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Nathalie Leborgne · Chantal Teulières
Bertrand Cauvin · Sébastien Travert
Alain Michel Boudet

Carbohydrate content of *Eucalyptus gunnii* leaves along an annual cycle in the field and during induced frost-hardening in controlled conditions

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Abstract The annual changes in frost hardiness were studied for three *Eucalyptus gunnii* genotypes. Frost resistance evaluated on leaf discs by the electrolyte leakage method reached a maximum in the coldest period and a minimum in summer demonstrating winter frost hardening. Genotype 634 exhibited a higher intrinsic resistance than the other genotypes both in the hardened and in the non-hardened stages. Plants of this genotype were also frost acclimated in controlled conditions by a progressive decrease of culture temperature (25 to 0 °C) but the degree of hardening appeared to be lower in these conditions. The carbohydrate patterns in leaves varied with acclimation. In controlled conditions the leaves of genotype 634 exhibited a rise in sucrose, fructose and raffinose concentration up to a temperature of 10 to 7 °C which subsequently decreased. In natural conditions a comparison of the three genotypes allowed us to correlate the higher intrinsic resistance of genotype 634 to a higher soluble sugar content. During acclimation fructose and raffinose changes were also correlated to an increase in cold resistance even though the kinetics of these changes differed in controlled and natural conditions. The starch content was very low in the various genotypes in the different conditions but oligosaccharides such as stachyose and possibly verbascose were detected. The results point out the relationships occurring between increased frost resistance and changes in fructose and raffinose concentration in *E. gunnii* leaves.

Key words Carbohydrates · *Eucalyptus gunnii* · Frost resistance · Hardening

N. Leborgne (✉) · C. Teulières · S. Travert · A. M. Boudet
Signaux et Messages Cellulaires chez les Végétaux,
URA CNRS n° 1457, Centre de Biologie et Physiologie Végétales,
Université Paul Sabatier, 118 route de Narbonne, F-31062 Toulouse,
France

B. Cauvin
Afocel région-Sud, 98 route de Tournefeuille, F-31270 Cugnaux,
France

Introduction

In the field, perennial plants become more resistant to low temperatures when progressively exposed to decreasing temperatures during autumn, a natural process known as cold hardening or cold acclimation (Levitt 1980). This process, which can also be artificially achieved by manipulating the conditions of culture, is a complex adaptative phenomenon correlated to many biochemical and physiological changes such as the accumulation of compatible osmolytes in the cell. For instance, plant acclimation to freezing temperatures is often paralleled by an accumulation of carbohydrates sometimes derived from starch hydrolysis (Sakai and Larcher 1987). In trees relationships between carbohydrate changes and cold acclimation have already been reported (Pomeroy and Siminovitch 1970; Sauter and Kloth 1987; Bonicel et al. 1987; Alberdi et al. 1989; Fischer and Höll 1991; Stushnoff et al. 1993). These carbohydrates could prevent intracellular ice formation by decreasing the freezing-point of intracellular fluids but could also act as cryoprotectants of cell structures and more particularly could stabilize the membranes (Santarius 1973) through interactions with the polar head groups of phospholipids (Anchordoguy et al. 1987).

Forty years ago, the first attempt was made to introduce *Eucalyptus* to France, an evergreen woody plant exhibiting high quality fibers and fast growth, two characteristics which make this genus particularly interesting for the pulp industry. *E. gunnii* is the most frost resistant species among several hundred species of the genus *Eucalyptus* (Potts et al. 1987). Some clones can survive at −17 °C. Nevertheless, the expansion of *E. gunnii* plantations in some southern regions of France is still limited by low winter temperatures.

In order to improve the frost resistance of this species we are engaged in a research program aiming to understand the biochemical and cellular mechanisms involved in its adaptation and survival at low temperatures.

In this paper we report the natural hardening and dehardening of different *E. gunnii* genotypes and the

corresponding seasonal changes in soluble carbohydrates in the leaves. In addition, for the most resistant genotype, we have investigated the effects of an artificial hardening on frost resistance and on soluble carbohydrate and starch content.

Materials and methods

Plant material

Trees in the field

The experiments were performed on 5-year-old *E. gunnii* trees originating from cuttings and grown in the south-west of France on an AFOCEL experimental field (Cugnaux, altitude 163 m above sea level, latitude 43°32′ N, longitude 1°22′ E).

