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Leaf anatomy, chloroplast organization and photosynthetic rate of hyperhydric *Eucalyptus saligna* Sm. material

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Morphological, anatomical and ultrastructural differences between leaf tissues of field-grown and normal and hyperhydric *in vitro*-cultured *Eucalyptus saligna* were investigated. Hyperhydric material showed abnormal, often discontinuous development of the epidermis and cuticle. Stomata were malformed. The leaf lamina appeared thickened and was characterized by poor differentiation between the palisade and spongy mesophyll. The chlorophyll content of hyperhydric material was significantly less than that of field-grown and normal cultured material. Hyperhydric leaves had a significantly lower chloroplast number per cell and chloroplasts showed reduced thylakoid stacking. The gross photosynthetic rate of hyperhydric tissue was less than that of normal cultured material.

Morfologiese, anatomiese en ultrastrukturele verskille tussen *Eucalyptus saligna* blaarweefsel versamel vanaf plante in 'n natuurlike staat en normale en hiperhidriese plante in weefselkulture, is ondersoek. Hiperhidriese materiaal het abnormale, dikwels gebroke ontwikkeling van die epidermis en kutikula getoon. Huidmondjies was misvorm. Die blaar-lamina het dikker vertoon en differensiasie tussen die palisade- en sponsmesofiel was onduidelik. Die chlorofil-inhoud van hiperhidriese weefsel was betekenisvol laer as dié van blare vanaf die natuurlike omgewing sowel as van normale blare wat in weefselkulture ontwikkel het. Hiperhidriese blare het betekenisvol minder chloroplaste per sel gehad en chloroplaste het verminderde thylakoïde stapeling getoon. Die tempo van fotosintese van hiperhidriese weefsel was laer as dié van normale *in vitro*-materiaal.

Keywords: Chloroplast, cuticle, epicuticular wax, *Eucalyptus saligna*, hyperhydricity (vitrification), leaf anatomy, photosynthetic rate, tissue culture.

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Introduction

Hyperhydricity (vitrification) is a physiological disorder that affects plantlets growing *in vitro* (Debergh *et al.* 1992). Hyperhydric plantlets are characterized by a translucent, glassy appearance (Ziv 1991). The thickened leaves are brittle and the tissues are often poorly differentiated (Vieitez *et al.* 1985). Cells in hyperhydric tissues have a high water content (Pierik 1987) due to deficient lignification (Kevers *et al.* 1987; Vieitez *et al.* 1985). As a consequence of these anomalies the survival rate of plantlets transferred into the soil is poor (Ziv *et al.* 1983). The occurrence and extent of hyperhydricity can be influenced by agar concentration (Ziv *et al.* 1983), low light and high temperatures (Pierik 1987), intensive sterilization (Pierik 1987), and poor gaseous exchange in the culture vessels (Hakkaart & Versluijs 1983). The production of ethylene which can occur in response to various non-wounding physical and chemical stresses, has also been implicated in exacerbating hyperhydricity in tissues (Kevers *et al.* 1984).

A commonly referred to symptom of hyperhydricity is chlorophyll deficiency (Ziv *et al.* 1983; Vieitez *et al.* 1985). Ziv *et al.* (1983) suggested that the lower chlorophyll and protein content of hyperhydric carnation leaves may contribute to the plantlets' low survival rate. A low chlorophyll content in hyperhydric leaves suggests that the occurrence and/or structural organization of the chloroplasts may be affected by hyperhydricity. This study investigated anatomical and ultrastructural differences in the leaf tissue of normal and hyperhydric *in vitro* material, as well as of field-grown material, of *Eucalyptus saligna* Sm. with emphasis on the chloroplasts.

Materials and Methods

Plant material

Plant material was obtained from an established clone bank of *E. saligna*. Explants from nodal shoots of juvenile coppice material were sterilized with 0.02% $HgCl_2$ for 15 min and then rinsed by several washes with sterile distilled water. Explants were cultured on conventional Murashige and Skoog (1962) medium, supplemented with vitamins (excluding glycine) and 1 g l^{-1} polyvinylpyrrolidone, 20 g l^{-1} sucrose and 0.1 mg l^{-1} BA. The pH was adjusted (5.8) before the addition of 2 g l^{-1} Gelrite. Cultures were placed in the dark for 5 days before being transferred to a room with a 24-h photoperiod, where light intensity was measured at a mean of 13.9 μmol $m^{-2}s^{-1}$. Shoots which developed from axillary buds were transferred to a shoot multiplication medium. This consisted of the initiation medium supplemented with 0.1 mg l^{-1} calcium pantothenate, 0.1 mg l^{-1} biotin and 0.1 g l^{-1} myo-inositol. The BA concentration was increased to 0.2 mg l^{-1}. The shoots were allowed to become hyperhydric naturally. Both hyperhydric and normal shoots occurred within the same culture vessel. Where possible, hyperhydric material (selected on the basis of the translucent nature of the leaves) and normal *in vitro* material were sampled from the same culture vessel.

