

## Acclimation to short-term low temperatures in two *Eucalyptus globulus* clones with contrasting drought resistance

F. COSTA E SILVA,<sup>1,2</sup> A. SHVALEVA,<sup>1,3</sup> F. BROETTO,<sup>3,4</sup> M.F. ORTUÑO,<sup>1</sup>  
M.L. RODRIGUES,<sup>1</sup> M.H. ALMEIDA,<sup>1</sup> M.M. CHAVES<sup>1,3</sup> and J.S. PEREIRA<sup>1</sup>

<sup>1</sup> Instituto Superior de Agronomia, Tapada da Ajuda 1349-017, Lisbon, Portugal

<sup>2</sup> Corresponding author (filipecs@isa.utl.pt)

<sup>3</sup> ITQB, Apt. 12 Oeiras 2784-505, Portugal

<sup>4</sup> Institute of Biosciences, São Paulo State University, Botucatu 18618-000, Brazil

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**Summary** We tested the hypothesis that *Eucalyptus globulus* Labill. genotypes that are more resistant to dry environments might also exhibit higher cold tolerances than drought-sensitive plants. The effect of low temperatures was evaluated in acclimated and unacclimated ramets of a drought-resistant clone (CN5) and a drought-sensitive clone (ST51) of *E. globulus*. We studied the plants' response via leaf gas exchanges, leaf water and osmotic potentials, concentrations of soluble sugars, several antioxidant enzymes and leaf electrolyte leakage. Progressively lowering air temperatures (from 24/16 to 10/−2 °C, day/night) led to acclimation of both clones. Acclimated ramets exhibited higher photosynthetic rates, stomatal conductances and lower membrane relative injuries when compared to unacclimated ramets. Moreover, low temperatures led to significant increases of soluble sugars and antioxidant enzymes activity (glutathione reductase, ascorbate peroxidase and superoxide dismutases) of both clones in comparison to plants grown at control temperature (24/16 °C). On the other hand, none of the clones, either acclimated or not, exhibited signs of photoinhibition under low temperatures and moderate light. The main differences in the responses to low temperatures between the two clones resulted mainly from differences in carbon metabolism, including a higher accumulation of soluble sugars in the drought-resistant clone CN5 as well as a higher capacity for osmotic regulation, as compared to the drought-sensitive clone ST51. Although membrane injury data suggested that both clones had the same inherent freezing tolerance before and after cold acclimation, the results also support the hypothesis that the drought-resistant clone had a greater cold tolerance at intermediate levels of acclimation than the drought-sensitive clone. A higher capacity to acclimate in a short period can allow a clone to maintain an undamaged leaf surface area along sudden frost events, increasing growth

capacity. Moreover, it can enhance survival chances in frost-prone sites expanding the plantation range with more adaptive clones.

*Keywords:* antioxidant capacity, chilling, dehydration tolerance, freezing, solute accumulation.

### Introduction

*Eucalyptus globulus* Labill. plantations continue to increase annually and worldwide to cope with the increasing needs for paper and also due to their high growth rate and pulping properties (Carbonnier 2004). However, this need has resulted in a tendency to include sites with less than optimal climatic conditions for planting such as those with more frequent frost conditions. Even in Mediterranean areas episodic occurrences of below-zero temperatures are important, limiting the expansion of *E. globulus* plantations. Moreover, because young *Eucalyptus* plants are less tolerant to extreme environmental conditions than the adult plants, the degree of frost tolerance can determine the successful establishment and thereby limit species/genotype distributions to certain regions or microsites. In addition, with the predicted increase in weather variability induced by global climate change (IPCC 2007), it is expectable that plants will be subjected to sudden frost events with variable hardening possibilities.

Plants face three major problems when exposed to low temperature: an alteration in the spatial organisation and biophysical properties of the cell membranes, a slowing down of their chemical and biochemical reactions and, under freezing conditions, changes in water status and availability (Sakai and Larcher 1987). The alterations induced by low temperatures comprise changes in the concentrations of a wide range of metabolites, including sugars, protective proteins, as well as modification of cell membranes, changes in hormone levels and alterations in gene expression

(Zhu et al. 2007). Moreover, exposure to low temperatures may cause mild oxidative stress, which generates and accumulates reactive oxygen species (ROS) capable of causing oxidative damage to proteins, DNA and lipids (Apel and Hirt 2004). Generally, cold acclimation ensures protection to plants through enzymatic ROS-scavenging mechanisms (Wise 1995). However, when plants are rapidly subjected to low temperature without acclimation, damages to the enzymatic ROS-scavengers might be too high and excess ROS can initiate cell death. Furthermore, because mild below-zero temperatures can be lethal even for the more hardy species in the unacclimated state, the speed of acclimation is crucial for plant survival in a given area, sometimes independent of the tolerance level to be acquired.

A large amount of research on cold stress and tolerance mechanisms was accumulated in the last decades (Levitt 1980, Sakai and Larcher 1987, Basra 2001) although there were not many published data on the frost tolerance of *E. globulus* (Almeida et al. 1994, Volker et al. 1994, Tibbits et al. 2006). Recently, it has been shown that winter-frost tolerance is a trait with considerable variation within *E. globulus* with the most tolerant families tolerating late-winter temperatures of 1.4 °C colder than the overall families average (Tibbits et al. 2006). Thus, it is expected that contrasting genotypes respond differently to low temperatures in the process of cold acclimation that takes place on the time scale of days or weeks as a result of a combination of physiological and metabolic changes under decreasing temperatures. Moreover, plant responses to low temperatures show many similarities with responses to water deficits, suggesting that cold-resistance and drought-resistance mechanisms often share the same pathways (Sung et al. 2003, Atkin et al. 2005, Beck et al. 2007).

