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Cold tolerance, water relations and accumulation of osmolytes in *Bituminaria bituminosa*

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Abstract

We determined the cold (freezing) tolerance of five Spanish populations of the perennial shrub *Bituminaria bituminosa* (L.) C.H. Stirton (*Fabaceae*), as the temperature at which 50 % of leaf electrolytes are released (LT_{50}) using leaves of field-grown plants, obtained in two winters and one spring. The freezing tolerance was greater in winter and reflected the minimum temperatures at the original sites from which the populations were obtained. Tolerance *in vitro* was related to osmotic adjustment in the leaves; more negative osmotic potential values and more positive pressure potential values (MPa) were associated with greater tolerance. Tolerance and osmotic potential were not related to leaf cation contents but to leaf amino acids, soluble sugar and proline contents.

Additional key words: amino acids, cations, freezing, osmotic adjustment, proline, sugars.

Introduction

Bituminaria bituminosa (L.) C.H. Stirton (syn. *Psoralea bituminosa* L.) (*Fabaceae*) is used in Mediterranean environments and the Canary Islands to provide hay or forage for livestock (Sternberg *et al.* 2006). It also synthesises the furanocoumarins angelicin and psoralen, used in sun-tan products and for photochemotherapy of vitiligo and psoriasis (Pecetti *et al.* 2007), and certain pterocarpans of pharmaceutical interest (Maurich *et al.* 2006). Although its N_2-fixation and drought tolerance make it suitable for low-input, sustainable production systems, its growth and distribution are limited by sub-zero winter temperatures.

Freezing damage arises mainly from destabilisation of cell membranes and the formation of ice in intercellular spaces, and the consequent cellular dehydration, due to movement of intracellular water to the extracellular space (Xin and Browse 2000, Uemura *et al.* 2003, Kosová *et al.* 2007). To minimise the cellular damage caused by freezing, during cold acclimation, which occurs naturally in autumn and early winter in response to shortening days and declining temperatures, the cold tolerance of herbaceous plant species increases, *via* mechanisms some of which seem to be ABA-dependent (Gusta *et al.* 2005). These include modifications of the cell wall and cell membranes (lipid composition), alterations in the anti-oxidant defence systems and changes in growth rate and morphology, regulated by a wide range of cold-induced genes (McKersie *et al.* 1999, Wanner and Junttila 1999, Xin and Browse 2000, Uemura *et al.* 2003). The physiological factors related to plant cold tolerance also include osmotic adjustment (OA), in which solute accumulation produces lower (more negative) osmotic potentials. Raised tissue concentrations of compatible solutes such as proline, other amino acids and soluble sugars (Thomas and James 1993, Wanner and Junttila 1999, Xin and Browse 2000, Kosová *et al.* 2007) contribute to the OA and also protect the structural integrity of cell membranes and proteins, but do not have negative effects on plant metabolism even when present at high concentrations in the cytoplasm (Hare and Cress 1997, Xin and Browse 2000, Prášil *et al.* 2007).

The seasonal variation in cold (freezing) tolerance and underlying mechanisms are described for populations of *B. bituminosa* from the Canary Islands and mainland

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Abbreviations: DM - dry mass; EC - electrical conductivity; FM - fresh mass; LT_{50} - temperature at which 50 % of leaf electrolytes are released; OA - osmotic adjustment; RWC - relative water content; WSM - water saturated mass; Ψ_π - leaf osmotic potential; Ψ_p - leaf pressure potential; Ψ_w - midday leaf water potential.

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Spain. The idea was to incorporate traits conferring cold tolerance into our breeding programme, to produce cultivars suitable for cold-winter Mediterranean areas and having high biomass yield (for livestock production) or high yields of compounds of pharmaceutical interest.

