

Ultrastructural and biochemical aspects of normal and hyperhydric eucalypt

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Summary: Hyperhydricity was observed throughout *in vitro* multiplication phase of a *Eucalyptus grandis* clone. Ultrastructural approach of tissue and cell differentiation, isoenzyme patterns, binding protein (BiP) expression, and pigment content were performed. Hyperhydric tissues showed a reduction in cell wall deposition, reduction of membranous organelles, higher cell vacuolation, and more intercellular spaces than its normal counterpart. Additionally, several vesicles were present in hyperhydric cells suggesting the occurrence of organelle autophagy by autophagic vacuole. Lower pigment content, intercellular spaces on the epidermis and the induction of a molecular chaperone (BiP) were observed in hyperhydric phenotype. Evidences of schizolysigenous process of intercellular space formation are compatible with a stress condition. Although plastoglobuli were observed in normal and hyperhydric chloroplasts, they were more evident in the normal ones. Abnormal stomata also reflected a disruptive situation and morphogenesis disturbances which would difficult plant acclimatization. Further observation of the epidermis ultrastructure allows us to conclude that the presence of intercellular spaces on its surface may be constraining the recovery and development of hyperhydric plants. Similarly to BiP, other proteins such as esterase (EST), acid phosphatase (ACP), malate dehydrogenase (MDH) and peroxidase (POX) are possible to be used as stress markers in *in vitro* conditions. Our results confirm earlier findings about negative effects of hyperhydricity on *in vitro* plant morphogenesis and ultrastructure, which in eucalypt is associated with a stressful condition contributing to lower propagation ratios.

Key words: BiP, hyperhydricity, isoenzymes, micropropagation, pigment, stress

Introduction

Eucalyptus is an important genus of Myrtaceae, valuable for timber, pulp and paper production. For these reasons, it is widely propagated throughout the world. Additionally, vegetative propagation of eucalypt is easily accomplished, what facilitate breeding strategies aiming to produce, capture and develop superior genotype combinations (Eldridge et al., 1993; Assis et al., 2004).

Micropropagation techniques allow the rescue, the rejuvenation, or the early multiplication of elite genotypes (Watt et al., 2003; Alfenas et al., 2004; Assis et al., 2004). The most commonly used explant for the eucalypt micropropagation are seedlings and axillary buds from field-grown plants, although, other explants are also employed. Further applications are available as well, such as adventitious regeneration, germplasm conservation, and genetic modification (Watt et al., 2003). Despite the prospect

of *in vitro* tissue culture, there are some obstacles such as recalcitrant genotypes, somaclonal variation, and hyperhydricity.

Hyperhydricity is well reported for several plants such as *Origanum vulgare* (Ueno et al., 1997), *Citrullus lanatus* (Thomas et al., 2000), *Malus* sp. (Chakrabarty et al., 2005), *Malus prunifolia* (Lucyszyn et al., 2005), *Solanum melongena* (Picoli et al., 2001), *Capsicum annuum* (Fontes et al., 1999), *Eucalyptus* (Jones et al., 1993; Louro et al., 1999, 2003; Whitehouse et al., 2002), *Dianthus caryophyllus* (Piqueras et al., 2002; Saher et al., 2004, 2005), *Pyrus* (Kadota et al., 2001; Kadota & Niimi, 2003), *Prunus* (Pérez-Tornero et al., 2001), *Simmondsia chinensis* (Mills et al., 2004), *Gypsophyla paniculata* (Paek et al., 1991), *Arabidopsis* (Delarue et al., 1997). It encloses physiological disorders, anatomical and ultrastructural abnormalities affecting *in vitro* cultured plants. Moreover, there is also evidence that hyperhydric plants display metabolism

changes related to oxidative stress (Piqueras et al., 2002; Kevers et al., 2004; Franck et al., 2004; Saher et al., 2004, 2005). Recently we have shown that hyperhydricity is associated with the induction of the ER resident, stress-related protein BiP (Fontes et al., 1999; Picoli et al., 2001). The up regulation of BiP synthesis has also been proved to be an efficient approach to monitor intracellular stress in plants (Fontes et al., 1991; Cascardo et al., 2001; Alvim et al., 2001).

Other alterations such as deficiencies in cell wall and cuticle deposition (Phan & Hegedus, 1986; Andarwulan et al., 1999; Chakrabarty et al., 2005), deficiency of chlorophyll accumulation (Franck et al., 2001, 2004; Saher et al., 2004; Chakrabarty et al., 2005), stomata abnormalities (Miguens et al., 1993; Ziv & Ariel, 1994; Fontes et al., 1999; Picoli et al., 2001; Chakrabarty et al., 2005), watery tissue and excessive hydration (Gribble et al., 1998; Saher et al., 2004), poor morphogenesis and low dry weight content (Ziv, 1991; Jones et al., 1993; Saher et al., 2004, 2005) are equally observed.

All these symptoms may result from headspace relative humidity and composition (Paek et al., 1991; Thomas et al., 2000; Mills et al., 2004; Saher et al., 2005; Makunga et al., 2006), the salt composition or element concentration in the culture medium (Paek et al., 1991; Kadota et al., 2001), gelling agent (Thomas et al., 2000; Kadota et al., 2001; Pérez-Tornero et al., 2001; Franck et al., 2004), growth regulator used and its concentration (Kadota & Niimi, 2003; Mills et al., 2004), among others. The ultimate result is low acclimatization rates and plant loss (Werker & Leshem, 1987; Debergh et al., 1992; Olmos & Hellin, 1998).

