasdir and Maamoura were the most freezing-tolerant (more-negative LT_{50} values). For populations from the same location, Maamoura, *A. halimus* showed greater tolerance than *A. canescens*, but overall the species effect was not significant ($P=0.987$). Salinisation of the soil decreased the LT_{50} values significantly ($P<0.05$) for *A. halimus* populations El Biodh and El Kasdir. All plants frozen at -15°C or lower died within 12 days, but for plants subjected to -5 or -10°C , the order of damage (1 month after the assays) was: all *A. canescens* populations and *A. halimus* population Oran (75-95% of leaves necrotic) > *A. halimus* El Kheiter, Maamoura and El Biodh (\pm NaCl) (30-50%) > *A.*

Table 2. Mean values of LT_{50} ($^{\circ}\text{C}$) ($n=4$), determined by electrolyte leakage assays for *Atriplex halimus* and *Atriplex canescens*. *A. halimus* populations El Biodh and El Kasdir were grown without (-) or with (+) soil salinisation (addition of 350 mL of 0.6 M NaCl to 3.5 kg of soil in the pots one month before the assays)

Species	Population	Soil \pm NaCl	LT_{50} ($^{\circ}\text{C}$)
<i>A. halimus</i>	El Biodh	-	-16.0
		+	-23.5
	El Kasdir	-	-15.6
		+	-20.6
	Maamoura	-	-15.0
		-	-9.5
<i>A. canescens</i>	Maamoura	-	-9.9
	Ain El Ha I	-	-9.5
	Ain El Ha II	-	-11.4
LSD ($P=0.05$)			4.6

halimus El Kasdir (\pm NaCl) (< 25% damage). This order is in agreement with that obtained in the electrolyte leakage assays, indicating their viability for estimation of freezing tolerance. Plant death was probably not related to root damage, as the measured soil temperatures did not fall below -2°C .

Excluding the plants grown in salinised soils, the leaf Ca concentration (Table 3) was significantly higher in *A. halimus* (overall mean of 4.57 mM) than in *A. canescens* (3.67 mM) (ANOVA, $P<0.05$). The leaf K level was significantly higher in *A. canescens* (267 mM) than in *A. halimus* (138 mM) and differed significantly among populations of both species. 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. However, there was no relationship between tissue K and Na levels of the plants grown in the non-salinised soil (Table 3) and the soil EC or soluble K and Na concentrations at the original sites at which the studied populations grow (Table 1); for example, *A. halimus* populations from saline sites (El Kheiter and Oran) did not have higher tissue concentrations of K or Na. The leaf Mg differed significantly among populations of *A. halimus*. The percentage dry matter content of the leaves was not affected by the soil salinisation and did not differ significantly between populations (data not shown), so this was not a factor contributing to differences in tissue sap cation concentrations.

Salinisation of the soil for *A. halimus* populations El Biodh and El Kasdir increased greatly the soil EC and soluble Na and doubled the soluble Ca and Mg (Table 4). Presumably in order to lower plant water potential and thus maintain water uptake when confronted with these higher concentrations of salts in the soil solution, leaf K increased significantly for *A. halimus* population El Biodh, but not El Kasdir, and leaf Na increased significantly for both populations in the salinised soil (Table 3).

The flow cytometry analyses (Table 5) show that all the Algerian populations of both *A. halimus* and *A. canescens* are tetraploid, with no significant variation in nuclear DNA content among populations of the same species.

Discussion

Although population Oran, from the coastal site having the mildest winter (Table 1), was the most freezing-

Table 3. Mean leaf sap cation concentrations ($n=4$) for *Atriplex halimus* plants grown for four months in pots before cold tolerance assays. Populations El Biodh and El Kasdir were grown without (-) or with (+) the addition of 350 mL of 0.6 M NaCl to 3.5 kg of soil in the pots one month before the freezing-tolerance (LT_{50}) assays. For Na, the statistical analyses refer to the \log_{10} -transformed values, given in parentheses