Materials and methods

Plants: Five populations of *B. bituminosa* were used: Boca de Tauce (Tenerife, Canary Islands, Spain), Tenerife (Tenerife, Canary Islands, Spain), Famara (Lanzarote, Canary Islands, Spain), Llano del Beal (region of Murcia, Spain) and La Perdiz (region of Murcia, Spain). In 2001, seedlings were planted in a field plot at La Alberca, Murcia (Spain) (37°56′N, 1°08′W), where the minimum temperature in the coldest months of the year ranges from 0 to -3 °C, sufficient to cause shoot and bud death in *B. bituminosa*. Temperature data for the period of the experimental work were obtained from a meteorological station located 50 m from the plants. The plants received drip-irrigation and were arranged in a randomised layout within an area of 600 m^2.

Freezing tolerance assays: In winter (December 2003 and January 2005) and in spring (April 2004), the freezing tolerance of detached leaves was determined by measuring the freezing-induced electrolyte leakage (Warren *et al.* 1996). The assays were performed on three days within the same week, at a final temperature between -2 and -10 ºC. For each population, three samples were used on each day, each comprised of equal masses of leaves from three plants. The leaves were rinsed with deionised water, dried, cut into 75-mm^2 pieces, weighed (approximately 2.5 g), rinsed again and placed in glass tubes, which were sealed and placed in a freezer. The temperature was lowered to -1 °C and maintained at this temperature for 1 h before being lowered at the rate of 2 °C h^{-1} to three different temperatures, according to the month of assay: -2, -6 and -10 °C in December 2003, -1, -5 and -8 °C in April 2004 and -3, -8 and -12 °C in January 2005. After the leaves had been maintained at each temperature for 30 min, one tube per population was removed from the freezer and left at 4 °C overnight. To each tube 10 cm^3 of deionised water was added and the tubes were shaken at 25 °C, for 4 h. The electrical conductivity (EC) was measured and the tubes were then heated to 95 °C for 30 min, before cooling and re-measurement of EC. The freezing damage (%) was calculated as: (EC after freezing/EC after 95 °C) × 100. The temperature at which 50 % of electrolytes were released (LT_{50}) was calculated by non-linear regression, using *SPSS version 11.5* software. The mean LT_{50} ($n = 3$) for each month was calculated using the individual LT_{50} values for each day of assay.

Water relations: The relative water content (RWC) of leaves was determined in discs of 5-mm diameter (1 disc for each of 12 leaves per plant). Their fresh mass (FM) was determined and the water saturated mass (WSM) was determined after the discs had been floating on deionised water for 24 h, in darkness at 20 °C. Dry mass (24 h at 65 °C) (DM) was measured and the RWC (%) calculated as: (FM - DM)/(WSM - DM) × 100. For three plants per population ($n = 9$ for each population-month combination), the midday leaf water potential (Ψ_w), osmotic potential (Ψ_π) and leaf pressure potential (Ψ_p) (MPa) were determined as described by Walker *et al.* (2008).

Cations: The extract produced by maintaining the leaves in deionised water at 95 °C for 30 min in the freezing assays was used to measure the concentrations of Ca^{2+}, Mg^{2+}, K^+ and Na^+ ($n = 9$ for each population-month combination) by inductively-coupled plasma-optical emission spectrometry (*Vista-MPX*, *Varian*, Mugrave, Australia).

Determination of organic osmolytes: For each of the three plants per population used each day in the LT_{50} assays, leaves collected at the same time were frozen immediately, lyophilised and ground ($n = 9$ for each population-month combination). Proline, soluble sugars, amino acids and starch were determined as described in Walker *et al.* (2008).

Statistical analyses: *ANOVA* was performed, using *SPSS version 11.5* software, to determine, for the studied parameters, if there were significant ($P < 0.05$) effects of the *B. bituminosa* populations and the month of assay, and whether there were population-month interactions. Where such effects existed, Tukey′s test ($P = 0.05$) was used to compare mean values. Pearson correlation coefficients (r) were determined for the relationships between LT_{50} and the other plant parameters ($n = 45$).

