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RESEARCH PAPER

Differential freezing resistance and photoprotection in C_3 and C_4 eudicots and grasses

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Abstract

Globally, C_4 plants dominate hot, open environments, but this general pattern is underpinned by important differences in the biogeography of C_4 lineages. In particular, the species richness of C_4 Poaceae (grasses) increases strongly with increasing temperature, whereas that of the major C_4 eudicot group Chenopodiaceae correlates positively with aridity. Freezing tolerance is a crucial determinant of biogeographical relationships with temperature and is mediated by photodamage and cellular disruption by desiccation, but little is known about differences between C_4 families. This study hypothesized that there is a greater risk of freezing damage via these mechanisms in C_4 Poaceae than Chenopodiaceae, that freezing protection differs between the taxonomic groups, and that freezing tolerance of species is linked to arid habitat preference. Chlorophyll fluorescence, water relations, and freezing injury were compared in four C_3 and six C_4 species of Poaceae and Chenopodiaceae from the same Mongolian flora. Contrary to expectations, freezing-induced leaf mortality and photodamage were lower in Poaceae than Chenopodiaceae species, and unrelated to photosynthetic pathway. The freezing resistance of Poaceae species resulted from constitutive protection and cold acclimation and an ability to protect the photosynthetic apparatus from photodamage. Freezing protection was associated with low osmotic potential and low tissue elasticity, and freezing damage was accompanied by electrolyte leakage, consistent with cell-membrane disruption by ice. Both Chenopodiaceae and Poaceae had the potential to develop cold acclimation and withstand freezing during the growing season, which conflicted with the hypothesis. Instead, freezing tolerance was more closely associated with life history and ecological preference in these Mongolian species.

Key words: C_3 photosynthesis, C_4 photosynthesis, Chenopodiaceae, cold acclimation, freezing tolerance, photodamage, *Poaceae*, water relations.

Introduction

The CO_2-concentrating mechanism of C_4 plants gives them a competitive advantage over those with the C_3 photosynthetic pathway under high light and temperature and under CO_2 limitation caused by atmospheric CO_2 depletion or water deficits (Sage *et al.*, 1999a). C_4 plants represent only 3% of terrestrial plant species, but dominate all tropical and subtropical grasslands, most warm temperate grasslands, deserts, and most disturbed landscapes in warmer regions of the world (Sage *et al.*, 1999b). Together, these ecosystems contribute approximately 20% of terrestrial carbon fixation (Lloyd and Farquhar, 1994). The C_4 pathway is distributed across >60 lineages in 17 families, and about 7000–8000 species of flowering plants (Sage *et al.*, 2011). About 6000 C_4 species are grasses and sedges, with only about 1200 C_4 eudicots. Within eudicots, the family Chenopodiaceae (chenopods) contains the largest number of C_4 species (Sage *et al.*, 1999b).

C_4 grasses dominate the North American prairies (Ehleringer *et al.*, 1997), African savannas (Batanouny

et al., 1988), and desert and semidesert regions in Australia (Hattersley, 1983), while C_4 members of the Chenopodiaceae are infrequent. However across the vast Eurasian steppe region, exemplified by the Mongolian Plateau, the situation is quite different. Native Mongolian C_4 plants from the Chenopodiaceae and Poaceae make up 50 and 30%, respectively, of the total C_4 species. C_4 grasses are most common in the steppe zones, with C_4 chenopods found largely in the arid desert zone. Moreover, the species richness of these C_4 lineages show contrasting associations with environmental variables (Pyankov *et al*., 2000).

The distributions of C_4 Poaceae and Chenopodiaceae species across the Mongolian plateau follow patterns similar to those seen at the global scale (Ehleringer *et al*., 1997). The relative abundance of C_4 grass species declines strongly with falling temperature, whilst the equivalent correlation for C_4 chenopod species is weak (Pyankov *et al*., 2000). Indeed, C_4 chenopods may survive at high altitudes close to the permanent frost line. Rather than being limited by low temperature, C_4 chenopod abundance correlates most strongly with aridity, implying that drought and frost tolerance are linked in these species. In fact, frost and drought exert similar desiccating effects on leaf tissues because extracellular ice formation extracts water from cells, leaving behind high concentrations of solutes that damage cell membranes. Given this common mechanism of damage, drought-tolerant species should better tolerate freezing than species adapted to more mesic habitats. Constitutive protection mechanisms would be expected in any environment, whereas any drought acclimation responses would also need to be induced by low temperatures to protect plants from freezing.

Freezing is an important abiotic factor limiting the geographical distribution and growth of plants in many regions of the world (Levitt, 1980). Cold acclimation or freezing resistance via osmotic adjustment is induced by exposure to chilling temperatures in the range 0–15 °C (Larcher, 2003; Liu and Osborne, 2008), with failure to develop freezing resistance allowing ice to damage cellular membranes. The combination of water relations and electrolyte leakage therefore provides a good means of describing freezing resistance in plants (Neuner and Bannister, 1995; Pearce, 2001). Chilling and freezing temperatures also limit enzymic processes and the capacity of the photosynthetic apparatus for dissipating absorbed energy through photochemistry (Demming-Adams and Adams, 1992). If excess energy is not dissipated, the photosynthetic machinery can be damaged, resulting in impaired PSII activity (Huner *et al*., 1993). Therefore, protection of the photosynthetic apparatus in plant leaves is considered to be critical for low temperature tolerance.

Despite this general understanding of the physiological basis of cold tolerance, the mechanisms underpinning the differential geographical distributions of C_4 grasses and chenopods are not well understood. This study hypothesized that the observed differences in biogeography are associated with differences in known mechanisms of chilling and freezing resistance, including the protection of the photosynthetic pathway at low temperatures. By comparing Mongolian grass and chenopod species, four questions were addressed (1) Does freezing cause greater damage in Poaceae than Chenopodiaceae? (2) Is the freezing damage greater in C_4 than C_3 species? (3) Does the protection mechanism differ between Poaceae and Chenopodiaceae? (4) Given the known involvement of desiccation in freezing damage, do species originating from more arid habitats show greater freezing resistance?