For these reasons we hypothesised that, under a Mediterranean-type climate, *E. globulus* genotypes more resistant to dry environments might also exhibit higher frost tolerances than drought-sensitive plants. If this is true, it will allow a clone less susceptible to drought to maintain an undamaged leaf surface area along the frost periods, thus allowing those plants to enter spring with a higher capacity for growth than more drought-sensitive plants. In addition, detailed physiological information of the stress-response of clones is necessary for the development of breeding programmes and is essential to support decisions to allocate clones to different climatic regions. In a previous work (Costa e Silva et al. 2004, Shvaleva et al. 2006), the two clones under study were shown to differ in their sensitivity to water deficits (CN5 was drought resistant and ST51 was drought sensitive) and in their capacity of long-term acclimation to chilling (Costa e Silva et al. 2007, Shvaleva et al. 2008). Under chilling conditions, the better performance of clone CN5 was associated with the maintenance of root growth, higher water status and anthocyanin concentration compared with clone ST51. The aims of the present work were to: (1) evaluate the effect of rapid acclimation to chilling and freezing in physiological and biochemical properties of two clones of *E. globulus* with

contrasting responses to drought, (2) compare the responses to chilling and freezing in clones without acclimation and (3) test whether the drought-resistant clone is less affected by freezing than the drought-sensitive clone.

## Materials and methods

### Plant material and treatments

We studied two *E. globulus* clones (CN5, drought resistant and ST51, drought sensitive). Ramets produced by rooted cuttings of both clones were grown in plastic containers containing peat (60%) and styrofoam (40%), and were transplanted at 4 months to pots (1.5 l) filled with peat and vermiculite (2/1 v/v). One month after transplanting, 30 cuttings per clone were transferred from the nursery to a growth chamber with controlled conditions (24/16 °C, day/night) (control plants). Another 18 cuttings per clone were placed in a growth chamber subjected to an acclimation period of 14 days with a gradual temperature decrease (1 °C per day and 1 °C per night during the first 10 days) from 24/16 to 10/6 °C (day/night) (acclimation treatment). After the acclimation period, the plants were subjected to a further decline in night temperature during 9 days and measurements were done at days 1, 5 and 9 with temperatures of 10/6, 10/2 and 10/−2 °C (day/night), respectively. In addition, another group of plants were measured in the same days, after transfer, 24 h earlier from the control to the low-temperature chamber (direct chilling/freezing treatment) (Figure 1). Both growth chambers had similar lighting systems (ca. 220  $\mu\text{mol m}^{-2} \text{s}^{-1}$  at the canopy level), a photoperiod of 12/12 h (day/night) and a relative humidity of about 60%. To avoid the effects caused by microenvironmental differences (light and temperature gradients), the plants were sorted by treatment and moved to the neighbouring position every other day. The experiment was carried out during January 2007. All plants were watered to the point of runoff in the first day and then watered twice per week (Mondays and Fridays) according to evapotranspiration values.

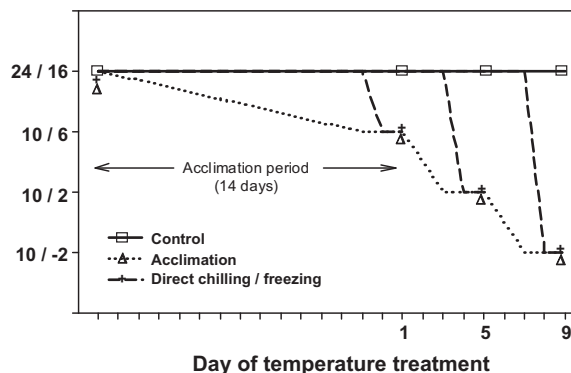


Figure 1. Day and night air temperature of control, acclimation and direct chilling/freezing treatments throughout the experiment.

Given that both shoots and roots were subjected to low temperatures, we can expect some low root temperature influence on leaf metabolism as generally observed: e.g., on stomatal conductance (Almeida et al. 1994). However, an unrealistic drought during the day can be dismissed since our low day temperatures prevented high evaporative demands. On the other hand, a 10 °C gradient between soil and air temperatures is a likely event in clear winter days of the Mediterranean climate due to slow soil warming.

#### *Water relations*

Leaf xylem water potential was measured at predawn ( $\Psi_{pd}$ ) with a Scholander-type pressure chamber (PMS Instruments, Corvallis, OR) in one leaf from four plants per treatment. Soon after measuring  $\Psi_{pd}$ , leaf discs (7 mm diameter) were taken from each leaf, frozen in liquid nitrogen and stored at  $-80$  °C for later determination of osmotic potential ( $\Psi_{\pi}$ ). After thawing the samples at room temperature,  $\Psi_{\pi}$  was measured using C-52 chambers (2 h for equilibration) connected to a Wescor HR-33T dew-point microvoltmeter (Wescor, INC Logan, UTAH) operating in the dew-point mode. The chambers were calibrated with standard NaCl solutions. The prevailing room temperature during the measurements was  $20 \pm 1$  °C.

#### *Gas exchange and chlorophyll fluorescence*

Gas exchanges were measured with a LI-6400 portable photosynthesis system (Li-Cor, Lincoln, NE) in one fully expanded leaf from four plants per treatment at midday (solar time). Measurements took place under the light conditions of the controlled environment chambers and temperature was fixed at 15 °C in the low temperature treatments. Pre-dawn maximal photochemical efficiency,  $F_v/F_m$ , was assessed using a Mini-PAM fluorometer (Walz GmbH, Effeltrich, Germany) under chamber conditions. The same leaves used in gas exchange were measured, taking care to avoid the midrib.

