

## Resistance to freezing in three *Eucalyptus globulus* Labill subspecies

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The resistance to low temperatures was assessed in seedlings of three subspecies of *E. globulus* (*ssp globulus*, *ssp bicostata* and *ssp maidenii*) of two different provenances. Lethal temperature 50 (LT<sub>50</sub>) was obtained by measuring the electrolytic conductivity, nucleation and freezing temperatures were obtained by thermal analysis and the total soluble carbohydrates concentration was determined through the phenol-sulphuric method. Results showed that the most resistant provenance corresponded to Bolaro Mountain of *ssp maidenii* with a LT<sub>50</sub> lower than -9°C. The provenance Moogora of *ssp globulus*, had a LT<sub>50</sub> of -8.47°C, which situates it, as the second most resistant to low temperatures. According to the nucleation and freezing temperatures, the results indicate that all the provenances analyzed evaded the formation of ice, except for Bolaro Mountain of *ssp maidenii* which was tolerant to freezing. Finally, an inverse correlation ( $r = -0.89$ ) between the content of total soluble carbohydrates and the LT<sub>50</sub> was found, indicating the cryoprotection effect of these in the resistance to low temperatures.

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*Eucalyptus globulus* Labill *ssp globulus* is the second most important specie in forest plantations of Chile, after *Pinus*

*radiata* D. DON, due to its fast growth and fiber quality (Doughty, 2000; Infor, 2004). However, there are some restrictions for its establishment, due to the freezing sensitivity of the young plantations (Skolmen et al. 1990), limiting the lands where it can be cultivated within Chile (Infor, 2004). The survival of this specie during a frost strongly depends on both its constitutive tolerance and acclimatation capacity.

Cold, salt or drought stresses are some of the main environmental factors that strongly affect growth, productivity and plant development (Alberdi and Corcuera, 1991; Nilsen and Orcutt, 1996). According to Larcher (1995) low temperatures can be the direct cause of damages to plant cells, causing a type of water-deficit stress (Griffith and Antikainen, 1996). The resistance or sensitivity to the stress depends on the species, the genotype, and the development age of the plant (Palva et al. 2002).

The resistance to freezing can be defined as the lowest temperature, under the freezing point to which a plant can be exposed without being damaged (Duryea and McClain, 1984). It is frequently defined as the minimum temperature at which half of the seedlings are damaged, and is expressed as lethal temperature 50 or LT<sub>50</sub> (Glerum, 1985;

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Larcher, 1995).

The resistance to frost in seedlings is given by mechanisms of evasion and tolerance (Larcher, 1995; Nilsen and Orcutt, 1996). The first corresponds to a mechanism in which seedlings avoid direct contact of its tissues with freezing temperatures, which means, they avoid the formation on ice in its tissues.

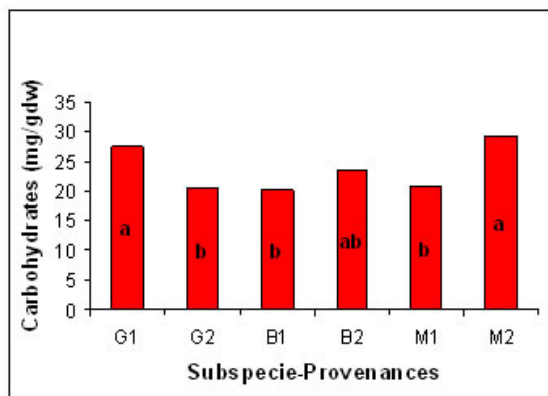


Figure 1. Average values of total soluble carbohydrates.

The tolerance mechanism includes the process that allows the icing in plant tissues without lethal consequences, making this mechanism act constitutively or induced (Larcher, 1995; Nilsen and Orcutt, 1996). Enhancement of freezing tolerance is also accompanied by accumulation of compatible solutes such as soluble sugars and amino acids (Palonen and Junttila, 1999). These solutes are believed to protect cellular membranes and enzymes from irreversible damage caused by freezing.

Therefore, the information that the nursery owner handles is important, specially regarding the external factors that influence or are involved in cold-resistance, as well as the internal physiological and biochemical changes that are associated to low temperature resistance in plants. This is basically related to the freezing produced at intra and intercellular level and the resulting injury (Glerum, 1985).