Materials and methods

Plant materials and environmental conditions

Plant species of Poaceae and Chenopodiceae used in this experiment were from Inner Mongolia in the south of the Mongolian Plateau (39° 6'–49° 48' N 101° 36'–122° 24' E). Seeds of *Haloxylon ammodendron* were collected from the desert ecosystem at Dengkou Xian (40° 57′ N 106° 26′ E, altitude 1037 m) (Table 1). Meteorological data from this area (1960–2010) show a desert climate, with mean annual temperature and average January and July temperatures of 7.8, –11.6, and 26.6 °C, respectively. The frost-free period is about 168 days, and mean annual precipitation is 103 mm. Seeds of the other nine species were collected from steppe vegetation at Zhenglan Qi (43° 56' N, 116° 08' E, altitude 1103 m) (Table 1). Meteorological data from this region (1960~2010) show a temperate arid and semi-arid climate, with mean annual temperature and average January and July temperatures of 1.8, –17.9, and 18.7 °C, respectively. The frost-free period is about 120 days, and mean annual precipitation is 378 mm. The main habitats in this ecosystem are shifting dune, fixed dune, lowland meadow, and wetland.

Collected seeds were germinated in sterile nutrient agar for 1 week, and 16 plants of each species were transferred to pots (height × diameter, 18 × 13 cm) containing four parts high nutrient compost (Levington M3, Scotts UK Professional, Suffolk, UK) to one part sand (Play Sand, William Sinclair Horticulture, Lincoln, UK) and one part Perlite (Esoteric Hydroponics, Guildford, UK). The plants were grown in a controlled environment chamber and watered daily with a 40% solution of Long Ashton solution to maintain a moist, nutrient-rich soil continuously thorough the experiment. Plants were maintained under a 14/10 hour light/dark cycle with a photosynthetic photon flux density (PPFD) of 600 μmol m^{-2} s^{-1} measured at plant height, day/night temperatures of 25/15 °C, and 60/80% relative humidity. These control conditions were maintained for 14 weeks. At the initiation of the experiment, eight plants of each species were transferred to an identical growth chamber with a day/night temperature of 15/5 °C for 20 d (chilling pre-treatment), and the other half were maintained under pre-treatment, control conditions. The transfer of plants was staggered, so that one replicate of two species was transferred each day. Plants were exchanged between cabinets midway through the experiment, so that any effect of cabinet was not confounded directly with treatment. The design meant that, on average overall, half the replicates for each species received the chilling treatment in one cabinet and half in the other.

Maximum quantum efficiency of PSII

On the 20th day of the chilling pre-treatment, chlorophyll fluorescence was measured using an integrated open gas-exchange and chlorophyll fluorescence system (LI-6400–40, LI-COR Biosciences, Lincoln, NE, USA). After 15 min of dark adaptation, the leaves were exposed to a weak modulated beam to determine the zero fluorescence level (F_0), and then a saturating pulse to obtain the maximum fluorescence level (F_m). Variable fluorescence (F_v) is the difference between F_m and F_0, which was used to calculate F_v/F_m, the maximum quantum efficiency of PSII.

Pressure and volume measurements

Pressure/volume curves were generated for four individuals per species from each treatment at the end of the 20-d pre-treatments using

Table 1. Species involved in current experiments: A, annual; F, forb; G, grass; P, perennial; S, shrub.

Scientific name	Abbreviation used in figures	Photosynthetic pathway	Life history and life form	Common habitats on the Mongolian Plateau
Chenopodiaceae				
Agriophyllum pungens (Vahl) Link ex A. Dietr.	*Ap*	C_3	A, F	Shifting sand dunes in sandy grassland
Ceratoides arborescens (Losina-Losinskaja) C. P. Tsien and C. G. Ma.	*Ca*	C_3	P, S	Sandy steppe, sandy slopes
Corispermum heptapotamicum Iljin	*Ch*	C_3	A, F	Shifting and semi-shifting sand dunes in sandy grassland
Haloxylon ammodendron (C. A. Mey.) Bunge	*Ha*	C_4	P, S	Desert, in saline sands
Kochia prostrata (Linn.) Schrad.	*Kp*	C_4	P, S	Sandy grassland, especially in sandy and loamy soils or stony soils
Salsola collina Pall.	*Sc*	C_4	A, F	Disturbed land
Poaceae		C_4		
Chloris gayana Kunth.	*Cg*	C_4	P, G	Naturalized exotic species, grown in pastures
Pennisetum clandestinum Hochst. ex Chiov.	*Pc*	C_4	P, G	Naturalized exotic species, grown in pastures
Cleistogenes squarrosa (Trin.) Keng	*Cs*	C_4	P, G	Desert steppes, especially in sandy and loamy soils; also found scattered in rocky habitats
Leymus chinensis (Trin.) Tzvel.	*Lc*	C_3	P, G	Steppe, in saline meadows, sand beds of river valleys

a bench drying technique (Turner, 1981). The youngest fully mature leaves were sampled for all species except *H. ammodendron*, where leaves are reduced to scales and the stem is the main photosynthetic unit. Stems were first cut to at least 15–20 cm. Leaves or stems were then re-cut underwater, weighed, and placed directly into labelled vials containing deionized water in a lab sink. The sink was covered with a wet cloth with tap water dripping on it to allow leaf saturation overnight. Leaves or shoots were then either processed immediately or refrigerated in the dark overnight until processing the following day. Leaves or shoots were weighed to 0.001 g immediately before and after water potential was measured with a Scholander Pressure Chamber (model 600, PMS Instrument Company, Albany, OR, USA), and left to dry on the bench between measurements. Initial balancing pressures varied between 0.05 and 0.3 MPa, and the measurements were continued until 4–5 points had been recorded below the turgor loss point. Leaves or shoots were then placed in an oven at 70 °C for 48 h and reweighed to determine dry weight, relative water content (RWC), and dry matter content (DMC).

