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ORIGINAL PAPER

Acclimatization of secondary somatic embryos derived plants of *Eucalyptus globulus* Labill.: an ultrastructural approach

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Abstract This paper reports the complete process from secondary emblings (SE-derived plants) regeneration to acclimatization of *Eucalyptus globulus* and describes histocytological changes that occur in leaves from in vitro to ex vitro acclimatization for a 3-month period. After elongation, plants were transferred to pots with sterilized peat:perlite and acclimatized in a phytotron, with progressive reduction of RH and increase of light intensity. Histocytological analyses were performed in fixed material using light microscopy and ultrastructural changes followed by electron microscopy (SEM and TEM). The protocol used allowed the successful acclimatization of the emblings. Plants looked morphologically normal and FCM screening revealed no ploidy or DNA content abnormalities. Histocytological analyses showed significant changes along time, mostly in stomata shape and aperture, starch reserves, chloroplast morphology and mesophyll differentiation. This is the first report concerning emblings acclimatization to ex vitro conditions in *Eucalyptus*. It was clearly demonstrated that during acclimatization emblings suffered profound changes in leaf morphology in order to successfully adapt to ex vitro conditions.

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Introduction

In the last decades somatic embryogenesis (SE) has been developed for rapid multiplication of many plants in a way that changed from simple laboratory curiosity to real industrial interest. The success of any in vitro propagation process on a commercial scale depends on the ability to regenerate a high number of plantlets (at a low cost) and to transfer them to ex vitro conditions with high survival rates (Barry-Etienne et al. 2002; Hazarika 2006). However, acclimatization continues to be a major bottleneck in the commercial application of SE protocols and a high percentage of plantlets are lost or damaged when transferred to ex vitro conditions (Pospisilova et al. 1999).

In a general way, plantlets obtained by SE (emblings) are regenerated in vitro in a semi-solid medium and later acclimatized once they have a few leaves and a root system. In vitro, these plantlets grow in a unique aseptic microenvironment with special conditions in often air-tight cultivation vessels, under low light intensity, on a medium containing sugar and nutrients to allow heterotrophic growth and in an atmosphere with a high level of humidity (Pospisilova et al. 1999; Hazarika 2003, 2006). These conditions frequently result in the formation of plantlets with an abnormal morphology, anatomy and physiology (Hazarika 2006). In fact, these plants are often characterized by poor photosynthetic efficiency and retardation in development of the cuticle and of a functional stomata apparatus (Pospisilova et al. 1999; Hazarika 2006).

Concerning *Eucalyptus,* the loss of in vitro-formed plantlets (obtained by shoot multiplication) during the acclimatization process is also a common problem (Louro 1994) often due to the formation of hyperhydric plants (Louro et al. 1999). Absence of palisade cells, large intercellular spaces and large chlorophyll-rich cells are also common features of in vitro-grown plantlets (Jones et al. 1993; Louro et al. 1999). The high number and volume of chloroplasts in palisade parenchyma, high number of thylakoids per granum and volume of starch grains were the main changes verified during acclimatization of *Eucalyptus saligna* (Jones et al. 1993).

With respect to *Eucalyptus* emblings, most works are restricted to germination frequency, conversion into plantlets and survival rates during acclimatization (Muralidharan et al. 1989; Watt et al. 1991; Pinto et al. 2002a; Prakash and Gurumurthi 2005; Pinto et al. 2008a, b), with little focus on the underlying morphological and physiological processes. Indeed there are no available data on histocytology and on the variability of plantlets obtained from *Eucalyptus* somatic embryos.

Besides SE has the potential to produce large number of genetically identical plants with reduced cost, there are still technical limitations that need to be removed before the process can be used in tree improvement programmes. Understanding the physiological and morphological behaviour of in vitro plants and the changes that occur during the acclimatization process should facilitate the development of an efficient acclimatization protocol.

Also, the production of "true-to-type" plants is a prerequisite for mass clonal propagation. Therefore, it is important to determine the level of clonal fidelity throughout all the process of SE. Genomic mutations sometimes affect the number of chromosomes, and these changes can be detected by flow cytometry (FCM) or by chromosome counting (Tremblay et al. 1999; Pinto et al. 2004). Considering the advantages of FCM, this technique has recently been preferred to screen for genomic changes in SE-derived plantlets (e.g. Pinto et al. 2004; Loureiro et al. 2005).