Three selected genotypes coming from seeds collected in different areas of Tasmania were studied: 870634 from Breona, 870622 from Mont Ruffus and 870626 from Pine Lake. Apices and leaves (first and third rank) were collected once a month from October 1992 to September 1993 (except in May and June) between 2.00 and 4.00 pm from the same tree. One part of the sample was used directly for the measurements of frost resistance with the electrolyte leakage method and the other part immediately frozen in liquid nitrogen and stored at −80 °C for carbohydrate studies. The apices include 4–6 small curled up leaves.

The daily minimum air temperatures were communicated by the French weather forecast from a station located nearby at Cugnaux-Francazal.

Plants in controlled conditions

Six-month-old plants of *E. gunnii* genotype 870 634 originating from cuttings were transferred from the greenhouse to a controlled chamber at 25 °C (45 W. m^{-2}, 14 h day/10 h night) 2 weeks before the experiments and were then grown at decreasing temperatures. Cold acclimation was achieved by decreasing the temperature weekly (25, 16, 13, 10, 7, 4, 0 °C) over 7 weeks with a constant photoperiod. The experiment was performed on ten plants. Every week, 30 leaves were harvested from one of the three main branches of three different plants 6–8 h after the beginning of the *hemero* period.

Methods

Determination of frost resistance of trees

Frost resistance was evaluated by the electrolyte leakage method (Cauvin 1988). Ten leaf discs (8 mm diameter) from the third rank leaves from the apex (for trees in the field or plants in controlled conditions) were first immersed in 25 ml deionized water in a 50-ml test tube for 16 h. At that time, the starting conductivity (SC) was measured with a conductimeter (digital conductimeter consort K520 Bioblock scientific). After pre-incubation of the different tubes for 30 min at 0 °C in a cryostat (Lauda RKP 20), the temperature was decreased at the rate of 2 °C/h and samples were collected at −12 and −15 °C (in our conditions freezing occurred at −9 to −10 °C). Then the samples were thawed at room temperature in the dark for 16 h and the conductivity was measured again (test conductivity: TC). For the final conductivity measurement (FC) the tubes were placed at −80 °C for 24 h and then transferred to room temperature in the dark for 24 h. The relative conductivity (RC) equalled TC-SC/FC-SC.

Soluble sugar extraction and analysis

Five hundred milligrams of fresh material was ground with pestle and mortar in the presence of 50 mg PVPP for 1 min at 4 °C. The sugars were then extracted from the ground sample with 10 ml deionized water containing 0.6% polyethyleneglycol 6000 (PEG). The supernatant was collected after centrifugation at 5000 × g for 15 min. The samples were stored at −20 °C before measurement.

The individual sugar analysis was performed using a DIONEX chromatographic unit including an anion-exchange column [CarboPac AS-6 PA1 (4 × 250 mm)] equipped with a precolumn (CarboPac PA1 10-32). The samples were filtered through a 0.22 µm filter, diluted (5 or 10 times) and injected into a separating column. Elution was performed at room temperature for 12 min with 150 mM NaOH (70 bar pressure, 1 ml/min flow rate). Sugars were detected by an amperometrical method and the results were analysed by computer (AI450, Dionex).

Starch extraction and measurement

The solid starch-containing centrifugation pellet, resulting from the treatment of 2 g of fresh material with the extraction soluble sugars procedure, was washed twice with 20 ml absolute ethanol and then lyophylised for 24 h. One hundred milligrams of residue was resuspended in 2 ml 100 mM acetate buffer at pH 4.5 containing 1% PEG 6000 and 5 mM DTT and autoclaved for 20 min at 120 °C. The resulting suspension was centrifuged at 5000 g. Supernatant (400 µl) was hydrolysed with 4 units of amyloglucosidase (EC 3.2.13, Sigma) in 600 µl 100 mM acetate buffer at pH 4.5 containing 2% PEG and 10 mM DTT for 1 h at 55 °C. The reaction was stopped at 100 °C for 4 min and then the medium was cooled on ice. Released glucose was measured spectrophotometrically (λ 585 nm) with the "glucose Trinder Sigma kit" in 15 µl supernatant.

Results

Hardening in the field

In the south-west of France winter 1992–1993 was particularly mild, temperature minima (−3.6 and −5.8 °C) were observed in early January and late February (Fig. 1A). Between these minima, the air temperature returned to warm.

The relative conductivity (RC) due to electrolyte leakage from leaf discs of the three genotypes was measured during the year at two artificial freezing temperatures (−12, −15 °C; Fig. 1B–C). A high conductivity is indicative of significant cold injury to cell membranes and therefore of high cold sensitivity.