Key tissue water relations parameters were determined from plots of $-1/\Psi_{leaf}$ versus RWC, using a curve-fitting routine based on the template of Schulte and Hinckley (1985). A straight line was fitted via the stepwise addition of points from the lowest RWC to higher RWC to obtain the linear response of osmotic potential to RWC. This permitted calculation of osmotic potential at full turgor (ψ_π, MPa) by extending the regression line back to the $-1/\psi$ axis. Relative water content and leaf water potential at the turgor-loss point (RWC_{tlp} and ψ_{tlp}, respectively) were obtained at the intersection of the plots describing $-1/\Psi_{leaf}$ versus RWC and osmotic potential versus RWC (Turner, 1981). Turgor pressures across the observed range of 1 – RWC were estimated from the difference between values of ψ_{leaf} on the non-linear curve and fitted data on the linear $1/\psi$ versus 1 – RWC plot. Bulk modulus of elasticity (ε_{max}, MPa) was estimated from the slope of the linear regression of turgor pressure and symplastic relative water content (R_s) values over the full range of positive turgor pressures (Tyree and Jarvis, 1982) using:

$$\varepsilon = \Delta P/\Delta RWC^*(Rs)$$

where ΔP is the change in turgor pressure and ΔRWC is the change in RWC. The maximum value of ε across the observed values of 1 – RWC was taken as ε_{max}.

High-light freezing treatment and chlorophyll fluorescence measurement

After pressure/volume curve measurements, all plants were exposed to a high-light freezing treatment to test for constitutive freezing resistance (control pre-treatment plants) and to investigate whether cold acclimation had developed (chilling pre-treatment plants). The freezing treatment was designed to simulate a frost during the growing season in the natural environment on the Mongolian Plateau and was applied using a walk-in controlled environment cabinet (BDW 40, Conviron Controlled Environments). The treatment was applied to a single block of plants (one of each species × pre-treatment combination = 16 plants), and replicated on five consecutive days.

The protocol for each freezing event followed Osborne *et al.* (2008). Briefly, the root system of each plant was prevented from freezing by wrapping each pot in polythene, and immersing it in a water bath held at 15 °C. The plants were put into the cabinet at 14:00 h and exposed to 15 °C at a PPFD of 600 μmol m^{-2} s^{-1} until 19:00 h. The lights were then turned off, and the temperature was lowered gradually overnight at a rate of 4 °C per hour until 0 °C, after which the temperature was lowered more slowly to reach a minimum of –5 °C at 07:00 h, before dawn. The temperature was held constant at this level for 1 h, then the lights were turned on at 08:00 h to deliver a PPFD of 400 μmol m^{-2} s^{-1}, and both temperature and PPFD were then ramped gradually upwards to reach 15 °C and 1200 μmol m^{-2} s^{-1} at 13:30 h.

After the high-light freezing treatment, three leaves for each plant were dark adapted for 15 min, before making F_v/F_m measurements

using an integrated open gas-exchange and chlorophyll fluorescence system, as already described.

Electrolyte leakage

Leaf injury caused by freezing was also assessed by the electrolyte leakage method described by Colombo *et al.* (1984). After the high-light freezing treatment, 15 freshly cut leaf discs (0.5 cm^2 each) were rinsed three times (2–3 min) with demineralized water and subsequently floated on 10 ml demineralized water. Electrical conductivity of the water after the freezing treatment (C_f) was measured after 24 h at room temperature using a conductivity meter (PCM1, Jenway Products, Leeds, UK). Before each measurement, the test tube was shaken vigorously. Total conductivity (C_t) was obtained after keeping the flasks in an oven (90 °C) for 2 h to kill the leaves. The temperature was then gradually lowered to 20 °C and held overnight. The electrolyte leakage of heat-killed leaves was then measured. The degree of freezing damage to leaves was assessed using relative conductivity ($R_c = C_f/C_t$).

Leaf freezing mortality

After the freezing treatment, the extent of leaf injury within the canopy was assessed for all plants exposed to the high-light freezing treatment. All dead leaves were cut off prior to the treatment, and the plants were returned to the control growth conditions after freezing. Over the subsequent 1–3 d, the total numbers of dead (>2/3% necrotic) and green leaves were counted. Leaf mortality was calculated as the number of dead leaves divided by the total number of leaves. For *H. ammodendron*, leaves are reduced to scales which cover the stem and here the succulent green stems serve a photosynthetic function. In this species, the visual assessment of freezing damage was based on colour changes of stems, by dividing the length of necrotic stem by the total stem length.

Statistical analysis

Mixed linear regression models (SPSS 16.0, Chicago, IL, USA) were fitted using photosynthetic type, chilling pre-treatment, family, and the interactions of chilling pre-treatment × photosynthetic type and chilling pre-treatment × family as fixed factors, and species as a random effect. The models were used to test whether values of leaf water status traits (DMC, ψ_{tlp}, ε_{max}, ψ_π), F_v/F_m, and leaf freezing injury (R_c and leaf mortality) differed between chilling and control (chilling pre-treatment), C_3 and C_4 species (photosynthetic type), and Poaceae and Chenopodiceae (family). For freezing injury, the data from each replicate were transformed by taking their natural logarithms to stabilize heterogeneous variances.

Results

Maximum quantum efficiency of PSII

F_v/F_m was used to measure photodamage after 20 days of the chilling pre-treatment, but before the high-light freezing treatment. Values of F_v/F_m for the control plants were 0.79–0.85, indicating the presence of a fully functioning photosynthetic apparatus (Fig. 1A). There were no statistically significant decreases in F_v/F_m for the chilling pre-treatment or differences between families and photosynthetic types (Table 2). There were no considerable differences in the F_v/F_m of chilling pre-treatment plants among species, because the values of F_v/F_m for most species were above 0.78, suggesting no damage to PSII (Fig. 1A). Exceptional values of F_v/F_m for the chilling pre-treatment and the control *Salsola collina* plants were 0.74 and 0.76, respectively, with only a small difference between treatments (Fig. 1A). Neither chlorosis nor other leaf discoloration was observed in any species during exposure to chilling.