The aim of this work was to study the internal leaf anatomy of in vitro and ex vitro emblings of *Eucalyptus globulus* (previously screened for genomic stability using FCM) and to analyse the changes that occurred during the acclimatization process by histological and ultrastructural approaches.

Materials and methods

Plant material and plant regeneration from secondary somatic embryos

Eucalyptus globulus Labill. secondary somatic embryos were obtained according to Pinto et al. (2008b, c), and were maintained on MS medium for one year without growth regulators. Clusters of cotyledonary embryos were isolated and then transferred to elongation medium (Celbi internal report, Leirosa, Portugal) consisting of MS medium supplemented with 30 g l^{-1} sucrose and 2.5 g l^{-1} Gelrite®. The pH was adjusted to 5.8 prior to autoclaving and incubated under a photoperiod of 16 h with a light intensity of 23.5 µmol m^{-2} s^{-1}.

To improve root elongation, shoots were transferred to MS medium with 1 mg l^{-1} indole-3-butyric acid (IBA) for 10 days and incubated at 24 ± 1°C in the dark. After this period, emblings were transferred to the same medium without growth regulators and incubated under a photoperiod of 16 h with a light intensity of 23.5 µmol m^{-2} s^{-1}. Media and reagents were supplied by Duchefa (Haarlem, Netherlands).

Assessment of emblings ploidy stability by flow cytometry

In order to assess the putative occurrence of ploidy level variability in the emblings, ploidy analyses were performed using FCM according to Pinto et al. (2004). For this, nuclear suspensions from leaf tissue were prepared according to Galbraith et al. (1983) as described by Pinto et al. (2004). Samples were chopped together with a young leaf of the internal reference standard *Solanum lycopersicum* cv. Stupicke ($2C = 1.96$ pg DNA; kindly provided by J. Doležel, Laboratory of Molecular Cytogenetics and Cytometry, Institute of Experimental Botany, Olomouc, Czech Republic). The relative fluorescence intensity of PI-stained nuclei was measured by a Beckman Coulter® EPICS XL (Beckman Coulter®, Hialeah, FL, USA) flow cytometer and nuclear genome size of 24 *E. globulus* emblings was calculated according to the following formula:

$$E.\ globulus\ 2C\ \text{nuclear DNA content (pg)} = \frac{E.\ globulus\ G_0/G_1\ \text{peak mean FL}}{L.\ esculentum\ G_0/G_1\ \text{peak mean FL}} \times 1.96$$

Emblings acclimatization to greenhouse

Forty plantlets (minimum 2 cm long) were transferred to pots containing sterilized peat:perlite (3:2) and were weekly watered with a commercial solution of 5 ml l^{-1}Complesal-Calcium® (Agrevo). Occasionally, Previcur® (66.5% propamocarbe-hydrochloride, 1.5 ml l^{-1}, Bayer CropScience) and Derosal® (60% de carbendazime, 0.75 g l^{-1}, Bayer CropScience) were added to both plantlets and soil to prevent fungal infections. During acclimatization, plantlets were grown in a phytotron (Snijders) at 24°C with a photoperiod of 16 h and a light intensity of 480 µmol m^{-2} s^{-1}. Initial relative humidity (RH) was higher than 95%, and was weekly decreased until reaching 50–60% RH.

Histological and cytological characterization

For histological and cytological characterisation, samples ($n \geq 4$) were collected from: (a) leaves from in vitro-grown emblings; (b) leaves from emblings at different stages of acclimatization (3 h, 3 days, 1, 2 and 4 weeks and 3 months).

For transmission electron microscopy (TEM), samples were fixed and treated according to Pinto et al. (2008c, 2010) For light microscopy, semi-thin sections (0.5–1.5 μm) of the material embedded for TEM were obtained using a glass knife and stained with 0.1% (w/v) toluidine blue or by the periodic acid-Schiff (PAS) reaction. Samples were analysed in a Nikon Eclipse 80i light microscope (Nikon Corporation, Kanagawa, Japan) and digital photographs were taken using a Leica DC 200 digital camera (Leica Microsystems AG, Wetzlar, Germany).

For scanning electron microscopy (SEM) analysis, material preparation and fixation were performed as previously described by Pinto et al. (2002b). Briefly, leaf samples were fixed with 2.0% (v/v) glutaraldehyde in PIPES buffer, at 4°C for 16 h. The dehydrated samples were submitted to Critical point device (Baltec CPD 030) using CO_2 as transition agent. Samples were then fixed and coated with gold using a JEOL metalizer (FFC-1100) at 1,100–1,200 V, 5 mA during 10 min. Samples were observed in a SEM (Hitachi, S4100) at 20 kV. PIPES buffer was acquired in Duchefa (Haarlem, The Netherlands), while the remaining chemicals were purchased from Agar Scientific (Essex, UK).