Many factors have been identified to induce hyperhydricity, and although it is not predictable, stress is believed to be the major reason underlying the phenomenon (Kevers et al., 2004). There are several approaches to reduce hyperhydricity that include increasing carbohydrate levels in the medium (Zimmerman & Cobb, 1989; Ueno et al., 1997; Makunga et al., 2006), changing light intensity (Pérez-Tornero et al., 2001), modifying concentrations of gelling agents (Zimmerman & Cobb, 1989; Pérez-Tornero et al., 2001; Whitehouse et al., 2002; Franck et al., 2004; Lucyszyn et al., 2005), reducing humidity (Makunga et al., 2006), and bottom cooling (Pérez-Tornero et al., 2001; Piqueras et al., 2002; Saher et al., 2005), but successes have been limited.

During the micropropagation of eucalypt clones, some of the genotypes displayed the hyperhydric phenotype, with was prejudicial to the overall process. In the present work we reviewed the main aspects of hyperhydricity in *in vitro* cultured plants and characterized ultrastructural, anatomical and biochemical changes in hyperhydric eucalypt, in support to the research of the influence of stress and flask capping in tissue culture experiments conducted in our lab. Here we describe some of the hyperhydric eucalypt features that may help the understanding of the processes involved, and, compared to its normal counterpart, suggests that hyperhydricity is in fact associated with a stressful condition, besides or in addition to the *in vitro* culture.

Materials and methods

Plant material and establishment of aseptic plants

The clone CC8 was supplied by Companhia Nippo-Brasileira de Celulose S.A. (CENIBRA), located in Belo Oriente, Minas Gerais state, Brazil. The clone was derived from seven years-old trees that reached flowering stage that had been selected from a commercial plantation. Eucalypt shoots from clone CC8 were established *in vitro* and further micropropagated in test tubes (150 × 25 mm), one shoot per tube, capped with polypropylene closures (Beloc, USA) with MS basal medium (Murashige & Skoog, 1962), supplemented with White's vitamins (White, 1943), 800 mg l⁻¹ PVP (polyvinyl pyrrolidone), 100 mg l⁻¹ myo-inositol, 3% (w:v) sucrose, 0.05 mg l⁻¹ NAA (á-naphtaleneacetic acid), 0.1 mg l⁻¹ BAP (6-benzylaminopurine), pH 5.7, and solidified with 0.7% (w:v) agar (Sigma Chemical Co.). Subculture was performed every four weeks and cultures maintained under 16:8 h light: dark regime, 30 µmol m⁻² s⁻¹ light radiation provided by two fluorescent tubes (Luz do Dia Especial, 20 W, Osram, Brazil), whereas the culture room temperature was kept at 26 ± 2°C. The number of *in vitro* subcultures at the time of the experiments were set up was 9–10. Several tubes, presenting normal or hyperhydric plants were selected and leaves collected for further analyses at the end of the subculture period. Hyperhydric tissues were chosen based on the glassy appearance of leaves and shoots.

Ultrastructural analysis

Scanning electron microscopy (SEM) Leaf samples were collected from *in vitro* grown (45–60 days-old) normal and hyperhydric plants obtained from independent regeneration events. Five leaf samples were taken from fully expanded leaves below the terminal bud, and fixed with 3% (v:v) glutaraldehyde in 0.05 M cacodylate buffer pH 7.0, at 4 °C overnight. Samples were then washed three times over 1h in the same buffer, and then dehydrated in a routine, graded ethanol series. Leaf samples were critical-point-dried with CO₂ using a Balzer's Critical Point Dryer (CPD020) and fragments were positioned on stubs prior to gold sputtering in a Sputter Coater (Balzer FDU010). Following coating with a thin layer of gold (about 20nm) the specimens were observed and micrographed using a JEOL (JSM T-2000) SEM at 15 kV.

Ultrastructural analysis: transmission electron microscopy (TEM) Stem and leaf blade samples of normal and hyperhydric eucalypt were used for transmission electron microscopy. Five segments from different stems and leaves were immersed in 4% paraformaldehyde (v:v), 2.5% glutaraldehyde (v:v) in 1.25% Pipes buffer pH 7.2, following vacuum for two hours and washed in the same buffer. Samples were post fixed in 1% (v:v) osmium tetroxide in 1.25% Pipes buffer, after what were dehydrated in a graded acetone series and embedded in Spurr resin (Spurr, 1969), polymerized for 15h at 70 °C.

The resin blocks were cut with a Reichert Ultracuts Ultramicrotome (Leica Germany) with diamond blades. The cuts were recovered in copper grids and contrasted for 20 min with 1% uranyl acetate in absolute ethanol, and stained in lead citrate for 5 min. Samples were examined with a Philips CM 100 (Zeiss, Germany) transmission electron microscope at 60KV.