From July to October, the electrolyte leakage was globally high indicating that all the trees were very cold-sensitive during that time which corresponds to a "non-acclimation period". Electrolyte leakage was always lowest for genotype 634 scored as the most frost tolerant in the field by AFOCEL. Genotype 634 was called frost resistant and the other two frost sensitive.

In mid-autumn the frost resistance of all the trees increased significantly before the occurrence of the lowest temperatures. This natural frost hardening was very intense, with a decrease in RC of up to 60% being observed. The speed and magnitude of acclimation were similar for the three genotypes. The clones do not differ by the acclimation capacity since the same differences in conductivity (frost tolerance) between trees were observed along the acclimation and deacclimation periods. Nevertheless, following an increase of temperature at the end of January a partial deacclimation was observed for the sensitive clones while the tolerant clone did not significantly lose its tolerance.

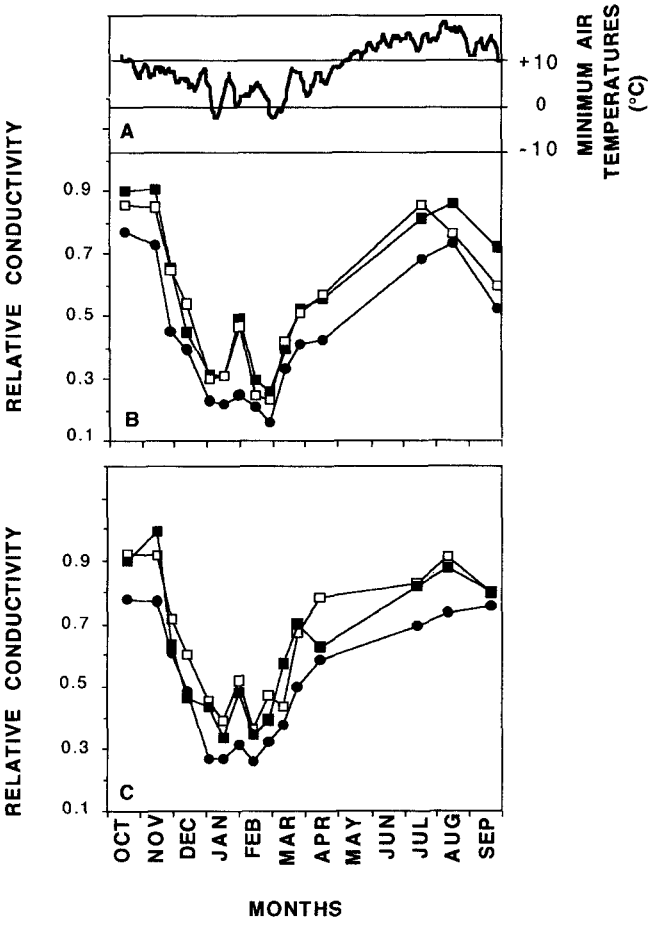


Fig. 1 Changes in frost resistance of trees during one year (□ 626, ■ 622, ● 634). The minimum air temperatures were measured daily by French Meteorology (**A**). Foliar relative conductivity was measured at –12 °C (**B**) and –15 °C (**C**)

During spring, when temperatures progressively increased, the three trees dehardened in the same way.

Acclimation in a controlled chamber

The acclimation of plants from genotype 634 (resistant) in controlled conditions was studied with the same method of electrolyte leakage (Fig. 2). For the control maintained at 25 °C, the relative conductivity was higher than for the non-acclimated trees grown in the field. The age of the plants (5 years for the trees and 6 months for the cuttings grown in controlled conditions), which has already been shown to influence cold tolerance (Battaglia and Reid 1993), could explain this difference. During the controlled cooling acclimation was progressively attained with decreasing temperature (from 25 to 0 °C) but to a lesser extent than the hardening observed in the field: only a 20% decrease of RC was obtained.