#### *Artificial freezing and membrane injury*

At day 9, three leaf discs per plant (10 mm in diameter) were punched from fully expanded leaves of six plants per treatment (control and acclimated) and placed in test tubes. The racks of test tubes were placed inside a freezer (Aralab, Lisbon, Portugal) in baths containing an aqueous ethylene glycol solution at 2 °C. A controlled freezing programme followed a constant cooling and thawing rate of 4 °C h<sup>-1</sup> and a 2 h exposure to five different target freezing temperatures ( $-2.6$ ,  $-3.4$ ,  $-4.6$ ,  $-6.2$  and  $-8$  °C). When the temperature of the bath was at  $-2$  °C, about 0.5 g of finely crushed ice (from deionised water) was added to each tube to make contact with the leaf disc.

Membrane injury was determined by measuring cell conductivity after artificial freezing. Electrolyte conductivity of 15 ml deionised water containing leaf discs was measured

after 24 h at 25 °C ( $T_1$ ) with a K220 conductivity metre (Consort, Turnhout, Belgium). The samples were then boiled in an autoclave at 120 °C for 10 min, held at 25 °C for 2 h and total electrolyte conductivity was measured ( $T_2$ ). Relative injury (RI) was expressed as a ratio of electrolyte conductivity measured after freezing treatment relative to maximum electrolyte conductivity,  $RI = (T_1/T_2) \times 100$ .

#### *Soluble sugars*

Soluble sugars in leaves were assayed by the anthrone method (Robyt and White 1987) as described in Shvaleva et al. (2006). Frozen leaf discs (0.02 g) were ground with a cold mortar and pestle in liquid N<sub>2</sub> with 1 mM of 70% (v/v) ethanol. The homogenate was thermomixed twice at 60 °C for 30 min, centrifuged at 14,000 g for 5 min and the supernatant was used for determination with a spectrophotometer (U-2001; Hitachi, Japan).

#### *Antioxidant enzymes*

Sample leaves were excised and immediately immersed in liquid nitrogen and stored at  $-80$  °C. The extract for enzymatic analyses was obtained by the suspension of the plant material (300 mg) in 5.0 ml of potassium phosphate buffer (0.1 M, pH 6.8). After centrifugation for 10 min at 20,000 g, the supernatant was collected and stored at  $-80$  °C. The concentration of soluble protein in the extracts was determined according to Bradford (1976) with bovine serum albumin (BSA) as protein standard. For the determination of glutathione reductase (GR, EC 1.6.4.2) and ascorbate peroxidase (APX, EC 1.11.1.11) activity in leaves (0, 5 g fresh mass) the general procedures of Foyer and Halliwell (1976) and Nakano and Asada (1981), respectively, were used with some modifications (Shvaleva et al. 2006). For GR, the assay medium contained 500 mM HEPES (Sigma Chemical) (pH 8.0), 0.25 mM EDTA (Sigma Chemical), 2 mM NADPH (Sigma Chemical), 20 mM oxidised glutathione (GSSG) and 100  $\mu$ l extract. Control rates were obtained in the absence of GSSG or NADPH. For APX, the assay medium contained 50 mM KH<sub>2</sub>PO<sub>4</sub>/K<sub>2</sub>HPO<sub>4</sub> (pH 7.0), 20 mM H<sub>2</sub>O<sub>2</sub>, 8 mM ascorbate and 100  $\mu$ l extract. Control rates were obtained in the absence of extract, ascorbate or H<sub>2</sub>O<sub>2</sub>.

The determination of the activity of superoxide dismutases (SOD, EC1.15.1.1) considered the capacity of the enzyme to inhibit the photoreduction of nitroblue tetrazolium chloride (NBT). The enzyme activity was determined according to Giannopolitis and Ries (1977) and Del Longo et al. (1993) by mixing 50  $\mu$ l of crude extract to a solution containing 13 mM metionine, 75  $\mu$ M *p*-nitro blue tetrazolium chloride, 100 nM EDTA and 2  $\mu$ M riboflavin in a 50 mM sodium phosphate buffer (pH 7.8). It was expressed as U mg<sup>-1</sup> protein, considering that one SOD unit (U) was defined as the amount of enzyme required to inhibit 50% of the NBT photoreduction.

Table 1. Statistical significance of the effects of clone (*C*), temperature regime (*T*) and their interaction as determined by two-way analysis of variance of leaf variables: predawn water potential ( $\Psi_{pd}$ ), osmotic potential ( $\Psi_{\pi}$ ), stomatal conductance ( $g_s$ ), net photosynthesis (*A*), pre-dawn maximal photochemical efficiency ( $F_v/F_m$ ), membrane relative injury (RI) and activities of glutathione reductase (GR), ascorbate peroxidase (APX) and superoxide dismutase (SOD) in two *E. globulus* clones. Symbols: \*, \*\*, \*\*\* represent statistical significance at  $P = 0.05$ ,  $0.01$  and  $0.001$ , respectively; ns = nonsignificant at  $P = 0.05$ .

Leaf parameters	Day	Clone	Temperature regime	$C \times T$
$\Psi_{pd}$	1	ns	***	ns
	5	ns	***	ns
	9	ns	***	ns
$\Psi_{\pi}$	1	ns	ns	ns
	5	ns	***	*
	9	*	***	ns
$g_s$	1	***	ns	ns
	5	***	*	ns
	9	ns	***	ns
<i>A</i>	1	*	ns	*
	5	***	***	ns
	9	**	***	ns
$F_v/F_m$	1	ns	***	ns
	5	ns	***	ns
	9	ns	***	ns
RI	9	ns	***	ns
	1	*	***	ns
	5	***	***	ns
GR	9	***	*	*
	1	ns	**	ns
	5	ns	**	ns
APX	1	ns	**	ns
	5	ns	**	ns
	9	ns	**	ns
SOD	1	ns	ns	ns
	5	ns	***	ns
	9	ns	**	ns

### Statistical analysis

Data were subjected to two-way analysis of variance (ANOVA) to test for the effects and interactions of temperature treatment and clone, using the STATISTICA (Version 6, StatSoft, Inc. 2001) data analysis software system. Whenever the mean value difference was significant, the Student–Newman–Keuls test was used to identify the differences between treatments. All variables were tested for normality and homogeneity of variances. Differences were considered statistically significant at  $P \leq 0.05$ .