Protein extraction and quantification, SDS-PAGE electrophoresis and immunoblot analysis

Protein extraction and quantification, SDS-PAGE electrophoresis and immunoblot analysis were carried out as described by *Fontes et al.* (1999). Normal and hyperhydric frozen leaf samples were ground in liquid nitrogen with a pestle and mortar and homogenised at 25 °C with extraction buffer (100 mM Tris-HCl, 50 mM NaCl and 1 mM PMSF, pH 7.5) at a ratio of 500 mg of tissue per 5 ml of extraction buffer. Cell debris was removed by centrifugation (14,000 rpm, 15 min) and the protein-containing supernatants retained. Protein quantification and gel electrophoresis were performed according to *Bradford* (1976) and *Laemmli* (1970), respectively. Protein extracts were incubated at 100 °C, for 3 min, with the sample buffer [10% (v/v) glycerol, 2.3% (v/v) SDS, 0.25% bromophenol blue, 5% 2-mercaptoethanol, 0.0625M Tris-HCl, pH 6.8], before loading the gels. Aliquots of protein extract (30 µl) were loaded onto 5% SDS-PAGE, electrophoresed for 16 h at 37 V in a running buffer (0.025M Tris-HCl, 0.2 M glycine, 1 mM EDTA and 3.5 mM SDS). The gel was stained with 40% (v/v) methanol, 7.5% (v/v) acetic acid and 0.01% Coomassie Brilliant Blue R-250 for 8 h and then de-stained in 10% (v/v) methanol and 7.5% (v/v) acetic acid.

After electrophoresis, the proteins were transferred to a nitrocellulose membrane using a blot apparatus (BioRad, USA) according to the manufacturer's instructions. Following transference (about 2 h; 700 mA) the membrane was incubated in a blocking solution (Blotting Grade Blocker Non-fat Dry Milk, BioRad USA) for 1 h at room temperature. The membrane was washed (three times, 15 min each) using TBS-T [0.01M Tris-HCl, pH 7.6, 1.5 mM NaCl and 0.1% (v/v) Tween 20] and incubated (2–4 h) with an anti-soybean BiP rabbit polyclonal antibody (*Figueiredo et al.*, 1997) using a 1 : 2000 dilution ratio. The membrane was repeatedly washed in TBS-T (three times, 25 min each) and incubated (2 h) with IgG alkaline phosphatase conjugate (Sigma, USA) at a 1:500 dilution. Following incubation, the membrane was washed intensively with TBS-T and then incubated (about 5 min) in enzyme buffer (0.1 M Tris-HCl, pH 9.8, 0.1M NaCl, 0.5M MgCl₂). Alkaline phosphatase activity was detected with NBT and BCPIP BRL substrates (Gibco BRL, USA).

Isoenzyme analysis

Isoenzyme extraction and quantification, and zymogram electrophoresis were carried out as described by

Alfenas et al. (1988). The following isoenzyme were used: alcohol dehydrogenase (ADH), glutamate oxaloacetate transaminase (GOT), isocitrate dehydrogenase (IDH), shikimate dehydrogenase (SKDH), esterase (EST), acid phosphatase (ACP), malate dehydrogenase (MDH), peroxidase (POX), phosphoglucose isomerase (PGI) and phosphoglucomutase (PGM).

The enzymes were extracted from hyperhydric and normal leaf tissue. In this process 20 mg of leaf tissue, 10 mg washed sand, 7 mg Polyvinyl pyrrolidone (PVP 40), 7 mg Polyvinyl pyrrolidone (PVP 60), and 200 µl extraction buffer number 1 (*Alfenas et al.*, 1988). Electrophoresis was performed in 13% (w/v) maize starch gel (penetrose 30), at 5 °C. The first 30 min of electrophoresis was conducted at 35mA, and subsequently at 40mA. The voltage was 120–180 volts and 140–210 in the electrode, and eight to ten in the gel, respectively during the same period. The electrophoresis was of approximately 8h and 30 min, when the marker bromophenol blue was at 7 to 8 cm from the start point. Subsequently the gel was divided in six slices with 1 mm each, and submitted to staining according to each enzyme.

Pigment content analysis

Pigment contents were quantified from samples of leaf tissue from both normal and hyperhydric plants. Pigments were extracted by homogenizing 1–2 g of fresh tissue with 80% acetone; the extract was filtered through filter paper Whatman 1. Carotenoid and chlorophylls *a* and *b* contents were estimated as described by *Lichtenthaler* (1987). Absorbances were determined at 451 and 503 nm for carotenoids, and 647 and 664.5 nm for chlorophyll.

Results

Ultrastructural and anatomical observations

Hyperhydric eucalypt displayed translucent turgid and brittle leaves, and stunted shoots, that under TEM displayed a reduction in cell wall deposition, less organelles and more intercellular spaces than its normal counterpart (Compare *Figures 1A* and *1C* with *1B*, *1D* and *1F*). Hyperhydric cells are conspicuously vacuolated (*Figures 1A* and *1C*), whilst non-hyperhydric cells exhibited electron dense substance in the vacuole (*Figures 1B* and *1H*). Possibly, these are factors which may be associated with cell burst (*Figure 1E*).

Starch accumulation in chloroplasts of hyperhydric plantlet was lower than in those of non-hyperhydric plants, and although plastoglobuli were observed in normal and hyperhydric chloroplasts, they were more evident in the normal ones (*Figure 1D*). Several vesicles are also exhibited in a hyperhydric cell (*Figure 1H*), what suggests the occurrence of organelle autophagy.

Stomata deformation, unexpected cellular interspaces (*Figure 2*), and a cell compromised wall deposition, as irregular outer cell wall from epidermis (*Figures 2B*, *2D* and *2F*), were also observed.