Results

There was a significant effect of the time of year ($P < 0.001$) on cold tolerance, measured as the LT_{50} for leaf electrolyte leakage (Table 1): tolerance was greater in winter (December 2003 and January 2005; overall mean LT_{50} values of -5.5 and -7.9 ºC, respectively) than in spring (April 2004; -4.5 ºC). In the field, freezing injury (tissue necrosis in February 2005, three weeks after two consecutive nights with minimum temperatures of

Table 1. Values of LT$_{50}$ (°C), determined by laboratory electrolyte leakage assays (mean values, $n = 3$), and water relations ($n = 9$) for leaves sampled from plants of *B. bituminosa* at the times shown. Different letters within a column represent significant differences ($P < 0.05$) according to Tukey´s test. The relative water content (RWC; no significant differences between means) and dry matter values were arcsin-transformed before analysis.

Month	Population	LT$_{50}$ [°C]	Ψ_w [MPa]	Ψ_π [MPa]	Ψ_p [MPa]	RWC [%]	Dry mass [%]
December 2003	Boca de Tauce	-5.9bcde	-0.579a	-1.085a	0.506bc	85.9	19.7d
	Tenerife	-4.7abc	-0.560a	-1.206ab	0.646ab	84.9	22.2cd
	Famara	-4.0ab	-0.540a	-1.201ab	0.661ab	87.6	24.5bc
	Llano del Beal	-6.1cde	-0.515a	-1.129ab	0.614ab	85.6	20.2d
	La Perdiz	-6.8cde	-0.625a	-1.250ab	0.625ab	82.7	20.1d
April 2004	Boca de Tauce	-5.3bcd	-0.660ab	-1.193ab	0.533abc	89.2	25.8ab
	Tenerife	-5.4bcd	-0.757abc	-1.287ab	0.530bc	82.0	26.1ab
	Famara	-2.7a	-0.577a	-1.241ab	0.664ab	83.6	25.1ab
	Llano del Beal	-4.4ab	-0.990bc	-1.070a	0.108d	88.4	21.1d
	La Perdiz	-4.8abc	-1.035c	-1.202ab	0.182cd	87.9	22.1cd
January 2005	Boca de Tauce	-7.8efg	-0.500a	-1.267ab	0.757ab	88.0	25.6ab
	Tenerife	-5.8bcde	-0.534a	-1.329bc	0.795ab	86.9	26.7ab
	Famara	-7.1def	-0.470a	-1.379b	0.919a	82.1	27.3a
	Llano del Beal	-9.4g	-0.522a	-1.391b	0.869ab	84.4	26.1ab
	La Perdiz	-9.2fg	-0.544a	-1.332ab	0.788ab	90.2	25.1ab