## Results

### Water relations

Low temperatures led to a significant ( $P < 0.001$ ) decrease in  $\Psi_{pd}$  in both cold treatments as compared to control plants (Table 1, Figure 2A). Plants in the acclimation treatment maintained stable  $\Psi_{pd}$  values throughout the experiment (ranging from  $-0.75$  to  $-0.99$  MPa) but much

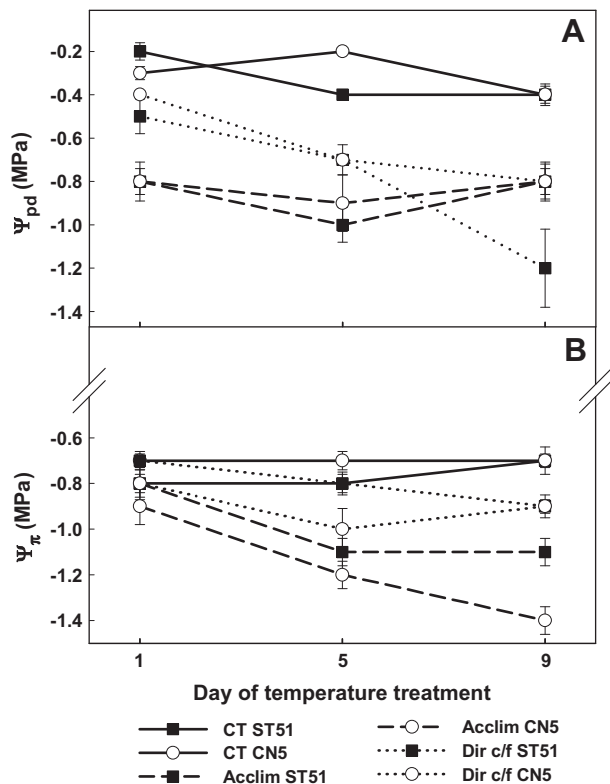


Figure 2. Predawn leaf water potential ( $\Psi_{pd}$ ; A) and leaf osmotic potential ( $\Psi_{\pi}$ ; B) in control (CT), acclimation (Acclim) and direct chilling/freezing (Dir c/f) treatments with plants belonging to a drought-sensitive clone (ST51) and a drought-resistant clone (CN5) of *E. globulus*. Data are means  $\pm$  SE ( $n = 4$ ).

lower than those of control plants (varying between  $-0.24$  and  $-0.41$  MPa). However, the direct chilling/freezing treatment showed a decrease in  $\Psi_{pd}$  with the decrease in temperature along the experiment. From  $10/6$  °C (day 1) to  $10/2$  °C (day 5)  $\Psi_{pd}$  declined on average from  $-0.47$  to  $-0.71$  MPa in both clones subjected to low temperatures without acclimation. With lower temperatures, i.e., at  $10/-2$  °C (day 9), a further decline to  $-1.16$  MPa was observed in ST51 clone, whereas in CN5 clone there was only a slight decline to  $-0.83$  MPa, a value similar to that presented by plants in the acclimation treatment.

Control plants of both clones presented similar and constant  $\Psi_{\pi}$  values throughout the experiment. Conversely, acclimated plants of both clones showed a decrease in  $\Psi_{\pi}$  at  $10/2$  and  $10/-2$  °C in comparison to control ( $P < 0.001$ ), although more marked ( $P < 0.05$ ) in CN5 than in ST51 plants (Figure 2B). In addition, CN5 subjected to direct chilling/freezing also exhibited a decrease in  $\Psi_{\pi}$  from  $10/6$  to  $10/-2$  °C, whereas ST51 decreased  $\Psi_{\pi}$  only at  $10/-2$  °C.

### Gas exchange and chlorophyll fluorescence

Stomatal conductance declined significantly ( $P < 0.05$ ) in both the clones and in all the treatments when temperatures

attained 10/2 °C (day 5). At 10/−2 °C (day 9) there was a further decrease ( $P < 0.001$ ), with acclimation and direct chilling/freezing treatments presenting  $g_s$  values corresponding to 18% and 7% from those of control plants, respectively (Table 1, Figure 3A). In days 1 and 5, clone ST51 exhibited higher values of  $g_s$  ( $P < 0.001$ ) than CN5 clone in all treatments.

Similarly for  $g_s$ , there was a significant effect ( $P < 0.001$ ) of low temperature in  $A$  of both clones at 10/2 °C (day 5) causing an average decrease of 23% in comparison to control either in acclimation or in direct chilling/freezing treatments (Table 1, Figure 3B). Moreover, at 10/−2 °C (day 9) there was a further decline in  $A$ , although with a clear effect of acclimation ( $P < 0.001$ ). Acclimated plants showed a 47% decrease in  $A$  as compared to control plants, whereas a higher reduction (79%) was observed in unacclimated plants of the direct chilling/freezing treatment. Throughout the experiment, clone ST51 showed higher  $A$  than CN5 clone either in control or in acclimation treatments. In response to direct chilling/freezing, CN5 plants showed higher  $A$  than ST51 plants at 10/6 °C ( $P < 0.05$ ), whereas at 10/2 °C it was clone ST51 that showed higher  $A$  ( $P < 0.001$ ).

Low temperatures led to a decrease of  $F_v/F_m$  ( $P < 0.001$ ) in both acclimation and direct chilling/freezing treatments

although within constant and high values ( $F_v/F_m > 0.75$ ) throughout the experiment indicating that no photoinhibition occurred (Table 1, Figure 4). There were no significant differences between the clones along the experiment.