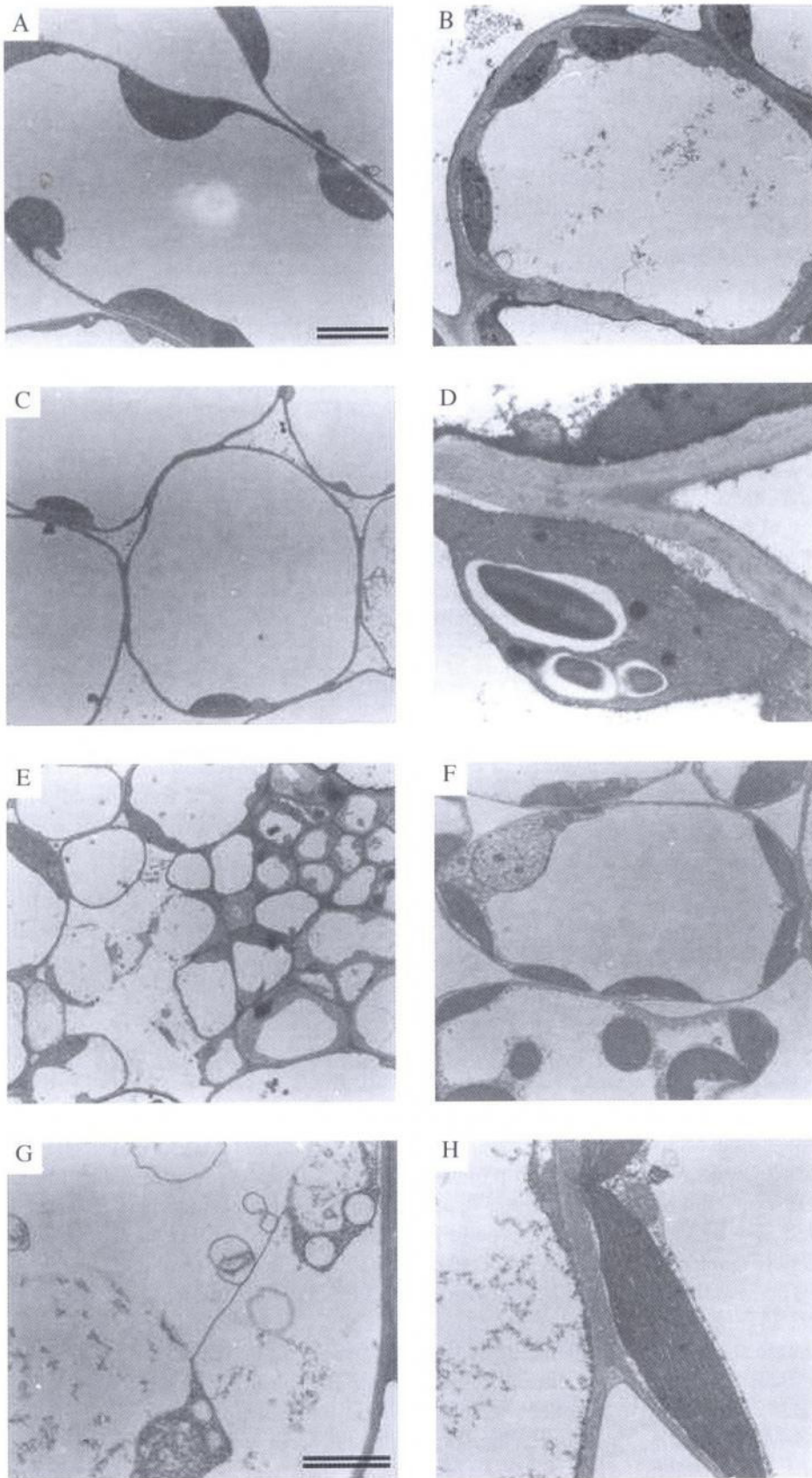


Figure 1 Transmission electron microscopy of hyperhydric (A, C, E and G) and non-hyperhydric (B, D, F and H) eucalypt. A – hyperhydric cell from leaf mesophyll; B – normal *in vitro* grown shoot cell; C – detail from hyperhydric shoot cells; D – detail of a chloroplast and plastoglobuli in a normal *in vitro* grown shoot cell; E – hyperhydric vascular tissue displaying a schizogenous/lysigenous intercellular space; F – a cell from a normal mesophyll tissue of a *in vitro* grown leaf; G – vesicles present in the cytoplasm of a shoot hyperhydric cell; and H – detail of a chloroplast of a normal leaf grown *in vitro*. Bars: A, B, D, F and H (2.0 μm); C, E and G (0.5 μm).

Pigments

Hyperhydric leaves displayed lower chlorophyll a and b content, contrary to carotenoid content (Figure 3).

Isoenzymes

It was not observed a pattern for alcohol dehydrogenase (ADH), glutamate oxaloacetate transaminase (GOT), isocitrate dehydrogenase (IDH) and shikimate dehydrogenase (SKDH) (data not shown). On the other hand, all samples displayed similar esterase (EST), acid phosphatase (ACP), malate dehydrogenase (MDH) and peroxidase (POX) activity (Figures 4A-4D).

Examining phosphoglucose isomerase (PGI) and phosphoglucomutase (PGM), it is observed a similar zymogram pattern, although the last samples displayed a slightly lower activity (Figures 4E and 4F).

Hyperhydric shoots accumulated BiP to a higher level than normal *in vitro* regenerated shoots

Hyperhydric eucalypt displayed significant BiP induction (Figure 5). BiP synthesis in hyperhydric and non-hyperhydric eucalypt shoots by immunoblotting assays with an anti-soybean BiP serum, and curiously, in hyperhydric samples, two other light bands were detected (white arrows).