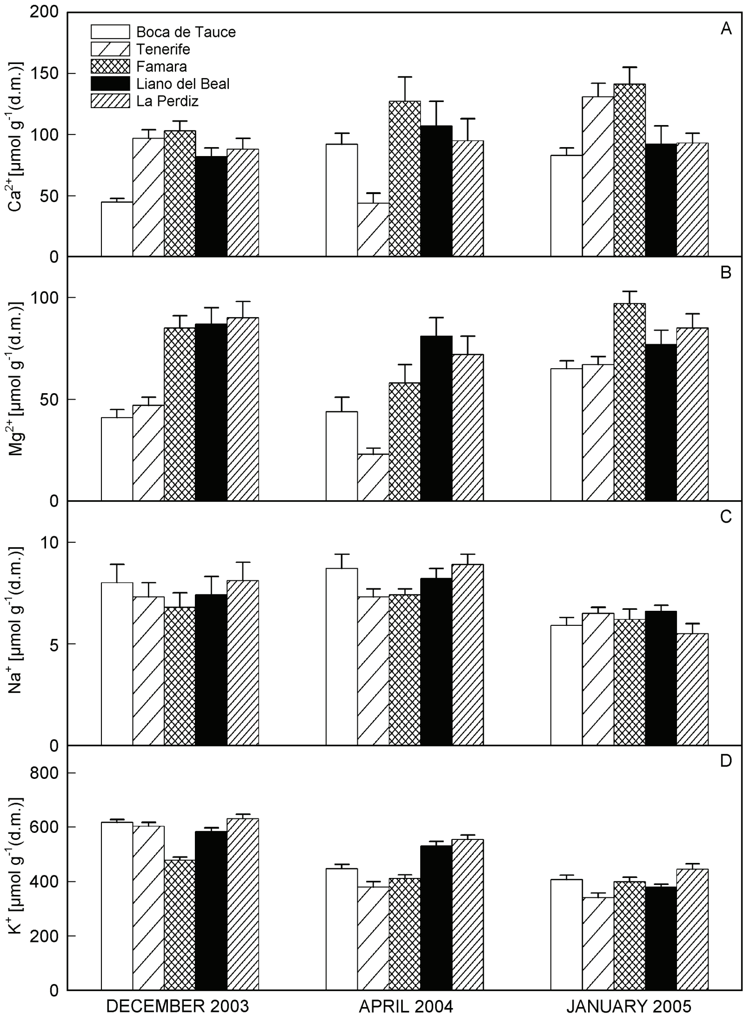

Fig. 1. Mean cation contents (*A* - Ca^{2+}, *B* - Mg^{2+}, *C* - Na^{+} and *D* - K^{+}) in *B. bituminosa* plants (populations Boca de Tauce, Tenerife, Famara, Llano del Beal and La Perdiz) sampled in December 2003, April 2004 and January 2005. *Error bars* are SE ($n = 9$).

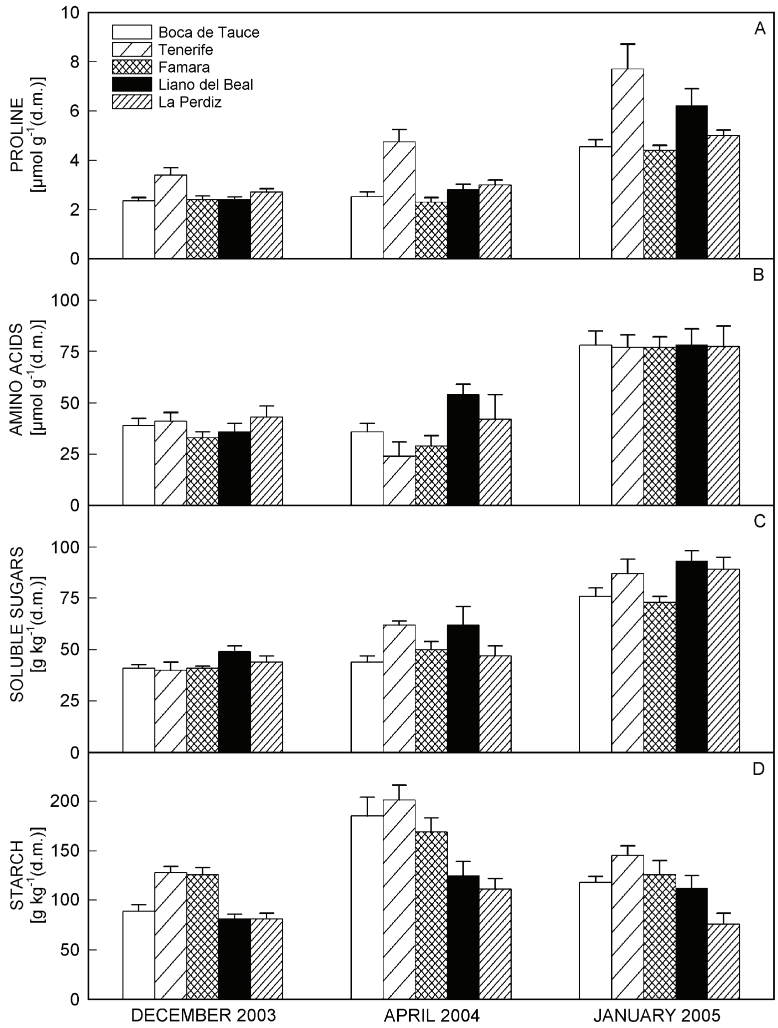

Fig. 2. Mean contents of proline (*A*), total amino acids (minus proline) (*B*), soluble sugars (*C*) and starch (*D*) in *B. bituminosa* plants (populations Boca de Tauce, Tenerife, Famara, Llano del Beal and La Perdiz) sampled in December 2003, April 2004 and January 2005. *Error bars* are SE ($n = 9$).