Chlorophyll determination

Chlorophyll determinations were made on 80% acetonic extracts of the material, using Arnon's (1949) equations to calculate total chlorophyll concentrations. In order to relate differences in chlorophyll content to the various treatments, leaf water content was determined.

Anatomical studies

Material was prepared for scanning electron microscopy using an SP-2000 'sputter-cryo' system interfaced with a Hitachi S570 scanning electron microscope. Pieces of leaf material of 3 mm^2 each were fixed by immersion in sub-cooled liquid nitrogen and viewed at 5 kV.

For light and transmission electron microscopy, pieces of tissue approximately 2 mm^2 were cut from similar positions in fully expanded leaves. Tissue was fixed in 3% glutaraldehyde in a 0.05M sodium cacodylate buffer which also contained 0.5% (w/v) caffeine to prevent the leaching of polyphenolics from the cell vacuoles (Mueller & Greenwood 1978). Material was post-fixed with cacodylate-buffered osmium-tetroxide, dehydrated through a graded epoxy-propane series (including uranyl acetate at the 70% ethanol stage) and embedded in low viscosity resin (Spurr 1969). Ultrathin sections (approximately 90 nm) obtained for electron microscopy were post-stained with lead citrate (Reynolds 1963). Thick (2 µm) transverse sections for light microscopy were stained with Ladd's (R) multiple stain.

For stereological and morphometric analysis, ultrathin sections were cut from 10 blocks (from 10 different plants) for each treatment. The volume fraction of the mesophyll cells occupied by chloroplasts was determined from counts of ordered dots on a transparent overlay (Wiebel & Bolender 1973; Hajibagheri *et al.* 1984). Measurements of chloroplast morphology were made from micrographs of known magnification using a digitizer linked to a computer. Cell area per treatment was determined in a similar manner.

To determine the number of chloroplasts in each cell, leaf tissue was fixed in 3% glutaraldehyde in a 0.1M phosphate buffer for 2 h and then incubated in 0.1M EDTA (pH 9) for 2 days at 60°C to separate the cells (Jellings *et al.* 1983). Dilute cell suspensions were placed on a microscope slide, and the coverslip was pressed down sufficiently firmly to rupture some cells and release the chloroplasts. Chloroplasts from approximately 100 cells per treatment were counted in order to establish the average number of these organelles per cell.

Photosynthetic activity

The photosynthetic rate of both normal and hyperhydric cultures was determined using an infra-red gas analyser. Six replicates of each treatment were incubated at 25°C and at a light intensity of 50 - 60 µmol $m^{-2}s^{-1}$ to simulate conditions in the growth room. The CO_2 content of the septum-sealed flasks was monitored at 15-min intervals by extracting 1×10^3 mm^3 of air from each flask, using a syringe. This procedure was repeated while flasks were incubated in the dark. The values obtained for net photosynthesis and respiration, respectively, were used to determine gross photosynthetic rates. The analysis was repeated six times, after which dry weight of the plant material was determined.

Statistical analysis

Statistical analysis was carried out using the Statgraphics statistical program. Where necessary, data were normalized by an arcsine conversion prior to a one-way ANOVA. When the ANOVA indicated statistical significance, a Tukey's multiple comparison test was used to distinguish differences among means between treatments.