After the high-light freezing treatment, the values of F_v/F_m displayed different patterns, with statistically significant effects of family and chilling pre-treatment ($P < 0.05$) (Fig. 1B; Table 2). For Chenopodiaceae species, the freezing treatment caused a smaller decrease in F_v/F_m in the plants exposed to the chilling treatment (4–22%), than in the control plants (9–45%), relative to the values before freezing (Fig. 1B). The equivalent values for grasses were 6–11% for the chilling pre-treatment and 8–23% for the controls (Fig. 1B). These patterns suggested that: first, the chilling pre-treatment protected PSII against the damaging effects of freezing; and secondly, this effect was most pronounced in the Chenopodiaceae, as evidenced by a statistically significant family × pre-treatment interaction for F_v/F_m after freezing, relative to the value before freezing (Table 2). Generally, PSII showed a higher degree of constitutive protection against freezing in the Poaceae, irrespective of whether there was a

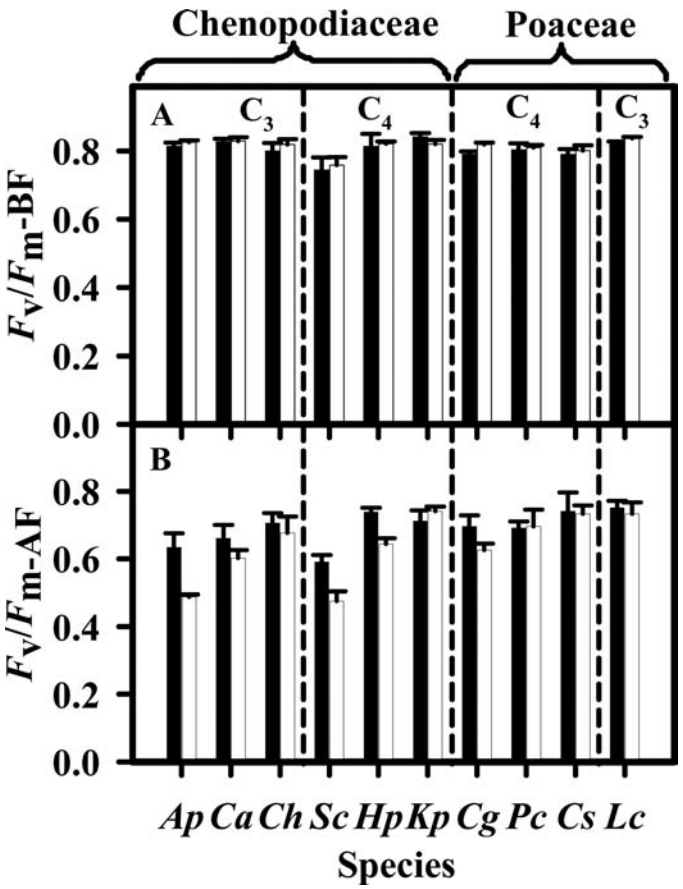

Fig. 1. Effects of the chilling pre-treatment on maximum quantum yield of PSII (F_v/F_m) after the chilling pre-treatment (A) and after the high-light –5 °C freezing event (B) for C_3 and C_4 species of Chenopodieace and Poaceae on the 20th day of chilling (filled bars) or control (open bars) pre-treatment. Abbreviations for species names are defined in Table 1. Values are mean ± SE for eight individual plants.

Table 2. Results of linear mixed-effects model analysis of the effects of chilling pre-treatment on: F_v/F_m, maximum quantum efficiency of PSII; AF, after freezing; BF, before freezing; ψ_π, osmotic potential at full turgor; ψ_{tlp}, water potential at turgor loss point; ε_{max}, maximum bulk modulus of elasticity; DMC, dry matter content; R_c, electrolyte leakage, and leaf mortality. Values are F(d.f.) significance. $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

Variable	Photosynthetic type	Chilling pre-treatment	Chilling pre-treatment × photosynthetic type	Family	Chilling pre-treatment × family
F_v/F_m-BF	1.44(1,7)	1.87(1,147)	0.64(1,147)	0.06(1,7)	0.03(1,147)
F_v/F_m-AF	0.53(1,7)	4.86(1,147)*	0.002(1,147)	4.94(1,7)*	0.53(1,147)
(F_v/F_m-AF)/(F_v/F_m-BF)	0.45(1,7)	1.92(1,147)	0.22(1,147)	0.75(1,7)	4.77(1,147)*
ψ_π	0.48(1,7)	6.79(1,87)**	0.062(1,87)	2.98 (1,7)	0.751(1,87)
ψ_{tlp}	0.26(1,7)	7.72(1,87)**	1.60(1,87)	2.67(1,7)	0.037(1,87)
ε_{max}	0.27(1,7)	5.89(1,87)**	0.67(1,87)	7.47(1,7)*	0.013(1,87)
DMC	0.06(1,7)	7.16(1,87)*	1.18(1,87)	1.03 (1,7)	0.34 (1,87)
Leaf mortality	1.67(1,7)	6.651(1,147)**	0.77(1,147)	14.79(1,7)**	0.19(1,147)
R_c	0.04 (1,7)	22.76(1,147)***	0.04(1,147)	0.79(1,7)	0.17(1,147)

chilling pre-treatment or not. There was no significant overall effect of photosynthetic pathway, nor an interaction with the chilling treatment (Table 2).

Pressure/volume curve traits

In leaves exposed to the chilling pre-treatment, osmotic potential at full turgor (ψ_π) decreased significantly to more negative values for all species, irrespective of photosynthetic pathway or family ($P < 0.01$) (Table 2, Fig. 2A). However, on average, across all species, ψ_π was only –0.1 MPa lower after the chilling pre-treatment than in control plants (Fig. 2A). There were no differences in ψ_π between families and photosynthetic types (Table 2). Leaf water potential at the turgor loss point (ψ_{tlp}) showed similar patterns to ψ_π, with an average decrease of –0.1 MPa ($P < 0.01$) in the chilling pre-treatment (Table 2, Fig. 2B). Patterns of bulk modulus of elasticity (ε_{max}) were significantly affected by the chilling pre-treatment ($P < 0.01$) and family ($P < 0.05$) (Table 2, Fig. 2C), but there was no effect of photosynthetic type and there were no interactions (Table 2). Average values of ε_{max} for Chenopodiaceae and Poaceae were 13.5 and 16.6 MPa in control plants but 10.5