#### Membrane injury

Both clones showed similar membrane RI when subjected to negative temperatures ranging from −2.6 to −8 °C (Table 1, Figure 5). Leaf discs of control plants grown at 24/16 °C and successively subjected to lower negative temperatures showed a gradual increase in membrane damage attaining an average RI of 50% in both clones at temperature  $-3.8 \pm 0.1$  °C. On the other hand, acclimation led to a significant ( $P < 0.001$ ) decrease in membrane damage in relation to control plants, with acclimated plants maintaining low RI up to −8 °C (<25%).

#### Soluble sugars

Acclimated plants showed an increase in soluble sugar concentration at 10/2 and 10/−2 °C ( $P < 0.001$ ) in both clones (Table 2). However, contrary to ST51, CN5 showed an earlier increase in soluble sugars at 10/6 °C ( $P < 0.05$ ) and, moreover, a significant higher concentration at 10/2 ( $P < 0.001$ ) and 10/−2 °C ( $P < 0.05$ ). In response to

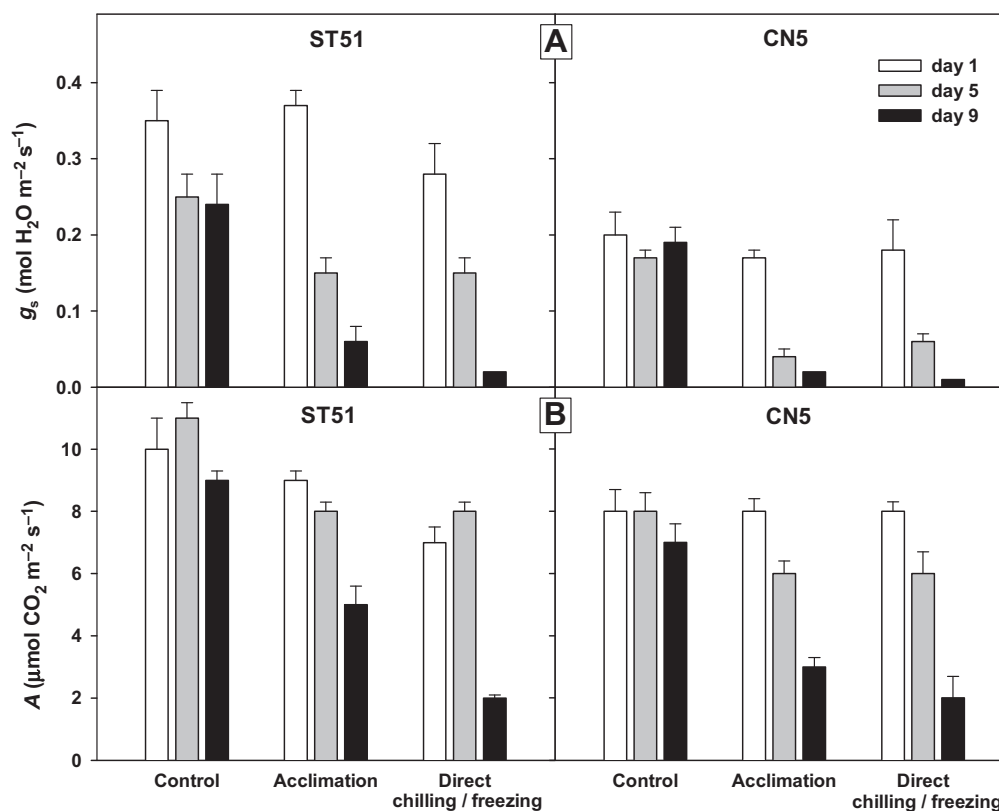


Figure 3. Midday stomatal conductance ( $g_s$ ; A) and net photosynthesis ( $A$ ; B) in control, acclimation and direct chilling/freezing treatments with plants belonging to a drought-sensitive clone (ST51) and a drought-resistant clone (CN5) of *E. globulus*. Control treatment was measured at 24/16 °C and acclimation and direct chilling/freezing treatments were measured at 10/6, 10/2 and 10/−2 °C in days 1, 5 and 9, respectively. Data are means  $\pm$  SE ( $n = 4$ ).

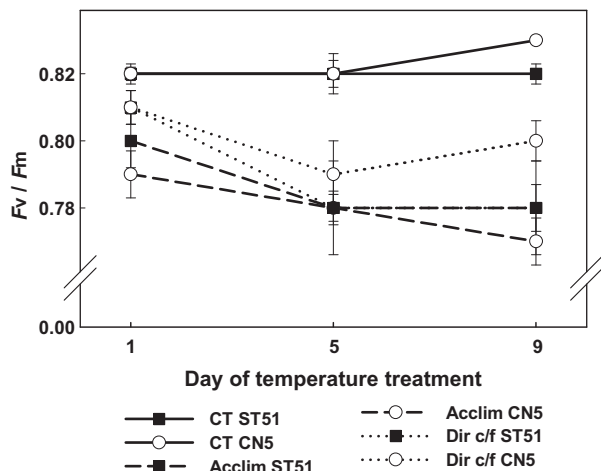


Figure 4. Predawn maximal photochemical efficiency ( $F_v/F_m$ ) in control (CT), acclimation (Acclim) and direct chilling/freezing (Dir c/f) treatments with plants belonging to a drought-sensitive clone (ST51) and a drought-resistant clone (CN5) of *E. globulus*. Data are means  $\pm$  SE ( $n = 4$ ).