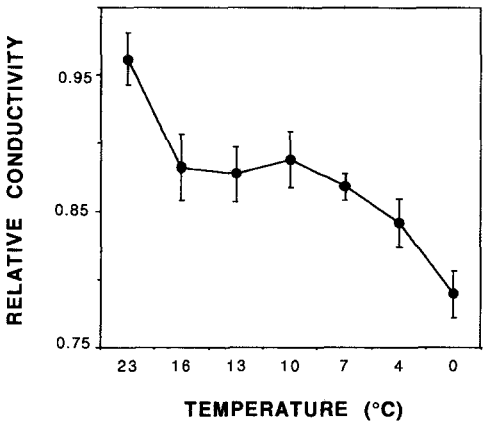


Fig. 2 Effect of cold acclimation in controlled conditions on freezing tolerance (–12 °C) of *Eucalyptus gunnii* plants of genotype 634. Freezing injury was estimated by determining relative conductivity of leaf discs. Each experiment was carried out in triplicate. *Error bars* indicate the standard deviation of the mean

Individual sugar content

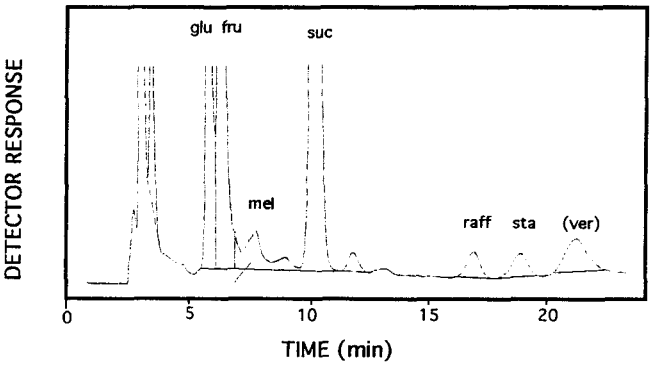
Individual soluble sugar concentrations were measured in apices and leaves of trees in the field along the year (with an interruption in May and June) and in leaves of plants in controlled conditions. A chromatogram showing the different carbohydrates identified in the leaves of the studied plants is shown Fig. 3.

The results obtained will be successively presented for the plants in controlled conditions, for which the environmental parameters were strictly controlled and for the trees in natura which face more complex climatic changes.

Plants of genotype 634 in controlled conditions

Among levels of the major carbohydrates, the glucose content of leaves did not exhibit significant variations during hardening in controlled conditions. In contrast, three other carbohydrates: fructose, sucrose and raffinose exhibited marked changes and very similar patterns. Their

Fig. 3 Soluble carbohydrate pattern from *E. gunnii* first leaves (genotype 634, February 1993) after chromatographic separation. *glu*: glucose, *fru*: fructose, *suc*: sucrose, *raff*: raffinose, *sta*: stachyose, *ver*: verbascose presumed



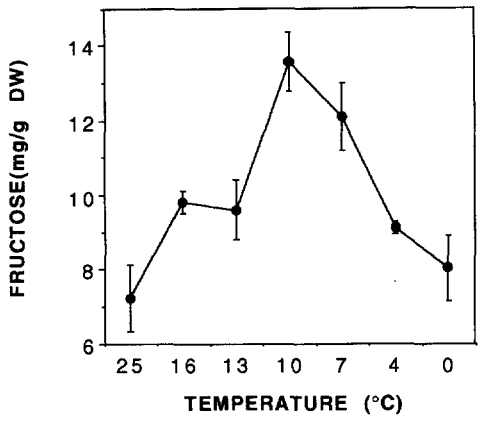


Fig. 4 Fructose content in leaves of genotype 634 *Eucalyptus* plants during a controlled acclimation

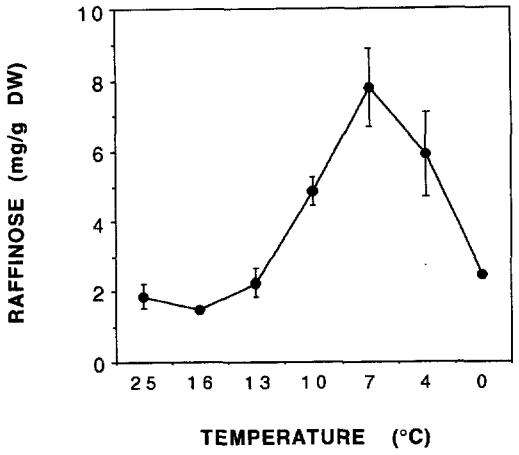


Fig. 6 Raffinose content in leaves of genotype 634 *Eucalyptus* plants during a controlled acclimation

concentrations increased with hardening up to 10 or 7 °C, depending on the sugar, and then decreased (Figs. 4–6).

The increase in sugar concentration was maximum for sucrose when absolute values are considered and maximum for raffinose when relative values are considered (the raffinose content rose 4-fold).