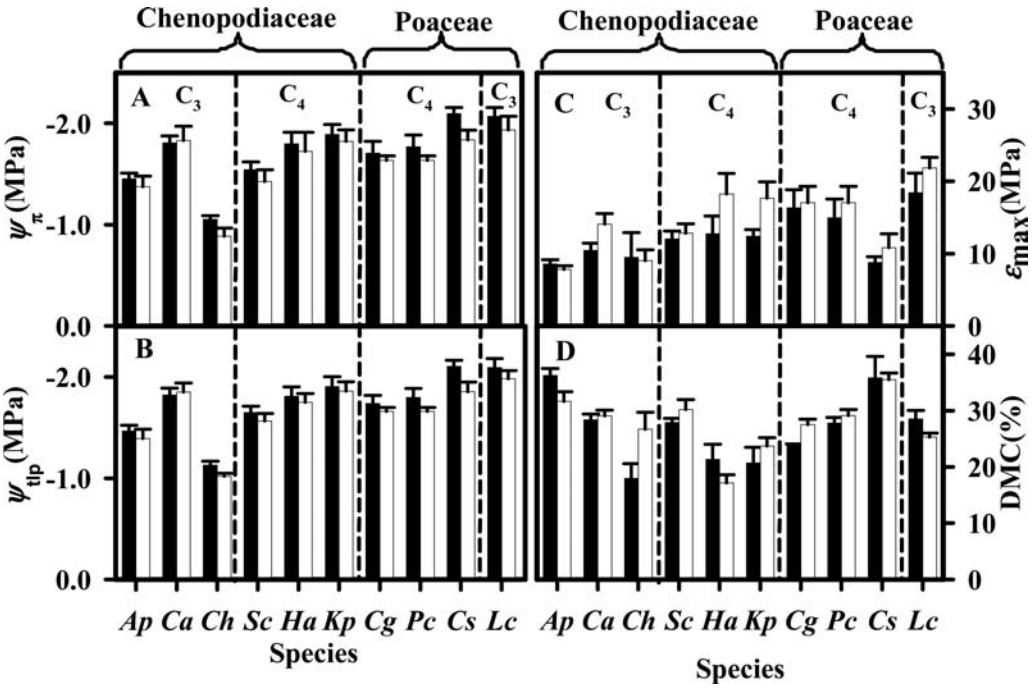

Fig. 2. Osmotic potential at full turgor (ψ_π) (A), water potential at the turgor loss point (ψ_{tlp}) (B), maximum bulk modulus of elasticity (ε_{max}) (C) and dry matter content (DMC) (D) in response to 20 d chilling (filled bars) and control (open bars) pre-treatments in leaves of C_3 and C_4 Chenopodiaceae and Poaceae species. Abbreviations for species names are defined in Table 1. Values are mean ± SE for five individual plants.

and 13.9 MPa after the chilling pre-treatment, with 17 and 22% reductions in Chenopodiaceae and Poaceae, respectively, in the chilled plants relative to the control ones. (Fig. 2C). The chilling pre-treatment decreased values of ε_{max}, and this is consistent with an increase in cell-wall elasticity under low temperature conditions (Fig. 2C). DMC was also significantly decreased by the chilling pre-treatment (Table 2; Fig. 2D). DMC was 7–33% and 1–14% less in Chenopodiaceae and Poaceae species in chilling pre-treatment plants compared to the control ones (Fig. 2D).

Leaf freezing injury

The high-light freezing treatment caused high leaf mortality in two C_3 species of Chenopodiaceae, *Agriophyllum pungens* and *Ceratoides arborescens*, killing 100 and 60% of the leaves, respectively, and in a C_4 species of Chenopodiaceae, *S. collina*, killing 75% of the leaf canopy (Fig. 3). For *Corispermum heptapotamicum*, a C_3 species of Chenopodiaceae, leaf mortality was 48 and 73% for chilling pre-treatment and control plants, respectively, displaying a cold acclimation effect (Fig. 3). By contrast, the C_4 Chenopodiaceae species *H. ammodendron* and *Kochia prostrata* and all of the C_4 Poaceae species exhibited significant resistance to the freezing treatment in both chilling pre-treated and control plants, with leaf mortality of 16–47%. The C_4 Poaceae species *Pennisetum clandestinum* and *Cleistogenes squarrosa* showed cold acclimation responses, with leaf mortality in the chilling pre-treatment 35% less than that in the controls. The C_3 Poaceae species *Leymus chinensis* displayed the lowest freezing leaf mortality in either chilling or control plants, at about 16% (Fig. 3). There were statistically significant effects of the chilling pre-treatment and family on leaf mortality, but no effect of photosynthetic type, and no interactions between factors (Table 2).

Electrolyte leakage rates (R_c) provided quantitative evidence of freezing injury to leaf cellular membranes when leaves were frozen to –5 °C in both chilling and control plants. If the photosynthetic type was ignored, there was a positive correlation between leaf freezing injury and R_c in Chenopodiceae and Poaceae species across both the control and chilling treatments, with R_c explaining 61% of the variance in freezing leaf mortality (Fig. 4A). There was a strong correspondence between the visual assessments of leaf freezing injury and F_v/F_m measured immediately after freezing for all species across both the control and chilling pre-treatments, with F_v/F_m after freezing explaining 70% of the variance in freezing mortality (Fig. 4B).

The correspondence between cellular membrane damage and leaf mortality indicated that membrane damage was a critical element of leaf canopy injury. The correlation between R_c and ψ_{tlp} was also close for all species in both the control and chilling pre-treatments, with ψ_{tlp} explaining 57% of the variation in electrolyte leakage rates ($P < 0.01$) (Fig. 5). Overall, there was a significant effect of the chilling pre-treatment on R_c, but none of the other effects were statistically significant (Table 2).

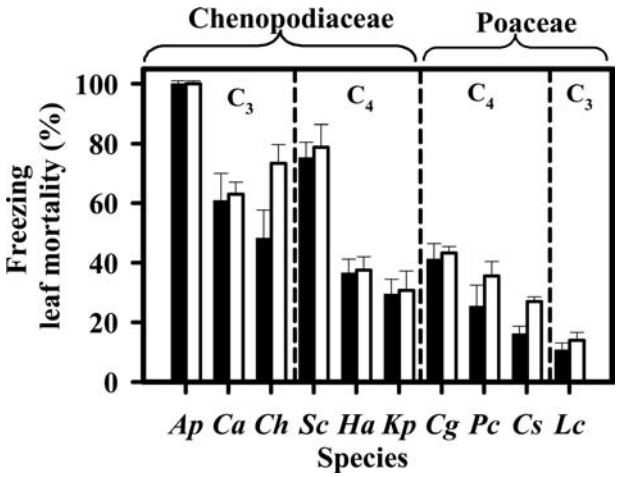

Fig. 3. Freezing leaf mortality of C_3 and C_4 plants after 20 d of chilling (filled bars) or control (open bars) pre-treatments followed by high-light –5 °C freezing events. Abbreviations for species names are defined in Table 1. Values are mean ± SE for eight individual plants.