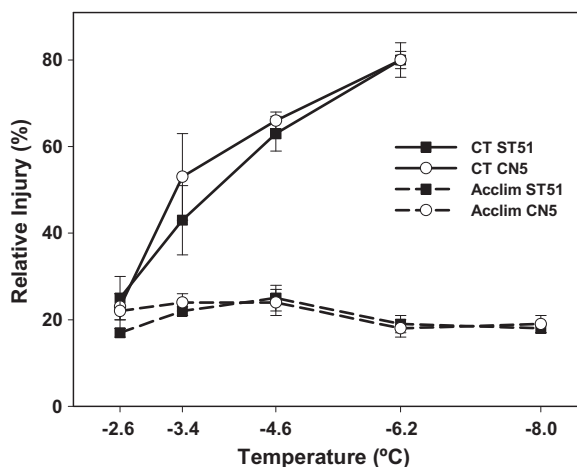


Figure 5. Membrane RI in control (CT) and acclimation (Acclim) treatments with plants belonging to a drought-sensitive clone (ST51) and a drought-resistant clone (CN5) of *E. globulus*. Data are means  $\pm$  SE ( $n = 6$ ).

direct chilling/freezing, the differences between the clones were clearer, with CN5 showing increases in soluble sugars of 45%, 69% and 34% at 10/6, 10/2 and 10/−2 °C, respectively, whereas ST51 clone slightly increased sugars (23%) at 10/−2 °C in comparison to control plants.

#### Antioxidant enzymes

Acclimation treatment to progressively lower temperatures of 10/6, 10/2 and 10/−2 °C led to similar responses of both clones with significant increases in all antioxidant enzyme activity in comparison to control plants (Table 1, Figure 6). From all enzymes, APX activity showed the larger increases in relation to control values, particularly at 10/6 and 10/2 °C

Table 2. Soluble sugar concentration in control, acclimation and direct chilling/freezing treatments with plants belonging to a drought-sensitive clone (ST51) and a drought-resistant clone (CN5) of *E. globulus* evaluated throughout the experiment. Control treatment was measured at 24/16 °C and acclimation and direct chilling/freezing treatments were measured at 10/6, 10/2 and 10/−2 °C in days 1, 5 and 9, respectively. Data are means  $\pm$  SE ( $n = 4$ ). Symbols: \*, \*\*, \*\*\* represent statistical significance at  $P = 0.05$ , 0.01 and 0.001, respectively; ns = nonsignificant at  $P = 0.05$ .

Temperature treatment	Soluble sugar concentration (mmol m <sup>-2</sup> )		
	Day 1	Day 5	Day 9
<i>Clone ST51</i>			
Control	31 $\pm$ 3.2	21 $\pm$ 1.9	30 $\pm$ 2.8
Acclimation	26 $\pm$ 2.1	38 $\pm$ 2.9	46 $\pm$ 4.2
Direct freezing/chilling	23 $\pm$ 2.0	25 $\pm$ 4.8	36 $\pm$ 2.5
<i>Clone CN5</i>			
Control	23 $\pm$ 2.5	26 $\pm$ 3.8	32 $\pm$ 3.7
Acclimation	33 $\pm$ 0.4	50 $\pm$ 4.7	55 $\pm$ 2.7
Direct freezing/chilling	33 $\pm$ 5.5	44 $\pm$ 3.5	43 $\pm$ 2.3
<i>Significance of two-way ANOVA</i>			
Clone (C)	ns	***	*
Temperature regime (T)	ns	***	***
C $\times$ T	*	ns	ns

(100%, on average). The only significant difference between the clones occurred in GR with ST51 plants showing higher activity than CN5 plants all along the experiment (Table 1, Figure 6A).

Clone responses to direct chilling/freezing were not so marked, with slight increases of antioxidant enzyme activity. Thus, both clones that were subjected to low temperatures without acclimation had increased the GR activity at 10/2 °C by 26%, on average, in comparison to control plants ( $P < 0.001$ ). Also, SOD activity in both clones significantly increased ( $P < 0.01$ ) at 10/−2 °C by 35%, on average, as compared to control plants (Figure 6C). There were no significant differences between the clones under direct chilling/freezing treatment although CN5 showed a clear increase in APX activity of 77% and 69% at 10/6 and 10/2 °C, respectively, in opposition to ST51 (Figure 6B).

#### Discussion

The water status of a plant influences its frost resistance via the cell sap concentrations and the degree of hydration of the protoplasm (Sakai and Larcher 1987). In our experiment, the relative water content was not altered by any treatment (data not shown). However, predawn leaf water potential exhibited significant changes with the decrease in growth temperature. Clone CN5 when subjected to freezing temperatures (10/−2 °C, day 9) without acclimation was able to maintain  $\Psi_{pd}$ , whereas ST51 did not (Figure 2A).