Stachyose was present at a maximum concentration during the non-acclimated stage (15 mg/g DW) and dropped at the beginning of acclimation and then increased again (data not shown). An additional sugar, tentatively identified as verbascose increased with hardening to 10 °C (2.5 mg/g DW) and then decreased. In the same way as fructose and raffinose the sum of the most abundant carbohydrates increased during controlled hardening to a maximum at 10 °C and then decreased to the initial level (Fig. 7).

Trees in the field in natural conditions

Beyond the changes in sugar content correlated to acclimation, this study allowed us to detect the changes associated with deacclimation and to perform comparative investigations on three genotypes exhibiting different frost resistances. For these plant materials the patterns observed are only partly similar to those previously described for the plants in controlled conditions. Thus, no significant differences in glucose content were detected through the year for the different genotypes (data not shown). The glucose content of leaves was however slightly higher during summer and mid-autumn. No accumulation was observed during hardening.

Concerning the other sugars, fructose, sucrose and raffinose, the trends observed in controlled conditions are not systematically reproduced, each individual sugar presenting specific time course changes during the year.

The fructose content was higher most of the year in the resistant genotype compared to the other two. This phenomenon was very clear for frost-sensitive organs: apices (Fig. 8), but also first rank leaves (data not shown). For the three genotypes, the fructose content was maximum during the winter when acclimation is acquired and also in summer and early autumn.

Fig. 5 Sucrose content in leaves of genotype 634 *Eucalyptus* plants during a controlled acclimation

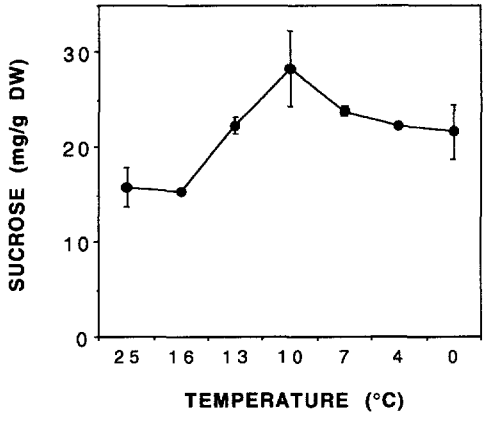
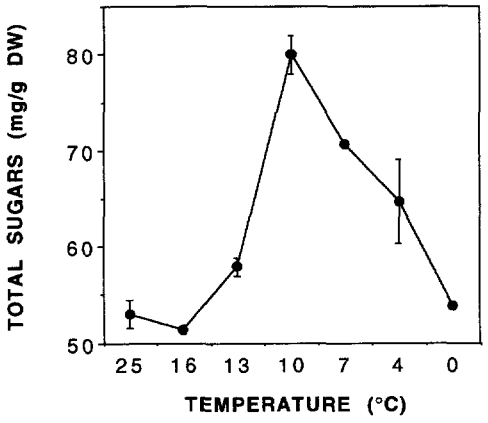


Fig. 7 Sum of the main soluble sugar content in leaves of genotype 634 *Eucalyptus* plants during a controlled acclimation



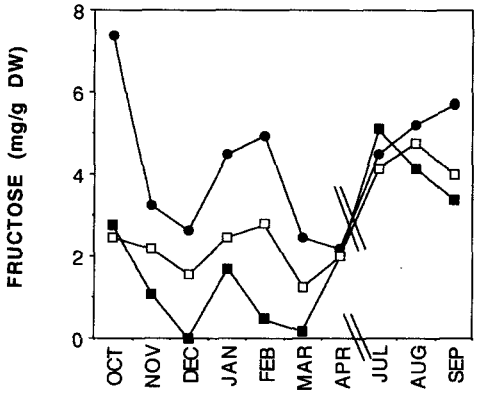


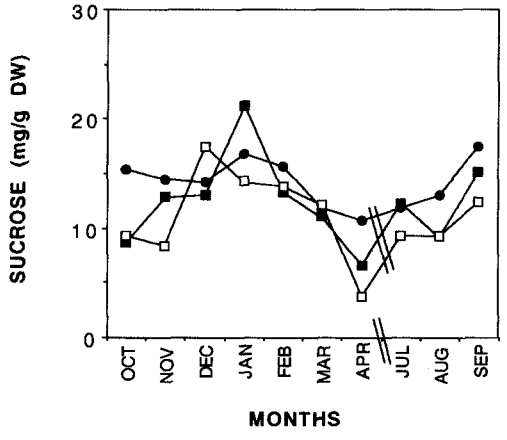
Fig. 8 Fructose content in apices of *E. gunnii* tree in nature. □ 626, ■ 622, ● 634

Sucrose was the most abundant carbohydrate in leaf tissues for the three *E. gunnii* genotypes (Fig. 9). The sucrose content changes in apices from the resistant tree (634) were very limited. In contrast, in 622 and 626 genotype apices, a rise in sucrose concentration in winter and a drop in April were observed. In first and third leaves the seasonal changes were identical for the three genotypes (data not shown).