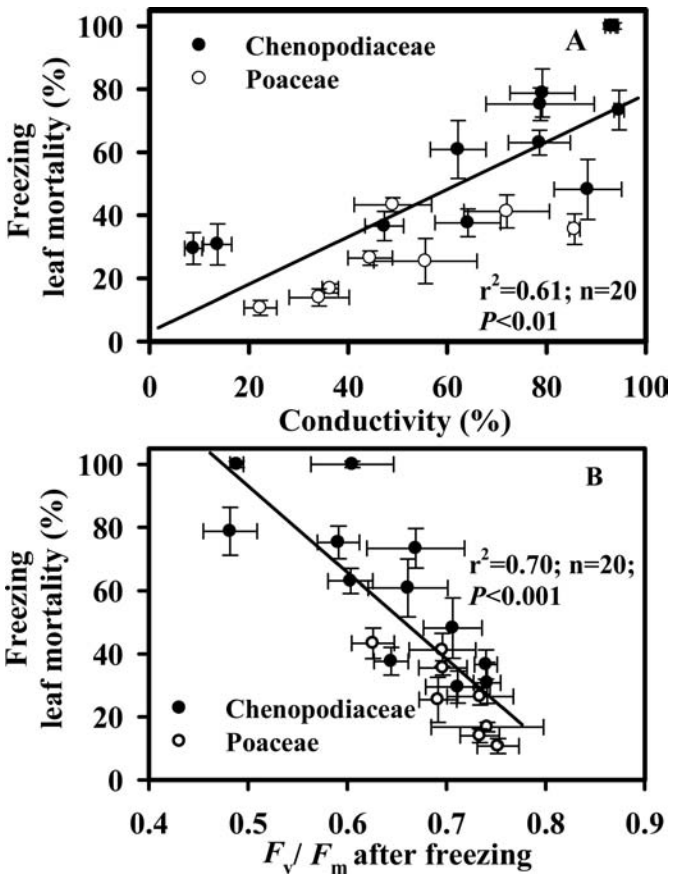

Fig. 4. Relationships of electrolyte leakage rates of leaf cellular membranes versus freezing leaf mortality ($r^2 = 0.61$; $n = 20$; $P < 0.001$) (A), and the F_v/F_m of leaves after the freezing treatment versus freezing leaf mortality (B) ($r^2 = 0.70$; $n = 20$; $P < 0.001$), for Chenopodiaceae and Poaceae species in the chilling compared with the control pre-treatment. Values are mean ± SE for eight individual plants.

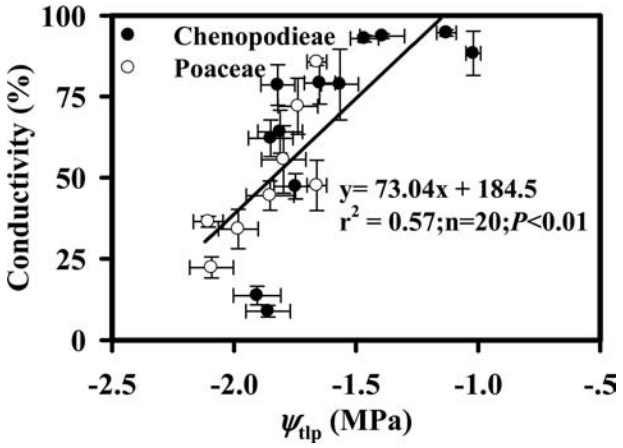

Fig. 5. Relationship of leaf water potential at the turgor loss point (ψ_{tlp}) versus electrolyte leakage rate (R_c) of cellular membranes ($r^2 = 0.57$; $n = 20$; $P < 0.01$), for Chenopodiceae and Poaceae species in the chilling and control pre-treatments. Values are mean ± SE for eight individual plants.

Discussion

Photodamage and leaf mortality

Photodamage and leaf mortality caused by freezing under high-light conditions were strongly associated across species. The exposure of plants to a chilling treatment drove photoprotection responses, which reduced photodamage during a subsequent high-light freezing event. This acclimation response was evident in both Chenopodiaceae and Poaceae species, irrespective of their photosynthetic type. However, when plants were grown under warm conditions prior to freezing and were not allowed to develop cold acclimation, the species of the Poaceae showed a greater degree of constitutive photoprotection. Chilling-induced photoprotection was more important within Chenopodiaceae. These results are consistent with prior reports of the effects of cold-acclimation on freezing responses (Strand and Öquist, 1988; Dai *et al*., 2007). However, there were no differences between species using C_3 and C_4 pathways.

Leaf freezing mortality was also higher in the Chenopodiaceae than the Poaceae, but exposure to chilling conditions induced a cold acclimation response that protected leaves against the subsequent high-light freezing event. As with photodamage, there were no differences in freezing damage between species using C_3 and C_4 photosynthesis. This finding, and the observation of greater leaf damage in Chenopodiaceae than Poaceae, conflicts with this study's *a priori* expectations. Rather, it supports an alternative hypothesis that freezing resistance in species is more closely related to their ecological preferences and life history. Notably, the two Chenopodiaceae species with greatest freezing resistance are both perennials of arid habitats and the Poaceae are all perennials.

Physiological mechanisms of freezing resistance

The strong association between visible damage to leaves and electrolyte leakage suggests that cellular damage caused by ice formation was the primary mechanism of freezing injury. Damage may be caused directly by the physical disruption of cellular membranes by intracellular ice crystals. Alternatively, it may be driven by the lower water potential over extracellular ice than water at the same temperature, which extracts symplastic water, causing cellular dehydration and (eventually) plasmolysis (Tao and Li, 1993; Mitchell *et al*., 2008). Consistent with this dehydration mechanism, the present study found a positive correlation of ψ_{tlp} with electrolyte leakage after the subsequent frost (Fig. 5); i.e. species with a low ψ_{tlp} suffered less freezing damage. Since osmotic potential is the major cause of interspecific variation in the turgor loss point (Bartlett *et al*., 2012), this result suggests that constitutive differences in leaf osmotic potential among species are important determinants of their differential freezing resistance.