In Chile, attending to the demands of the pulp industries, numerous alternatives have been investigated to overcome the freezing sensibility of *E. globulus*. Among these are identification of cold hardening genotypes of *E. globulus*, or the plantation of alternative species which are more cold

resistant. However, a possible alternative that has not been studied, it is the assessment of frost tolerance and the mechanisms involved in different subspecies of *E. globulus*. The subspecies *bicostata* and *maidenii* are of special interest, since they have been described by some authors as more resistant to frosts (Skolmen et al. 1990), and have a similar pulp yield to the subspecies *globulus* (Resquin, 2003).

The aim of this work is to assess the resistance to freezing and understand the mechanism used by plantlets of the subspecies *bicostata*, *globulus* and *maidenii*, of two different seed provenances from Australia. In addition, the total soluble carbohydrate concentration in plantlets was analyzed.

## MATERIALS AND METHODS

### Materials

Three of the four existing subspecies of *E. globulus* were used in this study. Each subspecies analyzed was obtained from seeds from two provenances of Australia (Table 1), which gave origin to plants produced in root covered and open sky conditions. Material tested was 9 months of age. Previous to the test, plantlets were hardened by a process that consisted of apical and lateral pruning, in addition to water stress (irrigation each 4 days, 40% container capacity).

### Determination of freezing resistance

LT<sub>50</sub> was determined by electrolytic conductivity, as described (Raymond et al. 1986). Leaf discs of 8 mm diameter were transferred to a cold air chamber and analyzed at temperatures of -3, -4.5, -6, -7.5 and -9°C. The electrolytic conductivity was recorded, and by interpolation the LT<sub>50</sub> was obtained. The nucleation and freezing temperatures were determined by thermal analysis slightly modified from the one described by Quamme (1991).

### Determination of the total soluble sugars

Carbohydrate concentration was determined through the phenol sulphuric method, for which leaf samples (0.3 g) were used. The absorbance was measured with a spectrophotometer at 485 nm. In order to obtain the values of the sugar concentrations a calibration curve was used.

Table 1. Background of the subspecies of *Eucalyptus globulus* Labill.

Subspecies	CSIRO code	Assay code	Provenance	State
globulus 16470		G1	Moogora	Tasmania
globulus 16417		G2	N Cape Banan Island	Tasmania
bicostata 16370		B1	M T Strathbogie	Victoria
bicostata 17965		B2	Tumbarumba	N.S. Wales
maidenii 17745		M1	Yurammie SF	N.S. Wales
maidenii 17769		M2	Bolaro Mountain	N.S. Wales

## Design and statistical analysis

The experimental design used was random simple for resistance to freezing and total soluble sugars, but in resistance to freezing was determined with bifactorial model (temperature and provenance) and 8 seedlings by treatment. For the analysis of the results a test of significance for the comparison of the averages of the treatments through the test of Tukey was made ( $\alpha = 0.005$ ).

## RESULTS AND DISCUSSION

The freezing resistance varied in a range between  $-7.1^{\circ}\text{C}$ , for the less resistant plants up to less than  $-9^{\circ}\text{C}$ , for the most resistant ones. In Table 2, the values of  $LT_{50}$ , nucleation and freezing temperatures are shown.

The provenance Bolaro Mountain of *ssp maidenii* with a  $LT_{50}$  below  $-9^{\circ}\text{C}$  was the most resistant sub-specie to freezing in this study. The Moogora provenance of *ssp globulus*, had a  $LT_{50}$  of  $-8.5^{\circ}\text{C}$ , was classified as the second most resistant sub-specie to freezing, and with a resistance level greater than the one commonly mentioned in the literature.

In Figure 1, the differences in the total carbohydrate concentration is shown, for the different subspecies from different provenances (intraspecific variation), being significant for the subspecies *globulus* and *maidenii*.

Also, an inverse correlation between the total soluble carbohydrate content and the  $LT_{50}$  ( $r = -0.89$ ) was found. The same is mentioned by Johnson and Cline (1991).

An approach being used in several countries is to study eucalypts hybrids for use in plantation forestry to incorporate frost tolerance into marketable species. Scott et al. (2002) made five large field trials at four altitudes in Tasmania, southern Australia to measure performance of *E. gunnii*, *E. globulus*, their hybrids and *E. nitens*, the species currently used at higher altitudes. Their results showed that *E. gunnii* x *E. globulus* F1 hybrids did not grow as rapidly as *E. nitens* and were more damaged by possums. This means that *E. nitens* should generally remain the species of choice for forestry at high altitudes in southern Australia.