In apices of trees grown in the field, raffinose was only found in the 634 resistant genotype (Fig. 10). In first rank leaves raffinose was also detected in the two other genotypes but at a lower concentration when compared to the resistant genotype. For both organs the raffinose content was maximum in October and then dropped dramatically during the hardening period of the resistant genotype to reach a low level during spring. In the first leaves of the other genotypes the raffinose concentration increased during winter.

When the sum of all these most representative sugars was plotted through the year, it was particularly interesting to notice that in apices the total sugar content was highest in the resistant tree particularly during early autumn (Fig. 11). The same difference, but to a lesser extent, was observed in

Fig. 9 Sucrose content in apices of *E. gunnii* tree in nature. □ 626, ■ 622, ● 634



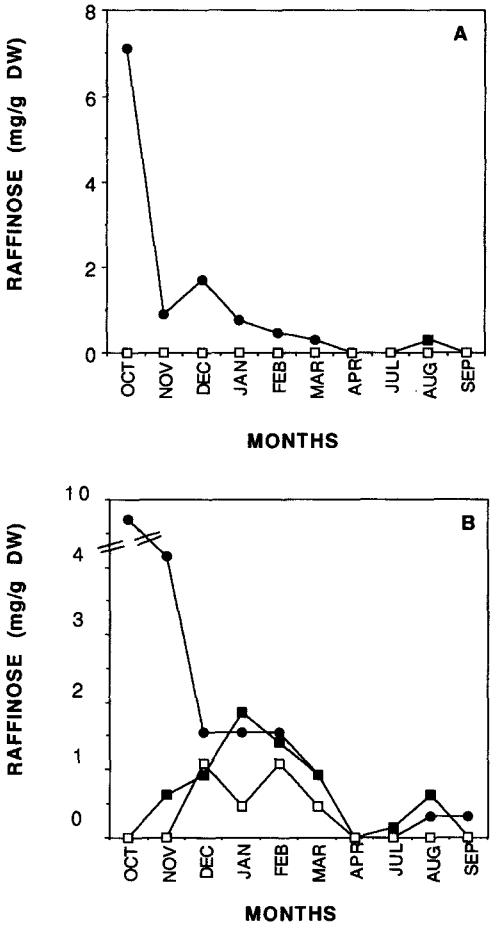
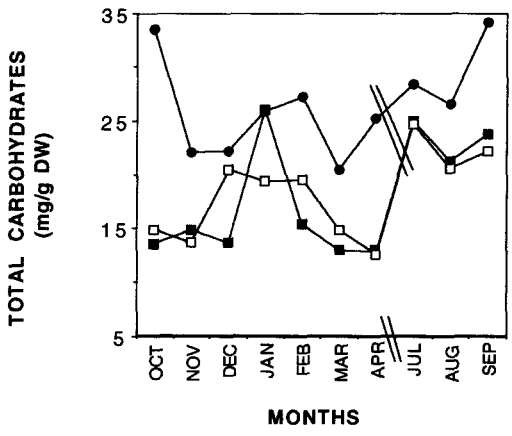


Fig. 10 Raffinose content in apices (**A**) and in first rank leaves (**B**) of *E. gunnii* trees in nature. □ 626, ■ 622, ● 634

first rank leaves (data not shown). Stachyose content was maximum (7 mg/g DW) during the non-acclimated stage. It dropped at the beginning of acclimation (data not shown).

The additional sugar, presumed to be verbascose, was detected during the winter months particularly in the organs

Fig. 11 Seasonal changes of the total main soluble carbohydrate content in the apices from three clones of *E. gunnii*: □ 626; ■ 622; ● 634. The dry weight was calculated from an aliquot of lyophilised fresh material



from the resistant genotype (data not shown). During summer this polysaccharide was found in the three genotypes, in first leaves in July and third leaves in August.