Cold acclimation also involved adjustments to leaf water relations, manifested as a decrease in ψ_{tlp} and associated with a lowering of ψ_{π}, ε_{max}, and DMC. However, these adjustments are unlikely to form a major component of the cold acclimation response, since full equilibration of apoplastic water with extracellular ice generates a water potential of approximately –6 MPa (Améglio *et al*., 2001), and the present study found an osmotic adjustment <0.25 MPa. Instead, alternative acclimation mechanisms such as changes to membrane composition, the accumulation of dehydrin and ice-interacting proteins, and the stabilization of membranes by fructans (in grasses) may be more important (Sandve *et al*., 2011). Osmotic adjustment associated with chilling has been reported in a previous experiment on Mongolian grasses (Liu and Osborne, 2008) and other studies (e.g. Roberts and Knoerr, 1977; Tyree *et al*., 1978; Grossnickle, 1992).

The high constitutive freezing resistance in the Poaceae and two Chenopodiaceae species (*H. ammodendron* and *K. prostrata*) was also associated with a high ε_{max}. High values of ε_{max} mean low tissue elasticity, which leads to large changes in leaf water potential for only small changes in water content. In the situation where leaf water potential is driven downwards by extracellular ice formation, this maintains tissue hydration as water potential declines (the 'cell water conservation hypothesis'; Valentini *et al*., 1990; Bartlett *et al*., 2012). Indeed, high ε_{max} and low ψ_{tlp} are considered key facets of desiccation tolerance (Bartlett *et al*., 2012). However, cell water conservation only protects leaves at water potentials above the ψ_{tlp} and at temperatures close to the freezing point (Valentini *et al*., 1990).

Ecology and photosynthetic pathway

Leaf freezing resistance for both C_4 grasses and C_4 Chenopodiceae species fell within the range of C_3 species across the two families, suggesting that the C_4 pathway presented no inherent barrier to freezing resistance in either family. Despite the general finding of higher leaf freezing mortality in Chenopodiaceae than Poaceae species, some C_4 Chenopodiaceae from the Mongolian Plateau (*H. ammodendron* and *K. prostrata*) had a high resistance to frost during the growing season. Since most species coexist in steppe

ecosystems at a similar latitude, differential freezing resistance cannot be attributed to climatic adaptation. Instead, two possible interpretations of these results may be put forward.

First, interspecific differences could represent differing ecological characteristics. The two Chenopodiaceae species with high freezing resistance were *H. ammodendron*, a perennial tree-like species distributed in the deserts of the Mongolian Plateau, especially in arid deserts with <100 mm annual precipitation (Pyankov *et al*., 2000), and *K. prostrata*, a perennial shrub-like species that naturally occupies sandy grassland or stony soils and has a high resistance to drought and salt. In contrast, the majority of remaining Chenopodiaceae species were annuals, which are opportunistic in ecosystems, taking advantage of pulses of water after rains or living in moist, disturbed habitats, and thus lack resistance to low water potentials.

Secondly, although cold acclimation is theoretically less effective in C_4 than C_3 plants due to the lower phenotypic plasticity of C_4 leaves (Sage and McKown, 2006), the present data have demonstrated that both C_4 Poaceae and Chenopodiaceae species can resist leaf freezing injury at temperatures of –5 °C. This result is consistent with several previous field observations and controlled experiments that have documented the development of frost protection in some C_4 grass species during exposure to chilling (Rowley, 1976; Sage and Kubien, 2007; Liu and Osborne, 2008). Thus this study furthers understanding of the phenomenon by providing evidence for the mechanism of freezing resistance in eudicots, through an investigation of some C_4 Chenopodiaceae species from the Mongolian Plateau.

A striking feature of perennial C_4 plants in deserts and grasslands of the Mongolian Plateau is their presence in areas with large daily and annual temperature fluctuations and extremely low temperatures during winter (Pyankov *et al*., 2000). By contrast, the annual C_4 chenopod species *S. collina* is a succulent halophyte, occurring in saline and arid environments in steppe or disturbed land, which survives the winter as seeds (Liu *et al*., 2009). The successful protection of leaves from frost in the C_4 Chenopodiaceae species could therefore represent an adaptive response of above ground parts of plants to early frost in autumn or extremely cold winters in the steppe environment. The present findings suggest that this mechanism may reflect physiological plasticity and is an ecologically relevant strategy.

In conclusion, contrary to *a priori* expectations, leaf mortality and photodamage during a high-light freezing event were lower in Poaceae than Chenopodiaceae species and were unrelated to the photosynthetic pathway. The freezing resistance of C_4 Poaceae species was associated with a low turgor loss point, a low osmotic potential, and an ability to protect photosynthetic apparatus from damage by energy dissipation. However, despite this general difference between plant families, some C_4 Chenopodiaceae species originating from arid habitats also showed constitutive freezing tolerance. Freezing damage to leaves was mediated by electrolyte leakage consistent with intracellular ice formation and the desiccating effects of extracellular ice formation in tissues. Both freezing resistance and photoprotection were enhanced by cold acclimation, which involved only a slight lowering of the turgor loss point. In contrast, the leaves of species with constitutive resistance to freezing typically had a low tissue elasticity, which minimizes cellular water loss during extracellular ice formation. It is hypothesized that ecotypic differentiation between the Chenopodiaceae and Poaceae species with respect to chilling and freezing sensitivity may be interpreted in terms of physiological and ecological divergence among the families. This study demonstrated that responses to freezing events during the growing season are better explained by taxonomic and ecological factors than by the differential limitation of species with different photosynthetic pathways.

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References

Améglio T, Ewers FW, Cochard H, Martignac M, Vandame M, Bodet C, Cruiziat P. 2001. Winter stem xylem pressure in walnut trees: effects of carbohydrates, cooling and freezing. *Tree Physiology* **21,** 387–394.

Bartlett MK, Scoffoni C, Sack L. 2012. The determinants of leaf turgor loss point and prediction of drought tolerance of species and biomes: a global meta-analysis. *Ecology Letters* **15,** 393–405.

Batanouny KH, Stichler W, Ziegler H. 1988. Photosynthetic pathways, distribution, and ecological characteristics of grass species in Egypt. *Oecologia* **75,** 539–548.