In our study, three different sub-species of *E. globulus*, from two provenances were assessed. Hardened seedlings

of all subspecies should have a higher resistance to low temperatures than in natural conditions. This would indicate that the process of hardening in nursery can induce a resistance equal or superior to the naturally more resistant species.

By the results obtained in  $LT_{50}$ , the process of plantation in zones with incidence of low temperatures (up to  $-7^{\circ}\text{C}$ ), should not to be a problem for plants properly hardened of any of the analyzed provenances, especially for Moogora and Bolaro Mountain. According to the nucleation and freezing temperatures, the results indicate that all the analyzed provenances evade the formation of ice in their leaves, suggesting that supercooling is the most probable mechanism conferring resistance to these plants. This is not observed in the provenance Bolaro Mountain of *ssp maidenii* which did tolerate freezing.

Almeida et al. (1995) tested some provenance trials of *E. globulus* in Portugal and observed that the effect of provenance is significant, especially on the sites with more unfavourable conditions and their results showed that *ssp bicostata* and *ssp maidenii* had a higher frost tolerance overall, and within the *ssp globulus*, provenances from regions where the subspecies is an exotic are more frost tolerant than the Australian provenances. These results agree with those found in our study, since there were differences within two provenances tested of each subspecies. For example, in the *ssp maidenii*, a provenance was more resistant to low temperatures, whilst the other had one of the lowest value of  $LT_{50}$ .

According to the results obtained for total soluble carbohydrate content, it is a good indicator of the resistance to low temperatures. If these results were correlated with the  $LT_{50}$ , an inverse relationship for all the provenances can be observed, indicating that as the level of soluble carbohydrates increases in the cell, they are able to resist lower temperatures, supporting the theory that soluble sugars function as cryoprotectants in plant tissues, especially at membrane level. This agrees with the results of Tinus et al. (2000) that found a near relationship between cold hardiness and the absolute concentration of soluble sugars.

Similar results were found, when treating callus cultures of wheat genotypes differing in frost tolerance (Kerepesi et al. 2004). On the other hand, Zuther et al. (2004) observed that

Table 2. Values of  $LT_{50}$ , nucleation and freezing temperatures.

Lot ssp	$LT_{50}$ ( $^{\circ}\text{C}$ )	Nucleation temperature ( $^{\circ}\text{C}$ )	Freezing temperature ( $^{\circ}\text{C}$ )
G1 ( <i>globulus</i> )	-8.47	$-8.76 \pm 0.29$	$-5.46 \pm 0.22$
G2 ( <i>globulus</i> )	-7.68	$-8.08 \pm 0.29$	$-5.61 \pm 0.18$
B1 ( <i>bicostata</i> )	-7.10	$-8.03 \pm 0.20$	$-5.34 \pm 0.44$
B2 ( <i>bicostata</i> )	-8.01	$-8.37 \pm 0.40$	$-5.02 \pm 0.17$
M1 ( <i>maidenii</i> )	-7.10	$-7.16 \pm 0.35$	$-4.35 \pm 0.49$
M2 ( <i>maidenii</i> )	< -9	$-3.77 \pm 0.33$	$-1.78 \pm 0.29$

raffinose did not have a direct effect on basic freezing tolerance or cold acclimation when using either transgenic lines or mutants of *Arabidopsis*. Dalmannsdottir et al. (2001), studied white clover in relation to frost tolerance, using different cultivars. They found that sucrose was the most abundant water-soluble carbohydrate, but during the month of September (spring) populations had similar levels of this carbohydrate, which decreased in January (winter) in one cultivar, the most frost tolerant, concluding that the selected population was more frost and ice-encasement than the original population in autumn. The same is found by Traver et al. (1997); they used cell-suspension culture hardened by exposure to lower temperatures obtained from hybrid of *Eucalyptus* spp. During the cold exposure the resistant cells accumulated soluble sugars, in particular fructose and sucrose, with a limited increase in cell osmolality. In contrast, the cell suspension that was unable to acclimate did not accumulate soluble sugars in response to the same cold treatment. Also Leborgne et al. (1995) made a comparison of soluble sugar content in various cell lines of *E. gunnii* exhibiting different freezing resistances revealed that the most resistant cell line contained the highest soluble sugar content.

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