Discussion

The occurrence of hyperhydricity varied among subcultures, what suggests that factors that trigger hyperhydricity in the observed genotype were present at random; although the relative humidity and plant hormones seemed to influence the frequency of hyperhydric shoots (data not shown). Hyperhydricity involves multiple factors that, depending on the specific physiological responses to culture conditions and the species studied, are expressed in various degrees of abnormal morphogenesis (Ziv, 1991). The augment of the intercellular spaces in the mesophyll, schizogenous or lysigenous, together with a thinner or

absent cuticle (Capellades et al., 1990; Olmos & Hellin, 1998; Chakrabarty et al., 2005), contribute to the observed anatomical and ultrastructural modifications, and/or are originated from this stress condition.

These are important remarks considering the existence of a relationship between hyperhydricity and lack of transpiration, and that decreases in relative humidity were able to reduce or prevent hyperhydricity (Saher et al., 2005). The present data are consistent with reports for other species since hyperhydricity is a symptom related to changes in cell water relations that lead to cell enlargement as observed in several reports (Olmos & Hellin, 1998; Delarue et al., 1997).

The water distribution in intercellular spaces in leaves provides clues that support the idea that hyperhydricity manifests itself with a range of severities and that all tissues *in vitro* suffer from this abnormality to a lesser or greater extent (Gribble et al., 1998). Furthermore, the lower dry mass of the eucalypt hyperhydric leaves compared with healthy leaves from *in vitro*-grown shoots indicated that the hyperhydric leaves contained more water than the healthy ones (Jones et al., 1993). These are a consequences of the reduced cell wall deposition and water accumulation into the vacuoles (Figure 1A and 1C) and in intercellular spaces. Werker & Leshem (1987) considered that the larger intercellular space was formed by decomposed laminae that resulted in the absence of cell contact. In the hyperhydric *S. melongena* cortex, large intercellular spaces tend to appear, lending the tissue a semi-disintegrated aspect (Picoli et al., 2001).

These observations might account for cell burst (Figure 1E) and for both the large intercellular spaces. Excessive water accumulation in plant tissue is the most characteristic symptom of hyperhydricity, which can result in depletion of cellular oxygen concentrations (Chakrabarty et al., 2005). Saher et al. (2004) also reported increased solute leakage in hyperhydric shoots compared to normal *D. caryophyllus* shoots. Nevertheless, it may also be associated with, or facilitate, physical damage in sample processing.

Anatomical studies demonstrated that hyperhydric leaves have an unorganized spongy mesophyll (Werker & Leshem, 1987). These same authors considered that the larger intercellular space was formed

by a schizolysigenous process where the laminae were decomposed providing the absence of cell contact. The reduced cell number and bigger cellular area of hyperhydric leaves show that the leaf growth and a thicker leaf was a result of the increased size of mesophyll cells. Figure 1E illustrate this process occurring in eucalypt vascular tissues. Autophagy is also a possible event associated with these observations (Figure 1H).

Conversely to Fontes et al. (1999), starch accumulation in chloroplasts of hyperhydric plantlet was lower than in those of non-hyperhydric plants, and although plastoglobuli were observed in normal and hyperhydric chloroplasts, they were more evident in the normal ones (Figure 1D). Damaged chloroplasts have also been previously observed in hyperhydric leaves of *P. avium* and *D. caryophyllus*, and interpreted as a manifestation of autophagy (Franck et al., 1998; Olmos & Hellin, 1998; Pérez-Tornero et al., 2001).