Results and Discussion

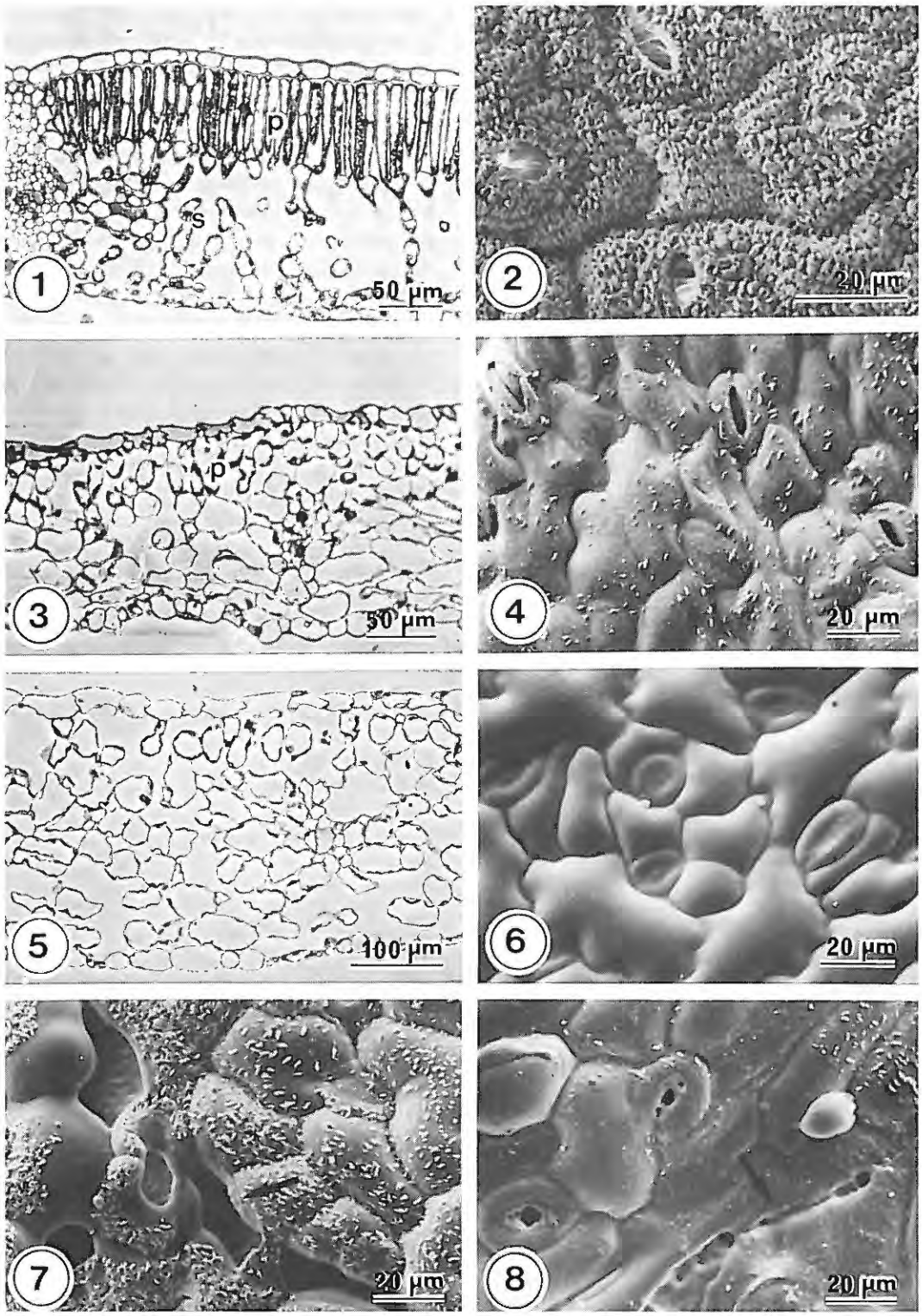
Leaf anatomy

The leaf lamina of field-grown *E. saligna* was characteristically bifacial and amphistomatous (Figure 1). The uniseriate adaxial and abaxial epidermis was covered by a well-developed cuticle and extensive epicuticular wax deposits (Figure 2). Cells of the palisade mesophyll of normal *in vitro* material were not as elongated as those of clone bank material, and extensive airspace development also occurred in this layer (Figure 3). Epicuticular wax on normal *in vitro* leaves was sparse (Figure 4). The thin-walled mesophyll cells of hyperhydric material were often isodiametric and the palisade mesophyll was barely distinguishable from the spongy mesophyll (Figure 5). Both the abaxial and the adaxial epidermal layers were characterized by a lack of, or patchy development of, the epicuticular wax (Figures 6 and 7) and stomata which were either incompletely developed or malformed (Figures 6 – 8). The epidermis also appeared discontinuous in hyperhydric leaves (Figure 7).