Starch

A very low starch concentration without significant variations during environmental changes was observed in *E. gunnii* plants both grown in the field or in the controlled chamber (1 to 4 mg/g DW). This level is very low compared to other *Eucalyptus* species such as *E. globulus* (up to 60 mg/g dry weight; S. Travert personal communication).

Discussion

In order to correlate carbohydrate changes with cold acclimation for different *E. gunnii* genotypes, we investigated the acclimation-deacclimation patterns and the corresponding sugar profiles in natural and controlled conditions. In all our experiments frost tolerance was estimated using the electrolyte leakage method. This procedure has been used to measure frost tolerance in many plants including *Eucalyptus* (Raymond et al. 1986; Cauvin 1988; Hallam and Tibbits 1988) and was shown to be correlated to leaf damage (Tibbits and Reid 1987).

In contrast to other evergreen trees such as gymnosperms, the evergreen *Eucalyptus* does not have true dormancy. Keeping intact leaves through winter, it grows at any time when environmental conditions are propitious. When frost resistance was measured through electrolyte leakage for trees in the field the three studied genotypes always behaved differently, the leaves of the genotype known as the most tolerant showing the lowest conductivity (best tolerance) through the year. This difference, already detected during the summer in the absence of any hardening, shows the involvement of an intrinsic component (capacity to resist without acclimation) in frost tolerance of this species. These data are in agreement with previous results published by our group showing different levels of frost resistance for cell suspension cultures grown at 23 °C originating from different genotypes (Teulières et al. 1989). Intrinsic resistance differences within *Eucalyptus* species have also been reported by Hallam and Reid (1988) on *E. delegatensis* and Raymond et al. (1992) on *E. nitens*. These differences were particularly obvious when genotypes from different geographical origins were compared as in our case. The three genotypes exhibited a high RC, indicative of frost sensitivity during the summer in the field or with warm temperature conditions in a controlled chamber. A dramatic decrease of RC (increase of tolerance) was observed either in the field when temperature was naturally decreasing or in a controlled chamber during a cooling programme. Our experiments, performed in controlled conditions, showed that changes in temperature alone (with a constant photoperiod and humidity) are sufficient to induce partial cold acclimation. However, the hardening was less intense than in nature. This difference could be due to the timing of the temperature decrease and/or to the involvement of additional factors in natural conditions. Cold acclimation, a general phenomenon in woody plants, has been already observed for other Eucalyptus species (e.g. Hallam and Reid 1988). According to Eldrige (1969) short photoperiods are not required for cold hardening of *Eucalyptus* species and Paton (1982) points out that *Eucalyptus* is essentially sensitive to temperature and hygrometry changes.

The characteristics of acclimation and deacclimation (speed and magnitude) and therefore the capacity of acclimation looked very similar for the different genotypes. These data differ from observations on other *Eucalyptus* species showing both an intrinsic difference and an acclimation ability difference in controlled conditions (Almeida et al. 1994), or in nature (Hallam and Reid 1988; Scarascia-Mugnozza et al. 1989). This behaviour of *E. gunnii* genotypes compared to the data obtained on other species of the same genus shows once again how complex are the mechanisms of cold tolerance.

The three genotypes studied behaved differently towards partial dehardening when a short increase of temperature occurred in nature at the end of the acclimation period. The resistant plant did not then lose its induced tolerance in contrast to the sensitive plants. This observation is particularly interesting as it is widely accepted that low temperatures both prior to hardening and after dehardening are more damaging for the trees (Koski 1985; Tibbits and Reid 1987). This reduced dehardening response could be another factor of tolerance for the resistant genotype, especially in this species with no real stoppage of growth.

The second part of the study dealt with the carbohydrate content changes through the year and with different culture conditions. We will discuss successively (1) the correlations between the carbohydrate pattern and the intrinsic resistance which were deduced from the comparative study of the three genotypes, and (2) the correlations between the carbohydrate changes and acclimation in controlled and natural conditions.

In the most resistant genotype (634) three main characteristics of the carbohydrate pattern can be underlined: a higher content in total soluble sugars which was observed all through the year, a higher content in fructose during fall and winter and finally the exclusive (apices) or preferential (leaves) occurrence of raffinose. Sucrose content was not significantly different in the three genotypes and can hardly be related to mechanisms explaining the differential resistance of the studied genotypes. Previous data from the literature have demonstrated correlations between fructose or raffinose content and frost resistance. Alberdi et al. (1989) showed that the fructose content was higher in the resistant *Nothofagus* growing at high altitude than in sensitive trees, and Rybka (1993) showed a higher accumulation of fructose in resistant winter wheat seedlings compared to sensitive ones during cold acclimation. It has also been reported that an invertase activity increased in plants subjected to low temperatures (Graham and Patterson 1982; Tronsmo et al. 1993). In a study comparing four

conifers and in agreement with our results, foliar concentration of raffinose was also reported to be higher in the most resistant species (Hinesley et al. 1992). Castillo et al. (1990) found an increase in galactinol synthase activity (enzyme allowing RFO precursor synthesis) in leaves of soybean and kidney bean when plants were exposed to low temperatures.