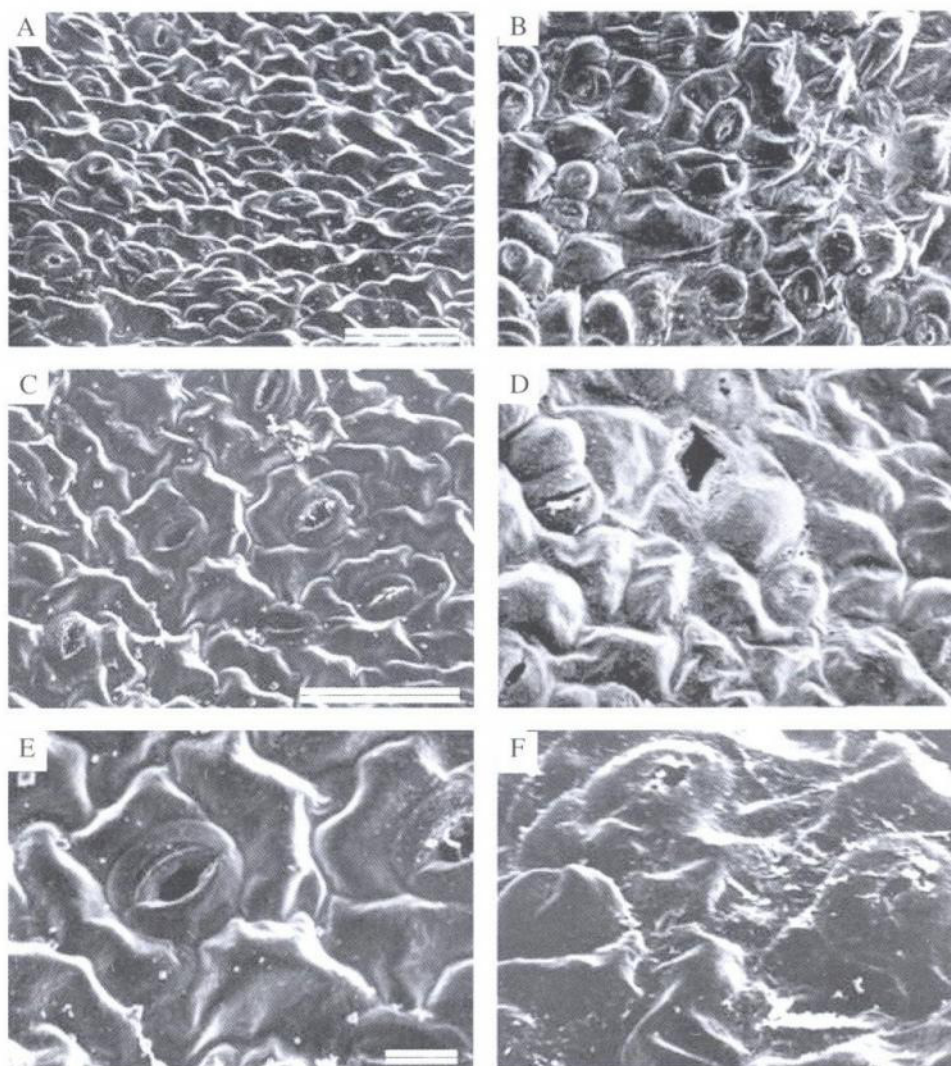


Figure 2 Scanning electron microscopy of the abaxial leaf epidermis showing numerous stomata of non-hyperhydric (A, C, E) and hyperhydric (B, D, F) eucalypt leaves. Overview of the epidermis, epidermal cells irregularity (A and B); guard cell deformation and intercellular spaces in the hyperhydric leaves (C and D); closer detail of stomata and epidermal cells in normal and hyperhydric leaves. Note the difference on cell wall bordering the stomata pore in guard cells (C, E). Bars: A and B (50 μ m); C and D (50 μ m); and E and F (10 μ m).

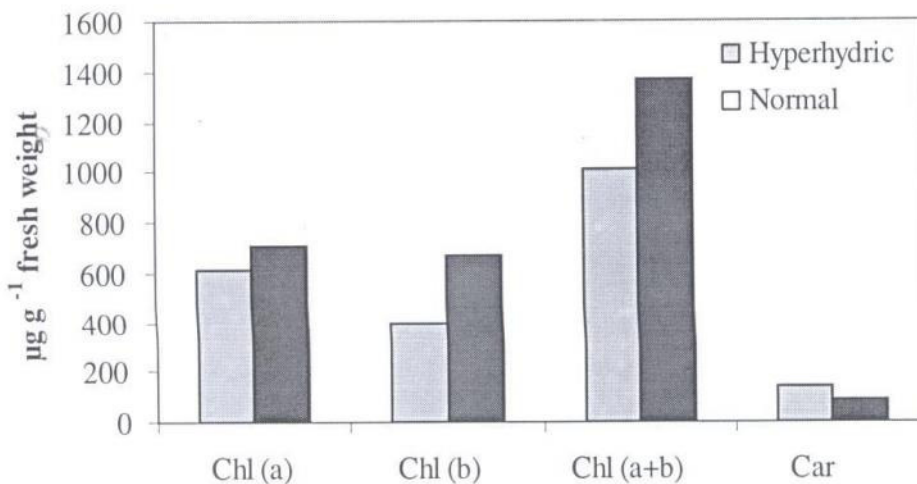


Figure 3 Pigment content in normal and hyperhydric leaves of *in vitro* cultured eucalypt. Chl (a) chlorophyll a, Chl (b) chlorophyll b, Chl (a+b) chlorophylls a plus b, Car – Carotenoids.

Despite the electron dense substance in non-hyperhydric cell vacuoles (Figures 1B and 1H), the ultrastructure of the hyperhydric leaves (Figure 1A, 1C, 1E and 1G) is a typical morphology of hypertrophic cells. A possible explanation is that these substances in the vacuole would be diluted, although eucalypt accumulates electron dense substances such as phenols, hyperhydric cells exhibit higher water content. Alternatively, the modification of some biochemical pathways in hyperhydric eucalypt could also result in a decreased synthesis of some phenols, as lower lignification (Piqueras et al., 2002; Saher et al., 2004) and enzyme activity (Phan et al., 1986; Franck et al., 2001) have been reported for hyperhydric plants.

The normal stomata morphology of non-hyperhydric plants (Figures 2C and 2E) and abnormal morphology for hyperhydric leaves reflect a stressful condition (Figures 2D and 2F). Similar results were reported for hyperhydric *C. annuum* (Fontes et al., 1999) and *Datura innoxiosa* (Miguens et al., 1993), *S. melongena* (Picoli et al., 2001), and *Malus* (Chakrabarty et al., 2005). Changes of this nature were accompanied by deformation of the guard cell walls due to loss of elasticity or to modifications in the pattern of deposition of cellulose microfibrils (Ziv & Ariel, 1994). Contrarily to the present results (Figure 2), normal and hyperhydric stomata of a *Eucalyptus* hybrid presented the same basic structure (Louro et al., 1999).

Hyperhydric leaves have a poor survival rate when transferred from *in vitro* to *ex vitro* conditions mainly due to water loss and desiccation (Debergh et al., 1992). The major cause of desiccation is non-functional stomata (Ziv & Ariel, 1994). Other reasons for this leaf malfunctioning are the absence of epicuticular wax and reduced development of palisade tissue (Debergh et al., 1992).

Similar to eucalypt (Figure 3), hyperhydric apple leaves had a significantly lower chloroplast number per cell and chloroplasts showed reduced thylakoid stacking compared with healthy ones (Chakrabarty et al., 2005). Additionally,

according to these authors, chlorophylls a and b and carotenoid concentrations were significantly lower in hyperhydric leaves than in healthy leaves. Jones et al. (1993) also reported a significantly lower photosynthetic rate and a decreased number of chloroplasts, thylakoids per granum and chlorophyll content in hyperhydric *Eucalyptus saligna*, compared to normal and *ex vitro* grown leaves.