Colombo SJ, Webb DP, Glerum C. 1984. Frost hardiness testing: an operational manual for use with extended greenhouse culture. *Forestry Research Reports* **110,** 14.

Dai F, Zhou MX, Zhang GP. 2007. The change of chlorophyll fluorescence parameters in winter barley during recovery after freezing shock and as affected by cold acclimation and irradiance. *Plant Physiology and Biochemistry* **45,** 915–921.

Demming-Adams B, Adams WW III. 1992. Photoprotection and other responses of plants to high light stress. *Annual Review of Plant Physiology and Plant Molecular Biology* **43,** 599–626

Ehleringer J, Cerling TE, Helliker BR. 1997. C_4 photosynthesis, atmospheric CO_2, and climate. *Oecologia* **112,** 285–299.

Grossnickle SC. 1992. Relationship between freezing tolerance and shoot water relations of western red cedar. *Tree Physiology* **11,** 229–240.

Hattersley PW. 1983. The distribution of C_3 and C_4 in grasses in Australia in relation to climate. *Oecologia* **57,** 113–128.

Huner NPA, Oquist G, Hurry VM, Krol M, Falk S, Griffith M. 1993. Photosynthesis, photoinhibition and low-temperature acclimation in cold tolerant plants. *Photosynthesis Research* **37,** 19–39.

Larcher W. 2003. *Physiological plant ecology*. Heidelberg: Springer.

Levitt J. 1980. *Responses of plants to environmental stresses* . Vol. I. Chilling, freezing and high temperature stresses. New York: Academic Press.

Liu MZ, Jiang GM, Yu SL, Li YG, Li G. 2009. The role of soil seed banks in natural restoration of the degraded Hunshandak Sandlands, Northern China. *Restoration Ecology* **17,** 127–136.

Liu MZ, Osborne CP. 2008. Leaf cold-acclimation and freezing injury in C_3 and C_4 grasses of the Mongolian Plateau. *Journal of Experimental Botany* **59,** 4161–4170.

Lloyd J, Farquhar GD. 1994. ^{13}C discrimination during CO_2 assimilation by the terrestrial biosphere. *Oecologia* **99,** 201–215.

Mitchell PJ, Veneklaas EJ, Lambers H, Burgess SSO. 2008. Leaf water relations during summer water deficit: differential responses in turgor maintenance and variation in leaf structure among different plant communities in south-western Australia. *Plant, Cell and Environment* **31,** 1791–1802.

Neuner G, Bannister P. 1995. Frost resistance and susceptibility to ice formation during natural hardening in relation to leaf anatomy of three evergreen tree species from New Zealand. *Tree Physiology* **15,** 371–377.

Osborne CP, Wythe EJ, Ibranhim DG, Gilbert ME, Ripley BS. 2008. Low temperature effects on leaf physiology and survivorship in the C_3 and C_4 subspecies of *Alloteropsis semialata*. *Journal of Experimental Botany* **59,** 1743–1754.

Pearce RS. 2001. Plant freezing and damage. *Annals of Botany* **87,** 417–424.

Pyankov VI, Gunin PD, Tsoog S, Black CC. 2000. C_4 plants in the vegetation of Mongolia: their natural occurrence and geographical distribution in relation to climate. *Oecologia* **123,** 15–31.

Roberts SW, Knoerr KR. 1977. Components of water potential estimated from xylem pressure measurements in five tree species. *Oecologia* **28,** 191–202.

Rowley JA. 1976. Development of freezing tolerance in leaves of C_4 grasses. *Australian Journal of Plant Physiology* **3,** 1086–1106.

Sage RF, Christin P, Edwards EJ. 2011. The C_4 plant lineages of planet Earth. *Journal of Experimental Botany* **62,** 3155–3169.

Sage RF, Kubien DS. 2007. The temperature response of C_3 and C_4 photosynthesis. *Plant, Cell and Environment* **30,** 1086–1106.

Sage RF, Li M, Monson RK. 1999b. The taxonomic distribution of C_4 photosynthesis. In: RF Sage, RK Monson, eds, *C_4 plant biology* . San Diego: Academic Press, 551–584.

Sage RF, McKown AD. 2006. Is C_4 photosynthesis less phenotypically plastic than C_3 photosynthesis? *Journal of Experimental Botany* **57,** 303–317.

Sage RF, Wedin DA, Li MR. 1999a. The biogeography of C_4 photosynthesis: patterns and controlling factors. In: RF Sage, RK Monson, eds, *C_4 plant biology* . San Diego: Academic Press, pp 313–373.

Sandve SR, Kosmala A, Rudi H, Fjellheim S, Rapacz M, Yamada T, Rognli OA. 2011. Molecular mechanisms underlying frost tolerance in perennial grasses adapted to cold climates. *Plant Science* **180,** 69–77.

Schulte PJ, Hinckley TM. 1985. A comparison of pressure-volume curve data analysis techniques. *Journal of Experimental Botany* **36,** 1590–1602.

Strand M, Öquist G. 1988. Effects of frost hardening, dehardening and freezing stress on in vivo chlorophyll fluorescence of seedlings of Scots pine (*Pinus sylvestris* L.). *Plant, Cell and Environment* **11,** 231–238.

Tao D, Li PH. 1993. Cell wall and freezing injury: a minireview. In: PH Li, L Christersson, eds, *Advances in plant cold hardiness*. Boca Raton: CRC Press, pp 195–201.

Turner NC. 1981. Techniques and experimental approaches for the measurement of plant water status. *Plant and Soil* **58,** 339–366.

Tyree MT, Cheung YNS, MacGregor ME, Talbot AJB. 1978. The characteristics of seasonal and ontogenetic changes in the tissue-water relations of *Acer*, *Populus*, *Tsuga*, and *Picea*. *Canadian Journal of Botany* **56,** 635–647.

Tyree MT, Jarvis PG. 1982. Water in tissues and cells. In: OL Lange, PS Nobel, CB Osmond, H Ziegler, eds, *Encyclopedia of plant physiology*. New series, vol. **12b**. Berlin: Springer-Verlag, pp 35–77.

Valentini R, Scarascia Mugnozza G, Giordano E, Kuzminsky E. 1990. The influence of cold hardening on water relations of three *Eucalyptus* species. *Tree Physiology* **6,** 1–10.