The higher light intensity (measured at 2250 µmol $m^{-2}s^{-1}$) under which field-grown material developed, possibly accounts for the extensive epicuticular wax development in comparison with the normal *in vitro* material, as both cuticle and wax development are affected by light intensity (Cutter 1979; Osborn & Taylor 1990). The poor epicuticular wax development in hyperhydric material in conjunction with aberrant stomata and epidermal perforations explain why hyperhydric plantlets succumb to the hardening-off process (Werker & Leshem 1987). The extreme similarity of the leaf surface abnormalities found in hyperhydric *E. saligna* and other hyperhydric species, especially carnation (Sutter & Langhans 1979; Leshem 1983), suggests that the metabolic changes leading to hyperhydricity are the same in most species.

Chlorophyll content

The chlorophyll content of hyperhydric leaves was significantly less than that of both field-grown and normal *in vitro* grown material on a fresh-weight basis (Table 1). Extrapolating from the known water content of the different treatments (Table 1), it was apparent that similar differences in chlorophyll content occurred on a dry-weight basis. The low chlorophyll content of the hyperhydric material on a fresh-weight basis therefore did not merely reflect the increased water content of the tissue. Low chlorophyll a : b ratios are characteristic of shade leaves (Boardman 1977). The low chlorophyll a : b ratios of the normal *in vitro* and the hyperhydric material in comparison with the field-grown material (Table 1) possibly reflect the difference in light intensity to which the material was exposed during development.



Figures 1 – 8 The surface and anatomy of leaf tissue from field-grown, *in vitro* normal and hyperhydric *Eucalyptus saligna*. **1.** Transverse section of a field-grown leaf showing distinct palisade (p) and spongy (s) mesophyll layers. **2.** The waxy abaxial surface of field-grown material with normal stomata. **3.** Transverse section of normal *in vitro E. saligna*. Cells of the palisade (p) are not as elongated as those of the field-grown material. **4.** Abaxial surface of normal *in vitro* material with functional stomata but sparsely distributed epicuticular wax. **5.** Transverse section of a hyperhydric *E. saligna* leaf showing poor differentiation of the palisade mesophyll. **6 – 8.** The surfaces of hyperhydric leaves have (6) non-opening stomata and no epicuticular wax (→) (adaxial surface), (7) aberrant stomata, sparsely distributed epicuticular wax and epidermal perforations (→) (adaxial surface), or (8) permanently open stomata, sparsely distributed epicuticular wax and epidermal perforations (adaxial surface).

Cell area, chloroplast number and chloroplast volume fraction

The volume fraction of the cytoplasm of the mesophyll cells occupied by the chloroplasts was considerably greater in the field-grown material than normal *in vitro* or hyperhydric material (Table 2). This is reflected by both a larger number of chloroplasts per cell (Table 2) and a smaller cell size/area (Table 2) found in field-grown material in comparison with

normal *in vitro* and especially hyperhydric material. Deficiency of both cellulose and lignin has been reported in hyperhydric plantlets (Kevers *et al.* 1984, 1988), creating changes in cell wall mechanical properties. These deficiencies would allow more water uptake due to reduced wall pressure, thus producing the very large cell size and high water content characteristic of hyperhydric material.

Chloroplast micromorphology

The chloroplasts of hyperhydric leaves appeared to be larger than those of the field-grown and normal *in vitro* tissue. However, there were no significant differences between the various treatments with respect to chloroplast area, perimeter, number of grana per chloroplast cross-section, number of grana per square metre or granum width (Table 3). Within treatments there were no apparent significant differences between the chloroplast parameters measured in the palisade and the spongy mesophyll, except that the starch content in the palisade mesophyll chloroplasts of the field-grown leaf material was higher than that of the spongy mesophyll (data for spongy mesophyll not presented). Thylakoid stacking within the grana was significantly reduced in normal *in vitro* and especially hyperhydric material (Figure 9) in comparison with field-grown material (Figure 10). Reduced thylakoid stacking is inconsistent with a shade response (Fagerberg 1988) but not with an elevated ethylene effect (Purvis 1980). There was significantly less starch in the chloroplasts of tissue-cultured material as opposed to field-grown leaves,

Table 1 Water and chlorophyll content and chlorophyll a:b ratio of leaf tissue

Water and chlorophyll content	Field	Normal	Hyperhydric
Water content (%)	81.20[a]	88.10[b]	91.20[c]
Chlorophyll a + b (mg g^{-1} FW)	0.95[a]	0.55[b]	0.29[c]
Ratio (chl a : chl b)	3.14[a]	2.38[b]	2.24[b]

[a],[b],[c] Treatment means followed by different letters are significantly different at the 0.05% level.