As observed in Figure 4, variation in enzyme activity is expected, as it is influenced by culture environment, resulting in various changes in metabolic processes, some either resembling plant responses under stress conditions (Ziv, 1991). Saher et al. (2004) observed an increase in

several enzymes antioxidant activities, although the activity from other enzymes such as PAL decreased significantly.

The EST, ACP, MDH and POX zymogram pattern (Figures 4A-4D) might be associated with an increase in these enzyme syntheses, considering that a dry matter reduction is expected in hyperhydric tissues. In fact, protein levels were lower in hyperhydric leaves (Ziv, 1991; Fontes et al., 1999) although some proteins were either present in hyperhydric plants, but not in normal leaves (Ziv, 1991; Chakrabarty et al., 2005) or detected in larger amounts in the former (Chakrabarty et al., 2005).

Peroxidases associated with lignin synthesis were detected in larger amounts in hyperhydric plants, contrarily; decreased lignification (Phan & Hegedus, 1986; Piqueras et al., 2002; Saher et al., 2004) has been attributed to the observed reduction of enzyme activity, as reported for *P. avium* (Phan et al., 1986; Franck et al., 2001), *Dianthus* (Saher et al., 2004) and *O. vulgare* (Andarwulan et al., 1999). Hyperhydric shoots of *Eucalyptus* were characterized by an increase in the relative density of ER, indicating that their metabolism is higher than that of the normal shoots (Louro et al., 1999).

Contrarily to a higher protein synthesis (Figure 4D), the reduction in cell wall deposition might be associated with a reduced enzyme activity, considering that peroxidases are associated with lignin deposition. On the other hand, the activities of antioxidant enzymes, such as superoxide dismutase, catalase, ascorbate peroxidase and glutathione reductase were higher in hyperhydric leaves than in healthy leaves, indicating that hyperhydricity was associated with oxidative stress (Chakrabarty et al., 2005; Saher et al., 2005).

Franck et al. (2001) observed a reduced metabolism and a slight reduction on the photosynthetic capacity of hyperhydric *Prunus* cultures. In hyperhydric shoots, lower activities of some enzymes involved in glycolysis (hexokinase, hexose phosphate isomerase, glycerol-3-