Morphometric studies

For morphometric studies, several features within selected regions in microphotographs of samples ($n \geq 10$) from the different acclimatization stages were measured using ImageTool for Windows (version 3.00, University of Texas Health Science Center, San Antonio, TX, USA). The following parameters were assessed: leaf thickness (μm), palisade parenchyma thickness (μm), spongy parenchyma thickness (μm), intercellular space area (μm^2), chloroplasts area (μm^2), number of plastoglobules and starch grains per cell.

Statistical analyses

Statistical analyses were performed using a one-way ANOVA on Ranks (SigmaStat for Windows Version 3.1, SPSS Inc., USA) to assay for possible differences in the morphometric measures among samples from different acclimatization stages. A multiple comparisons Dunn's test was applied to determine which groups were different.

Results

Plant regeneration from secondary somatic embryos

Eucalyptus globulus secondary somatic embryo clusters were maintained for 1 year on MS medium without growth regulators. In these clusters it was possible to follow the differentiation of somatic embryos until the cotyledonary stage (with well defined cotyledons, a vascular system and a root pole). Clusters with cotyledonary embryos were transferred to elongation medium, (data not shown). After this elongation stage it was possible to separate same plantlets from the cluster without damaging the roots (Fig. 1a). However, the separation of SE-derived plantlets from the clusters was often difficult to achieve due to both compactness of the embryogenic cluster and fragility of the plantlet root system (often consisting of only one thin, poorly elongated and phenolized root). In order to overcome these problems and improve root quality, isolated shoots were transferred to a root elongation medium before hardening. This strategy resulted in a higher number of plants that were ready for acclimatization.

Plantlets that were approximately 2 cm long were selected for acclimatization; these plantlets had round, opened stomata on both leaf sides (Fig. 1b). After 2 weeks in a phytotron these plants acquired longer oval stomata with prominent epicuticular wax (Fig. 1c). By this time, these plants (Fig. 1d) had a survival rate of approximately 50%. No obvious morphological differences were found (data not shown) between emblings and seedlings (Fig. 1e). With time, emblings showed a normal morphological development, with well developed leaves and internodes, no sign of stunting, hyperhydricity or chlorosis (Fig. 1f, g).

Assessment of emblings ploidy stability by flow cytometry

Emblings were screened with respect to ploidy stability prior to acclimatization. Emblings analysed by FCM presented a highly homogenous diploid genome size of 1.38 ± 0.02 pg with histograms presenting a typical G_0/G_1 dominant peak and a smaller G_2 peak (Fig. 2). Mean CV values of 3.05% and low background debris were obtained, suggesting a good quality of the obtained histograms. This data suggests that no major somaclonal variation had occurred among the analysed emblings. Therefore, these plantlets can be used for acclimatization.

Emblings acclimatization to greenhouse: histological and cytological characterization

In general, in vitro leaves had a thin cuticle and the mesophyll consisted of one single layer of poorly