Table 2 Cell area, chloroplast volume fraction and average chloroplast number of mesophyll cells in leaf tissue of *Eucalyptus saligna*

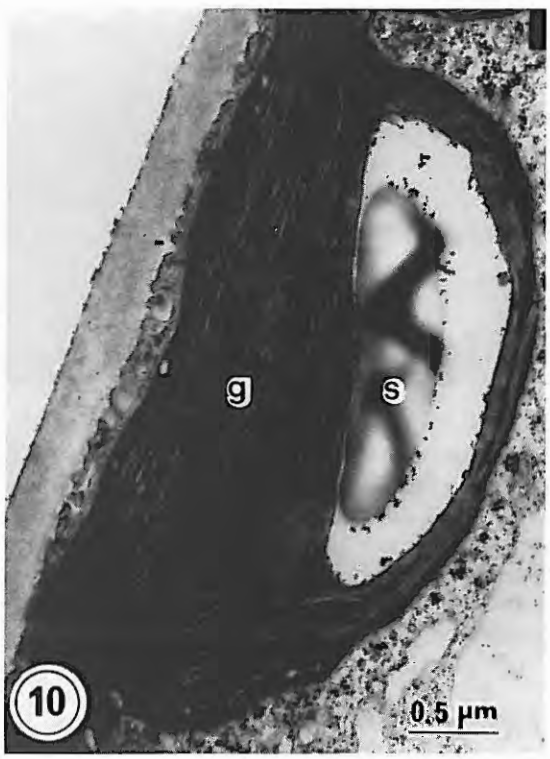
Mesophyll cell parameters	Field	Normal	Hyperhydric
Cell area (μm^2)	371[a]	408[a]	748[b]
Chloroplast volume fraction (as percentage of the cytoplasm)	47.3[a]	37.3[b]	17.3[c]
Chloroplast number	29.0[a]	18.0[b]	13.5[c]

[a],[b],[c] Treatment means followed by different letters are significantly different at the 0.05% level

Table 3 Palisade mesophyll chloroplast ultrastructure in leaves of field grown, normal and hyperhydric cultured *Eucalyptus saligna*

Chloroplast parameters	Field	Normal	Hyperhydric
Area (μm^2)	4.9[a]	5.3[a]	5.7[a]
Perimeter (μm)	10.0[a]	10.9[a]	12.0[a]
Number of grana per cross-section	19.2[a]	25.8[a]	27.6[a]
Number of grana/μm^2	0.19[a]	0.21[a]	0.21[a]
Granum width (μm)	0.38[a]	0.37[a]	0.36[a]
Number of thylakoids/granum	11.23[a]	7.47[ab]	5.55[b]
Starch (%)	27.6[a]	9.2[b]	2.9[b]

[a],[b] Treatment means followed by different letters are significantly different at the 0.05% level



Figures 9 & 10 Chloroplasts from hyperhydric leaves (9) have significantly fewer thylakoids per granum (g) and less starch (s) than those from field-grown leaves (10).

possibly reflecting the decreased photosynthetic efficiency of these tissues at low irradiances. Shading reduced the starch content of leaves of *Helianthus annuus* (Fagerberg 1988).

Photosynthesis

The gross photosynthetic rate of hyperhydric material under *in vitro* culture conditions was significantly lower than that of normal cultured material (Table 4). The low chlorophyll levels, reduced chloroplast numbers and thylakoids per granum in hyperhydric material must contribute to the reduced rate of photosynthesis. Photosynthetic inefficiency together with the described cellular malformations would reduce adaptability in *ex vitro* conditions.

The abnormalities in terms of stomatal abberations and epidermal perforations observed in *E. saligna* closely resemble symptoms of hyperhydricity described by other authors (Werker & Leshem 1987; Ziv 1991). Furthermore, the described chloroplast abnormalities are similar to those found in hyperhydric carnations (Drennan & van Staden 1986). These similarities between species suggest that while many factors in the *in vitro* environment may cause hyperhydricity, the consequent perturbation of the morphogenetic processes stems from a common ('key') event — probably ethylene production, as a result of various non-wounding stress conditions (Kevers *et al.* 1984).

Table 4 Gross photosynthetic rate of normal and hyperhydric cultured *Eucalyptus saligna*

	Normal	Hyperhydric
Photosynthetic rate ($mg\ l^{-1}\ CO_2\ g^{-1}\ l^{-1}\ min^{-1}$)	1433.223[a]	539.450[b]

[a],[b] Treatment means followed by different letters are significantly different at the 0.05% level

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