We also evaluated the carbohydrate changes occurring during acclimation since induced resistance (hardening) may involve mechanisms independent of and different from those of intrinsic resistance as already shown for the potato (Stone et al. 1993).

In controlled conditions the induction of acclimation was correlated with an increase in total soluble sugars mainly due to the accumulation of individual sugars such as sucrose, fructose and raffinose. These different sugars behave in the same way, their concentrations increase to an acclimation temperature of 10 to 7 °C and then decrease. The increase in total soluble sugars we observed at the beginning of induced acclimation has already been reported for woody species: *Nothofagus dombeyi* (Alberdi et al. 1989), citrus trees (Yelenoski and Vu 1992) and different *Eucalyptus* species (Almeida et al. 1994).

The decrease in soluble sugar content observed when acclimation reaches its maximum was not really expected. This phenomenon, suggesting either catabolism of the sugars, a binding or incorporation into cell components or translocation to other organs has also been reported in other species such as citrus (Yelenoski and Vu 1992) or *Arabidopsis thaliana* (Ristic 1993). Moreover during the cold acclimation of *E. gunnii* cell suspensions the same phenomenon was observed (N. Leborgne et al. 1995).

The results obtained in the field are more difficult to interpret particularly from a kinetic point of view, owing to the complexity of the changes in environmental parameters and the potential translocation and exchanges of sugars in different parts of the tree. However, they reflect the actual carbohydrate pattern of a specific tissue at a given time. A comparison of results obtained on trees grown in the field and on plants hardened in controlled conditions is important to facilitate the interpretation of the data and to identify the common changes in both conditions. In natural conditions it is possible to observe a slight rise in sucrose concentration during the winter period. An increase in sucrose levels during winter has been observed for different woody plants such as peach (Lasheen and Chaplin 1971), *Populus euramericana* (Jourdan 1980) and cloudberry (Kaurin et al. 1981).

Fructose and raffinose exhibit complex patterns. The concentrations of these sugars, which appear to be correlated to the intrinsic resistance of genotype 634, dropped during the acclimation period (October–December) and this trend was particularly obvious for the resistant genotype. This decrease in sugar concentrations contradicts results obtained during the beginning of acclimation in controlled conditions. However, it agrees with the observed results during the second phase of acclimation in controlled conditions for which a decrease in sugar content was detected. It is possible to suggest that these sugars are synthetised during an early period of acclimation and then converted or used for inducing increased resistance. Depending on the conditions (natural or controlled environmental changes) the rise in fructose and raffinose concentrations could show different patterns.

From the data in the literature, the kind of carbohydrates accumulated in winter or during acclimation appears to be specific to each plant studied. Consequently, it is difficult at the moment to assign a general role in cold resistance to a particular carbohydrate. Our data demonstrated at least a correlation between the accumulation of fructose and raffinose and frost resistance in *E. gunnii*. Determinations performed in parallel on cell suspension cultures from different *E. gunnii* genotypes have confirmed a correlation between fructose content and frost hardiness (N. Leborgne et al. 1995). In addition, experiments aiming to provide different exogenous sugars to *Eucalyptus* cells or protoplasts demonstrated the protective effect of fructose and raffinose against freezing damage (N. Leborgne et al. 1995). In a more general context, raffinose and sucrose have been suggested to be primary protectants against frost in plants (Levitt 1980). They would particularly be involved in the stabilization of membranes (Koster and Lynch 1992). For Caffrey (1988) raffinose plays a protective role for membrane lipids by inhibiting sucrose crystallization during extracellular ice formation in desiccated seeds.

All together, our data obtained in natural and experimental conditions underline the potential role of fructose and raffinose in the frost resistance of *Eucalyptus*. We are now studying the activity of key enzymes of carbohydrate metabolism and the expression of the corresponding genes during acclimation to identify potential targets for future genetic engineering experiments in order to improve frost resistance in *Eucalyptus*.

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