-2.5 and -2.7 ºC) broadly reflected the *in vitro* tolerance, being greater for the Canarian populations Tenerife and Famara. However, even the most-damaged plants recovered completely. The month of assay had a great effect ($P < 0.001$) on Ψ_w, Ψ_π and Ψ_p. The values of Ψ_w were most negative in April 2004 (overall mean of -0.794 MPa, compared to -0.570 and -0.522 in December 2003 and January 2005, respectively) and were slightly more negative for the more tolerant populations (Table 1). The Ψ_π values were lowest in January 2005 (overall mean of -1.341 MPa, compared with -1.174 in December 2003 and -1.194 in April 2004) ($P < 0.05$; Tukey test). The values of Ψ_p were highest in winter, particularly in January 2005 (Table 1). The RWC showed no significant variation according to month or population. The percentage of dry matter increased with time (Table 1) and, overall, was higher for the less cold-tolerant populations Famara (25.4 %) and Tenerife (24.4 %) than for the other populations (21.9 - 22.9 %) ($P < 0.05$) but showed no relationship with cold tolerance.

Leaf Ca^{2+} content (Fig. 1), averaged for all populations, was significantly higher ($P < 0.05$; Tukey test) in January 2005 [108 μmol g^{-1}(d.m.)] than in December 2003 [82 μmol g^{-1}(d.m.)] and April 2004 [93 μmol g^{-1}(d.m.)]. Averaged over all three months, K^+ showed the same order as the cold tolerance (La Perdiz > Llano del Beal, Boca del Tauce > Tenerife, Famara). However, when averaged over the five populations, K^+ was significantly lower in January 2005 (the month when the plants were most cold-tolerant) [395 μmol g^{-1}(d.m.)] than in December 2003 [583 μmol g^{-1}(d.m.)] or April 2004 [465 μmol g^{-1}(d.m.)]. Overall, tissue Mg^{2+} contents were higher in winter [71 - 78 μmol g^{-1}(d.m.)] than in April 2004 [56 μmol g^{-1}(d.m.)] ($P < 0.05$).

Proline contents were highest in population Tenerife, and in January 2005 ($P < 0.05$; Tukey test; Fig. 2*A*). The patterns of the total amino acids (without proline) and soluble sugars were similar, with the highest contents in January 2005 (Fig. 2*B,C*), while starch content was higher in spring (April 2004) than in the preceding or following winter (mean of all populations, $P < 0.05$; Fig. 2*D*).

Discussion

The cold acclimation in the autumn and early winter (in response to decreasing day length and temperatures) and de-acclimation in the spring observed here for *B. bituminosa* are general phenomena for species which do not shed their leaves in the autumn (Hou and Romo 1998, Kosová *et al.* 2007). Hekneby *et al.* (2006), studying annual Mediterranean legume species, found LT_{50} values and magnitudes of cold acclimation (ΔLT_{50}) similar to those reported here for *B. bituminosa*. The order of cold tolerance of the *B. bituminosa* populations reflects the mean temperatures in the coldest month of their original sites: 4.0, 11.7, 11.1, 9.7 and 5.9 ºC, respectively, for Boca de Tauce, Tenerife, Famara, Llano del Beal and La Perdiz. Canarian populations of *B. bituminosa* (*e.g.* Famara and Tenerife) which grow at low altitude are not exposed to sub-zero winter temperatures. The Canarian population Boca de Tauce is exposed to the lowest winter temperatures, but as a freezing-avoidance mechanism, sheds its leaves in winter. The current results indicate that it also possesses a relatively good degree of cold tolerance when this process is not triggered by sub-zero temperatures in early winter.