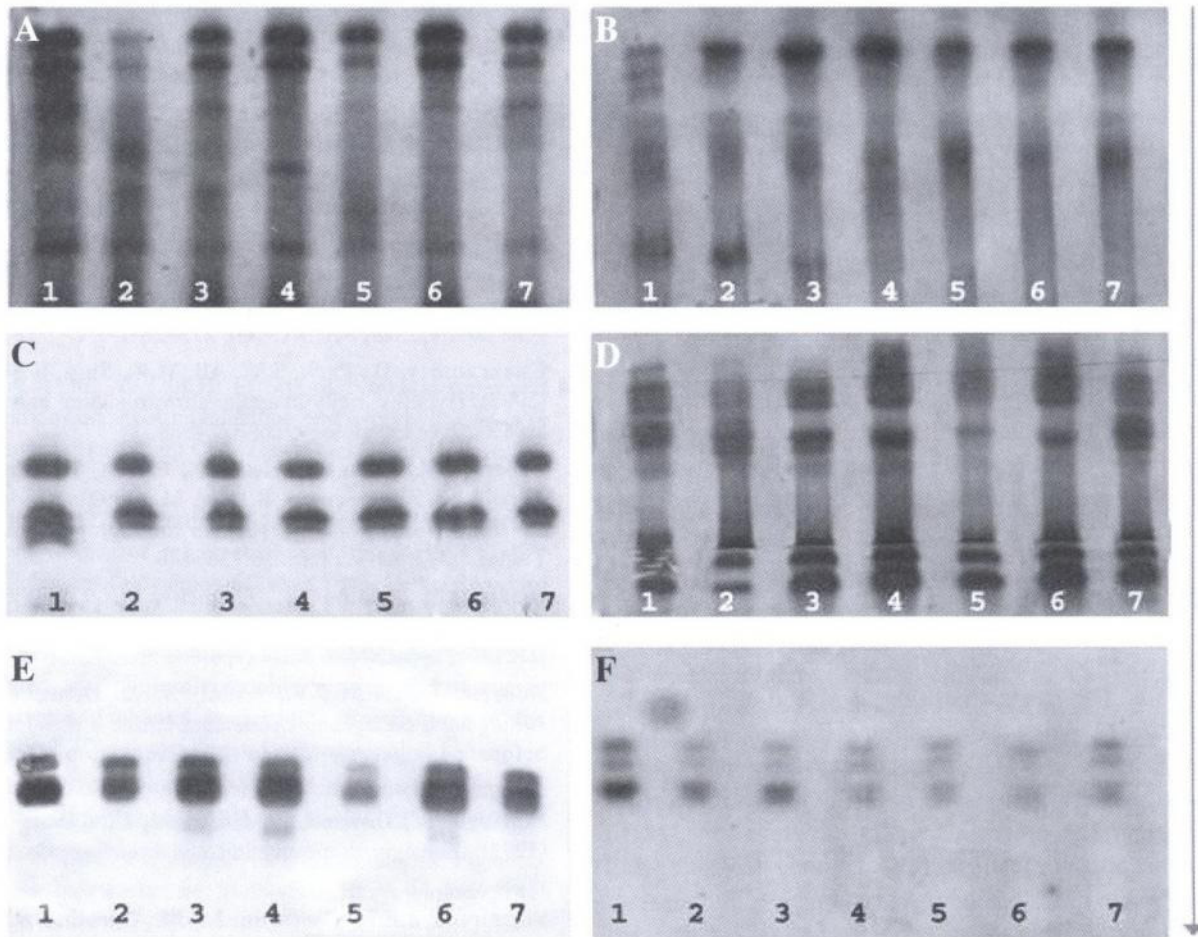


Figure 4 Zymograms of proteins extracted from normal and hyperhydric leaves of *in vitro* cultured eucalypt. A – Esterase (EST); B – Acid phosphatase (ACP); C – Malate dehydrogenase (MDH); D – Peroxidase (POX); E – phosphoglucose isomerase (PGI); and F – phosphoglucose mutase (PGM). The first three lanes correspond to samples obtained from normal leaves, whereas the subsequent four correspond to the hyperhydric ones.

phosphate dehydrogenase, phosphofructokinase) and OPP (6-phosphogluconate dehydrogenase, glucose-6-phosphate dehydrogenase) were observed in comparison to normal *Prunus* shoots. According to the authors, in hyperhydric shoots, the low activity of some enzymes involved in glycolysis suggests a general decrease of activity of these pathways. Hyperhydricity also resulted in a general decrease in concentrations of reduced and oxidized pyridine nucleotides, reflecting a reduction in metabolic activity.

Considering the significant BiP induction (*Figure 5*) and the high affinity of the antibody used, probably these proteins are very similar to the soybean binding protein, and expressed in a stressful condition such as hyperhydricity. Hyperhydricity was already associated with the induction of the ER resident, stress-related protein (BiP) in *in vitro* cultured plants (*Fontes et al., 1999; Picoli et al., 2001*).

As a member of the Hsp70 family, the synthesis of BiP is regulated by conditions of physiological stress that promote

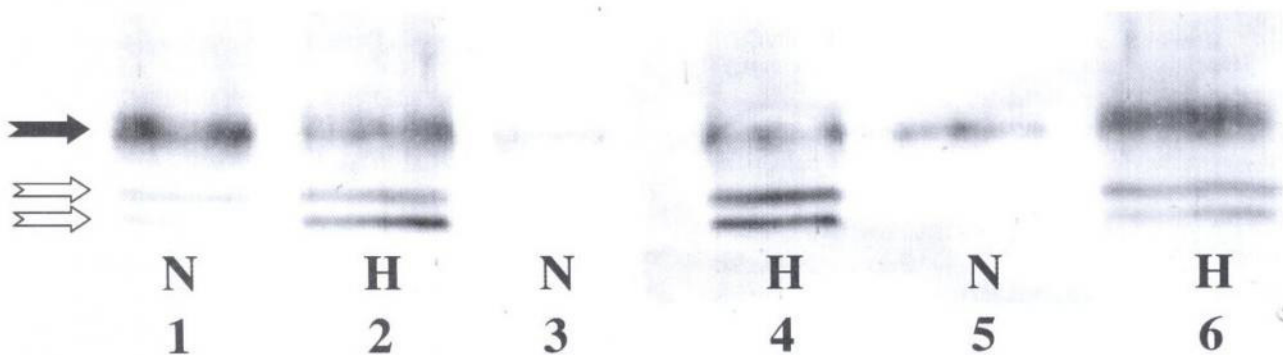


Figure 5 BiP synthesis in normal and hyperhydric eucalypt plants cultivated *in vitro*. Leaf soluble proteins from normal (N) and hyperhydric (H) regenerated shoots were fractionated by SDS-PAGE and the BiP protein was identified by immunoblotting using a polyclonal soybean BiP antibody. Approximately 30 mg total protein was applied to each lane. Frames 1 through 6 correspond to leaf extract from different individuals. Black arrow: expected band from immunoblotting of total protein using an anti-soybean BiP serum; white arrow: additional bands recognized by the same anti-serum.

an increase in cell secretory activity or in the accumulation of misfolded proteins in the ER (Fontes et al., 1991). The up regulation of BiP synthesis under stress condition has been proved to be an efficient approach to monitor intracellular stress in plants (Fontes et al., 1991; Cascardo et al., 2001; Alvim et al., 2001).

Plant BiP expression has also been shown to respond to a variety of abiotic and biotic stress conditions, such as water stress, pathogen attack, nutritional stress, cold acclimation, and elicitors of the plant pathogenesis response (Anderson et al., 1994; Fontes et al., 1999; Dooren et al., 1999; Alvim et al., 2001). Taken together the irregular hyperhydricity frequency in micropropagated eucalypt, BiP expression (Figure 5) and enzymes activity (Figure 4), the present results reinforce the idea of a gradient of stress; resulting in reduced growth. These differentiated protein expression might help detecting a stressful condition, and particularly for hyperhydricity even before the vitreous phenotype is observed.

Our results confirm earlier findings on the negative effects of hyperhydricity on *in vitro* plant morphogenesis and ultrastructure. The stress condition is indeed confirmed by the enhanced BiP expression. Additionally, proteins such as EST, ACP, MDH and POX are possible candidates to be used as stress markers in *in vitro* conditions. Further observation of the epidermis ultrastructure allows us to conclude that not only the reduction of cuticle deposition, but the development of intercellular spaces in the epidermis may be constraining the recovery and development of hyperhydric plants.

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