Species	Population	Soil \pm NaCl	Cation concentration (mM)				
			Ca	Mg	K	Na	
<i>A. halimus</i>	El Biodh	-	3.57	56.9	139	481 (2.682)	
		+	5.62	64.7	164	766 (2.884)	
	El Kasdir	-	4.50	64.5	162	500 (2.699)	
		+	4.62	55.3	182	675 (2.829)	
<i>P</i> values of the 2x2 factorial contrasts:							
Population (P)			0.951	0.835	0.033	< 0.001	
Soil salinisation (S)			0.055	0.884	0.023	< 0.001	
Interaction PxS			0.083	0.067	0.826	0.300	
<i>A. halimus</i>	Maamoura	-	3.37	47.9	127	465 (2.667)	
	El Kheiter	-	3.78	57.4	121	399 (2.601)	
	Oran	-	3.12	45.2	139	416 (2.619)	
	<i>A. canescens</i>	Maamoura	-	4.60	54.4	221	163 (2.212)
		Ain El Ha I	-	4.30	51.2	295	113 (2.052)
		Ain El Ha II	-	4.82	53.5	286	76 (1.882)
LSD ($P=0.05$)			1.47	9.8	23	0.097	

sensitive of the *A. halimus* populations, for neither species was there a correlation between the minimum

temperatures at the original sites of the populations and the LT_{50} values, indicating that factors other than the

Table 4. Mean ($n=6$) electrical conductivity (EC) values and cation concentrations for saturated paste extracts of the soil used to grow *Atriplex halimus* populations El Biodh and El Kasdir without (-) or with (+) the addition of 350 mL of 0.6 M NaCl to 3.5 kg of soil in the pots one month before the freezing-tolerance (LT_{50}) assays. The statistical analyses refer to the \log_{10} -transformed values, given in parentheses

Population	Soil \pm NaCl	EC (dS m ⁻¹)	Cation concentration in saturated extract (cmol kg ⁻¹ soil)			
			Ca	Mg	K	Na
El Biodh	-	5.6 (0.751)	0.58 (-0.251)	0.36 (-0.490)	0.10 (-1.026)	0.64 (-0.232)
	+	27.7 (1.442)	1.13 (0.041)	0.73 (-0.152)	0.16 (-0.805)	5.84 (0.760)
El Kasdir	-	5.3 (0.724)	0.53 (-0.277)	0.30 (-0.547)	0.09 (-1.028)	0.62 (-0.215)
	+	26.7 (1.427)	1.21 (0.076)	0.77 (-0.120)	0.15 (-0.839)	5.73 (0.757)
LSD ($P=0.05$)		0.128	0.199	0.345	0.233	0.271
<i>P</i> values of the 2x2 factorial contrasts:						
Population (P)		0.606	0.939	0.905	0.809	0.939
Soil salinisation (S)		< 0.001	0.001	0.007	0.007	< 0.001
Interaction PxS		0.870	0.625	0.684	0.684	0.907

Table 5. Mean 2C nuclear DNA contents ($n=6$) of the studied Algerian populations of *Atriplex halimus* and *Atriplex canescens*. For *A. halimus*, the ploidy^a was determined by comparison with the nuclear DNA contents, determined simultaneously, of populations of *A. halimus* (Cala Tarida and Butera) of known ploidy^b (Walker *et al.*, 2005).

Species	Population	Mean 2C nuclear DNA content (pg)	Ploidy
<i>A. halimus</i>	El Biodh	4.993	Tetraploid ^a
	El Kasdir	5.006	Tetraploid ^a
	El Kheiter	4.994	Tetraploid ^a
	Maamoura	4.920	Tetraploid ^a
	Oran	5.049	Tetraploid ^a
	Cala Tarida	2.460	Diploid ^b
	Butera	4.827	Tetraploid ^b
LSD ($P=0.05$)		0.214	
<i>A. canescens</i>	Ain El Ha I	3.145	Tetraploid
	Ain El Ha II	3.274	Tetraploid
	Maamoura	3.143	Tetraploid
LSD ($P=0.05$)		0.176	

adaptation of plants to their local conditions can influence freezing tolerance.