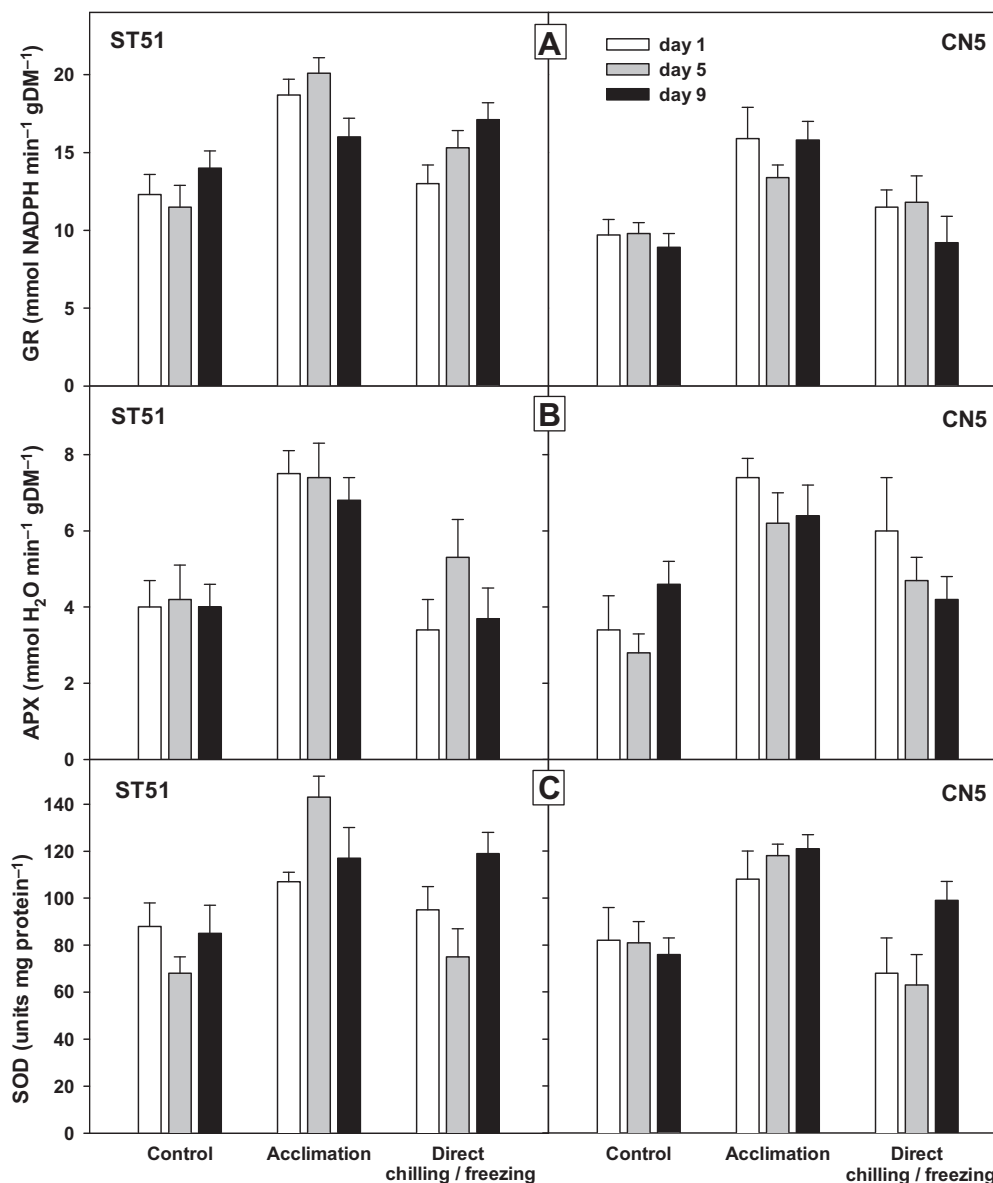


Figure 6. Glutathione reductase (GR; A), ascorbate peroxidase (APX; B) and superoxide dismutase (SOD; C) in control, acclimation and direct chilling/freezing treatments with plants belonging to a drought-sensitive clone (ST51) and a drought-resistant clone (CN5) of *E. globulus*. Control treatment was measured at 24/16 °C and acclimation and direct chilling/freezing treatments were measured at 10/6, 10/2 and 10/−2 °C in days 1, 5 and 9, respectively. Data are means  $\pm$  SE ( $n = 4$ ).

In addition, clone CN5 had a higher capability for osmotic regulation either in acclimation or direct chilling/freezing treatments along the progressively lower temperatures (Figure 2B). A decrease in  $\Psi_{\pi}$ , lowering the freezing point of tissues, can decrease the amount of ice formed, and therefore improve the avoidance of freeze-induced dehydration (Sakai and Larcher 1987). Good correlations between  $\Psi_{\pi}$  and frost resistances were found for *Eucalyptus* sp. (Valentini et al. 1990) although not always associated with a significant decrease in the temperature of ice formation but rather to an increased ability to endure extracellular ice formation (Almeida et al. 1994). In parallel with the decrease in  $\Psi_{\pi}$ , there was a significant increase in soluble

sugar concentration in leaf that was more noticeable in CN5 plants. Particularly, CN5 showed a rapid (24 h) increase of soluble sugars in unacclimated plants as compared to ST51 (Table 2).

A strong relationship between soluble carbohydrate accumulation in leaf and cold tolerance has been reported for conifers (Ögren 1997, Greer et al. 2000, Tinus et al. 2000, Repo et al. 2004) and for *Eucalyptus* sp. (Almeida et al. 1994, Leborgne et al. 1995a, 1995b). Furthermore, differences in cold tolerance between genotypes attributed to different carbohydrate metabolism and related to the effects of soluble sugar accumulation in cryoprotection have also been reported (Leborgne et al. 1995a, Bourion et al.

2003). The amount of soluble sugars present corresponds to a balance between the rate of photosynthesis, consumption by respiration and export to parts of the plant that are growing. A higher accumulation of soluble sugars in the drought-resistant CN5 clones despite its lower photosynthetic rates suggests a more efficient reprogramming of carbon metabolism under low temperatures in CN5 than in ST51. In addition, the significantly lower rates of respiration of CN5 clones under chilling temperatures (data not shown) may have contributed to this higher acclimation capacity as it was proposed by Ögren (1997) for several conifers, where sugar consumption led to a significant decrease in freezing tolerance.

Both clones showed similar membrane relative injury when subjected to freezing temperatures ranging from  $-2.6$  to  $-8$  °C (Figure 5). These results also indicate that acclimation resulted in fully acclimated plants as both clones maintained low values of electrolyte leakage until  $-8$  °C. In addition, we can conclude that both clones have the same inherent freezing tolerance before cold acclimation. The observed values of membrane injury of acclimated and unacclimated plants are in accordance with the ones reported in the literature for *E. globulus* (Almeida et al. 1994, Tibbits et al. 2006). Furthermore, given the observed differences in the time course of sugar accumulation between the two clones and its correlation with the development of freezing tolerance, we can speculate that CN5 clones can acclimate more rapidly and present higher tolerance for intermediate levels of acclimation than ST51 clones.