No significant correlations between tissue cation contents (individual or their sum) and *in vitro* or field cold tolerance ($P > 0.05$) were found. This reflects earlier findings for glycophytes (*e.g.* Thomas and James 1993), suggesting that cation accumulation as a mechanism of cold acclimation might be limited to halophytic species such as *Atriplex halimus* L. (Walker *et al.* 2008). However, Syvertsen and Yelenosky (1988) found that salinisation of the growth medium increased cold tolerance of *Citrus* species.

In glycophytes, osmotic adjustment involves the accumulation of organic osmolytes such as proline, other amino acids and soluble sugars. The resulting decrease in Ψ_π reduces the intracellular freezing temperature and minimises water movement from cells to extracellular spaces and thus cellular dehydration (Thomas and James 1993, Xin and Browse 2000, Kosová *et al.* 2007, Prášil *et al.* 2007). These compounds also protect the structural integrity of cell membranes and proteins (Xin and Browse 2000, Uemura *et al.* 2003). In the current work, there were significant relationships between *in vitro* cold tolerance (LT_{50}) and Ψ_π and Ψ_p (not shown). When the plants were exposed to lower-than-usual minimum winter temperatures in January 2005 (mean of 3.5 ºC), the greatest OA was found for populations La Perdiz and Llano del Beal (0.145 and 0.228 MPa, respectively), coinciding with the lowest LT_{50} values (-9.2 and -9.4 ºC, respectively). Significant correlations between Ψ_π (MPa) and the concentrations of soluble sugars (g kg^{-1}; Pearson correlation coefficient, $r = -0.716$, $P = 0.003$) and amino acids (µmol g^{-1}; $r = -0.620$, $P = 0.018$) demonstrate their contribution to the OA. Decreased and increased levels, respectively, of tissue starch and soluble sugars in cold-acclimated plants have been found for numerous species (Thomas and James 1993, Strand *et al.* 1999). In the current work, the starch concentration showed a negative relationship with cold tolerance, determined as LT_{50} ($r = 0.485$, $P = 0.067$). Soluble sugar contents were correlated significantly with the LT_{50} ($r = -0.697$, $P = 0.004$) and their contents were always highest in winter for the most tolerant populations (La Perdiz and Llano del Beal).

There was limited but significant variation in proline accumulation, which was greatest following the colder-than-usual period in January 2005, when leaf Ψ_π was below -1.25 MPa. The RWC values of *B. bituminosa* did not fall below 82 % (Table 1). Proline accumulation has been reported in plants which have become cold-acclimated without changes in water status, indicating a specific role in cold tolerance (Hare and Cress 1997). This role could be as a cytoplasmic osmolyte (Xin and Browse 2000), but proline also functions as an antioxidant and in the redox control of plant metabolism (Hare and Cress 1997).

In the winter months, there were no significant differences between the populations with regard to the measured parameters of water relations or solute concentrations, despite the differences in cold tolerance. This suggests the involvement of other factors in cold tolerance, such as leaf thickness (Hekneby *et al.* 2006), membrane lipid composition (Xin and Browse 2000, Uemura *et al.* 2003), leaf anatomy and extracellular ice formation (McCully *et al.* 2004), ice nucleation temperature (Hekneby *et al.* 2006) and the plant antioxidant system (McKersie *et al.* 1999).

The field freezing damage, unlike the LT_{50}, showed no significant relationship with any of the plant physiological parameters measured. This is probably because the electrolyte leakage assay shows the primary freezing damage to plant tissues, the disruption of cell membranes (Xin and Browse 2000, Uemura *et al.* 2003). Thus, it depends largely on the OA achieved by the cells. The more-slowly-appearing damage to whole plants or tissues is the result of secondary processes such as oxidative stress (McKersie *et al.* 1999).

On the basis of the results presented here, we have incorporated populations La Perdiz and Llano del Beal into our breeding programme to produce cold-tolerant ecotypes of *B. bituminosa* for pharmaceutical uses or livestock feeding.

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