Considering both *A. canescens* and *A. halimus* together, for all cations determined, the LT_{50} ($^{\circ}C$) was correlated significantly ($P<0.05$) only with leaf Na (mM) (Pearson correlation, $r=-0.817$, $P=0.004$) or Na + K (mM) ($r=-0.896$, $P<0.001$). Walker *et al.* (2008) reported significant correlations between leaf dry matter Na and K concentrations and the LT_{50} for field-grown *A. halimus*. These authors also found a good agreement between tolerance determined in laboratory freezing assays, using detached leaves, and whole-plant tolerance (visual damage) in the field. It appears that high soil salinity, which exists in both coastal and mountainous areas in Algeria (Table 1), can improve freezing tolerance in halophytic species such as *A. halimus* which are able to accumulate high tissue concentrations of salt. Since the cytoplasmic concentration of Na does not exceed 5-10 mM (Carden *et al.*, 2003), its accumulation in the vacuole could be the mechanism by which Na contributed to the increased freezing tolerance of the salinised plants of *A. halimus* populations El Biodh and El Kasdir. However, since freezing injury is caused mainly by cellular dehydration, due to loss of intracellular water to ice in the extracellular space, apoplasmic accumulation of Na could also have been involved - via a lowering of the temperature at which this ice forms (Xin and Browse, 2000; Uemura *et al.*,

2003). In order to control ionic strength in the cytoplasm, K is regulated at 100-150 mM in this intracellular compartment, with excess being stored in the vacuole (Walker *et al.*, 1996). So, the accumulation of K above this range of concentrations in the *A. halimus* populations grown in salinised soil probably had a role in osmotic adjustment (OA) in the vacuole. 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. The 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 4).

When *A. halimus* is exposed to drought or salinity, which can cause cellular dehydration, OA is achieved by an elevated vacuolar accumulation of inorganic ions and, in order to achieve osmotic balance across the tonoplast, cytoplasmic accumulation of betaine, proline and soluble sugars - "compatible" solutes whose accumulation to high concentrations does not affect metabolic processes (Bajji *et al.*, 1998; Martínez *et al.*, 2003, 2005). The present results indicate that elevated cation levels provide tolerance of freezing, as well as drought and salinity, in halophytic shrub species such as *A. halimus* and *A. canescens*. These species can achieve extremely negative osmotic potential values, 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 freezing injury (Xin and Browse, 2000). By contrast, in glycohytes, stress acclimation generally involves the vacuolar accumulation of organic solutes such as amino acids and hexose sugars (Xin and Browse, 2000; Nadwodnik and Lohaus, 2008), although salinisation of the growth medium can improve cold tolerance of glycohytes, at least in the short-term (Syvertsen and Yelenosky, 1988; Hinch, 1994). Zhang *et al.* (2004), reviewing abiotic stress tolerance in *Arabidopsis thaliana* (L.) Heynh., indicated that mechanisms relying on control of ion transport may be simpler since they do not depend on the coordinated transcriptional control of many genes.

Walker *et al.* (2008) showed that diploid ($2n=2x=18$) populations of *A. halimus* were more freezing-tolerant

than tetraploids ($2n=4x=36$). In the current work, all populations of both species are tetraploid, so ploidy was not a factor which could explain differences in freezing tolerance among them. For *A. canescens*, tetraploid populations (e.g. var. *occidentalis*) are more freezing-tolerant than diploid populations (e.g. var. *gigantea*) (Stutz, 1989) — the main reason for planting tetraploids in upland areas of Algeria. As far as we know, nuclear DNA contents have not been published before for *A. canescens*; the values reported here show its smaller genome size (0.786-0.818 pg) relative to *A. halimus* (1.200-1.285), despite the fact that the two species have the same base chromosome number ($x=9$) (Stutz, 1989; Sanderson and Stutz, 2001; Walker *et al.*, 2005).

The current findings, together with previous results, indicate that for *A. halimus* and *A. canescens*, soil salinity (*i.e.* the supply of ionic osmolytes) is an important factor determining freezing tolerance via its influence on tissue cation accumulation. This will be useful when selecting cold-winter sites suitable for plantations of these two species, of value for their fodder supply under semi-arid and/or saline conditions.

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