Low temperatures are known to inhibit rates of photosynthesis through limiting the activity of Calvin cycle enzymes. In addition, a light-dependent decrease and slowly reversible retardation in photosynthetic efficiency may occur following low temperature events, a process termed cold-induced photoinhibition. It has been shown that cold-induced photoinhibition and photodamage under high levels of irradiance affect *E. globulus* development after transplanting (Close et al. 2000). Moreover, when the environmental conditions do not promote carbon fixation, even moderate light may lead to high levels of photoinhibition (Govindachary et al. 2004, Close and Beadle 2005).

The reduction in photosynthetic rates caused by low temperatures is strongly influenced by the degree of acclimation of plant material (Weger et al. 1993, Greer et al. 2000, Davidson et al. 2004). In our experiment, there was a significant acclimation effect with acclimated plants maintaining higher net photosynthesis at  $10/-2$  °C than unacclimated plants. However, despite the decrease in photosynthesis with low temperature (Figure 3B) throughout the experiment, none of the clones, either acclimated or unacclimated, exhibited signs of photoinhibition assessed by photochemical efficiency evolution (Figure 4). Thus, *E. globulus* plants do not seem to suffer from cold-induced photoinhibition under moderate levels of light contrary to what has been observed in other species (Govindachary et al. 2004) or in

*Eucalyptus* sp. under high irradiances (Close et al. 2000, 2001, Egerton et al. 2000). Furthermore, mild frost temperatures alone do not seem to cause photoinhibition in *E. globulus* and we can assume that the observed decrease of photosynthetic rate was due to limitations either in stomatal or mesophyll diffusion. In addition, we can conclude that non-photochemical, heat-dissipation mechanisms were sufficient to deal with excess excitation.

Under optimal environmental conditions, light reactions and electron transport in photosynthesis lead to minimal production of ROS, which otherwise can cause photooxidative damage to chloroplasts, carotenoids and proteins. To cope with stress, the plants developed an enzymatic antioxidant defence system, the enhancement of which is often correlated with the acquisition of cold tolerance (Wise 1995, Tao et al. 1998, Verhoeven et al. 2005). In the present study, we examined whether antioxidant enzyme capacity is involved in cold tolerance and whether they differ between drought-resistant and drought-sensitive clones of *E. globulus*.

Acclimation to low temperatures led to similar responses of both clones with significant increases in GR, APX and SOD activity in comparison to control plants. Thus, the combined action of these three enzymes seems to have a protective role against chilling-induced active oxygen species. This enhancement in antioxidant capacity of both clones under low temperatures was not observed under drought stress (Shvaleva et al. 2006), where enzyme activity of leaf was not significantly altered. Nevertheless, the absence of clone differences in leaf antioxidant enzyme activity after full acclimation suggests that differences in cold tolerance between the clones are not associated with antioxidant capacity. On the other hand, we cannot disregard possible differences between the two clones in antioxidant capacities at intermediate levels of acclimation. In fact, the results of direct chilling/freezing treatment after 24 h of cold exposure showed a significant increase in APX activity only in CN5 clones which can consequently result in different antioxidant capacities between the two clones, or at least, suggest different resistance pathways in each clone when unacclimated.

When we compare the responses of both clones to low temperatures with responses to drought from a previous experiment (Costa e Silva et al. 2004, Shvaleva et al. 2006), some common trends arise. In response to low temperatures and to water deficit, the drought-resistant clone CN5 maintained higher leaf water status (higher predawn and midday leaf water potentials) and decreased  $\Psi_{\pi}$  significantly more than the drought-sensitive clone ST51 (Costa e Silva et al. 2004, Shvaleva et al. 2006). In addition, under drought and chilling conditions, CN5 ramets exhibited a lower inhibition of root growth than ST51 (Costa e Silva et al. 2004, 2007). The higher capacity to deliver water to the leaves given by a more extensive root system is an advantageous trait under water deficit conditions. It is worth mentioning that the higher growth rate of ST51



ramets in optimal conditions (Costa e Silva et al. 2004) can also be related to its higher cold sensitivity. In fact, strong tradeoffs often exist between growth and cold hardiness, even if these negative genetic correlations are weak and more variable within a population than among populations (Howe et al. 2003).

In summary, our data indicate that progressively lowering air temperatures to 10/−2 °C (day/night) led to acclimation of both *E. globulus* clones. Acclimated ramets exhibited higher photosynthetic rates and stomatal conductances when compared to unacclimated ramets under cold freezing treatments. Moreover, low temperatures led to lower membrane relative injuries, significant increases of soluble sugars and antioxidant enzyme activity (GR, APX and SOD) of both clones in comparison to plants grown at control temperature (24/16 °C). On the other hand, none of the clones, either acclimated or not, exhibited signs of photoinhibition under low temperatures and moderate light. Although comparing only one pair of genotypes there were consistent differences between them in the responses to low temperatures resulting mainly from the differences in carbon metabolism, including a higher accumulation of soluble sugars in the drought-resistant clone CN5 and a higher capacity for osmotic regulation as compared to the drought-sensitive clone ST51. Although membrane injury data suggested that both clones had the same inherent freezing tolerance before and after cold acclimation, the results also support the hypothesis that the drought-resistant clone had a greater cold tolerance at intermediate levels of acclimation than the drought-sensitive clone. A higher capacity to acclimate in a short period can allow a clone to maintain an undamaged leaf surface area along sudden frost events, therefore increasing growth capacity. Moreover, it can enhance survival chances in frost-prone sites expanding the plantation range with more adaptive clones.

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