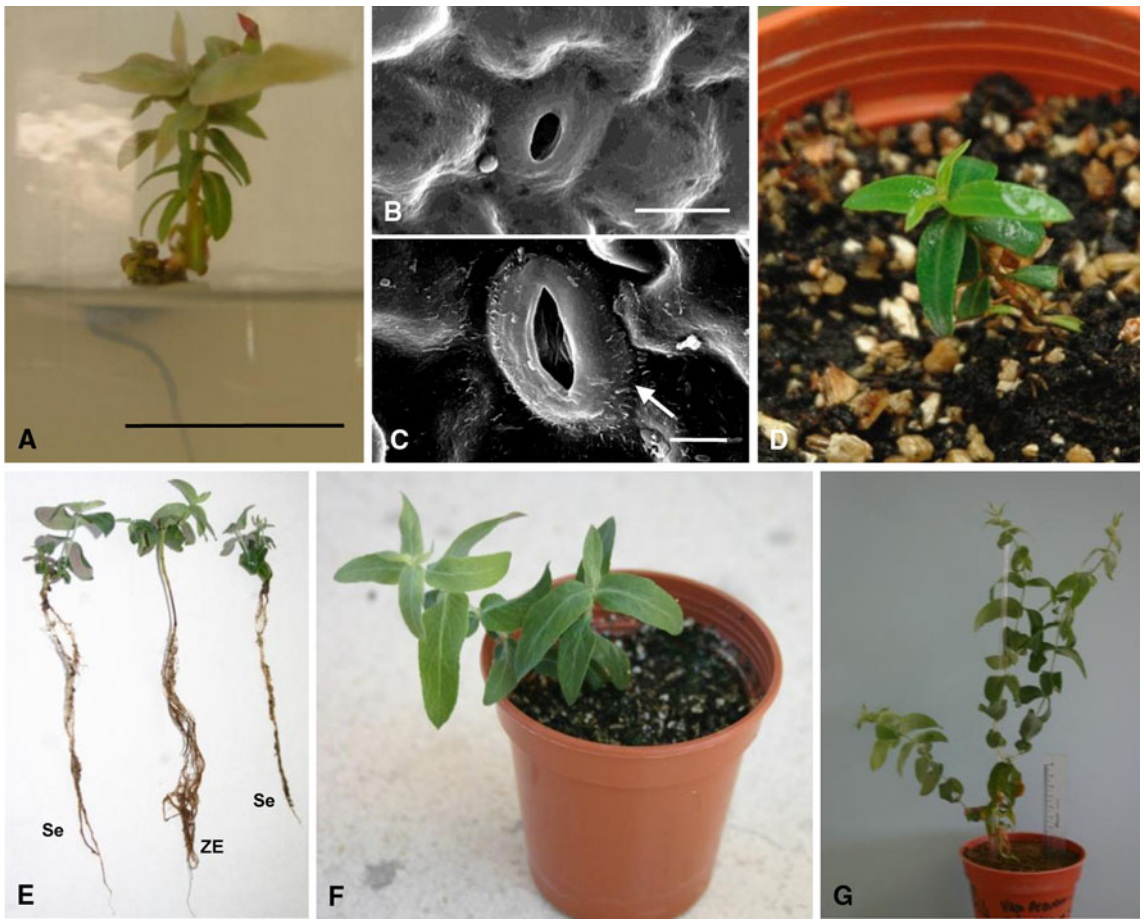

Fig. 1 **a** Embling selected for acclimatization studies (*bars* 1 cm). **b** Round and opened stomata from a in vitro embling (SEM, *bar* 10 μm). **c** Longer and oval stomata from one embling 2 weeks after acclimatization (SEM, *bar* 10 μm). **d** Embling 2 weeks after transfer to ex vitro conditions. **e** Plants derived from zygotic embryo (*ZE*) and somatic embryo (*Se*) conversion. **f** Embling 3 months after transfer to ex vitro conditions. **g** Embling before transfer to open greenhouse

differentiated palisade cells, averaging 25.0 ± 2.0 μm in length (Table 1). The spongy tissue had an average height of 64.0 ± 0.3 μm (Table 1) and consisted of 3–5 small cell layers (Fig. 3a). Chloroplasts were present in both palisade and spongy parenchyma with an average area of 6.50 ± 0.74 μm^2 (Table 1). These chloroplasts were mostly flat–convex and/or concave–convex, had poorly developed grana and small starch grains and were rich in osmiophilic bodies (plastoglobules) (Fig. 3b, c; Table 1). The absence and/or low content of starch grains (0.32 ± 0.09 grains/cell, Table 1) in these in vitro leaves was confirmed by PAS staining (Fig. 3a). Also, no lipid or protein reserves were detected in in vitro leaves at this stage. 3 h after transferring to ex vitro conditions, stomata were in general closed, even when exposed to light (Fig. 3d). Epicuticular waxes were already visible at this stage (Fig. 3e). No histologically significant changes were observed in mesophyll differentiation. Most significant changes ($P < 0.05$) were observed in starch grain density increase (Fig. 3e, 1.33 ± 0.19 grains/cell, Table 1). Some changes also occurred in chloroplasts which increased their size, showed more diverse shapes and less plastoglobules (Fig. 3f) than in the younger in vitro plants (Fig. 3c).

After 3 days, no significant differentiation in the mesophyll was noted (Fig. 3g). However, stomata were already partially opened (Fig. 3h) and starch granule density had decreased significantly ($P < 0.05$) (0.25 ± 0.07 grains/cell) (Fig. 3g, Table 1). Significant changes were also observed in chloroplasts, which were highly irregular and presented higher areas (18.20 ± 1.70 μm^2, Table 1) ($P < 0.05$); these chloroplasts also had highly irregular internal thylakoid membranes and maximal deposition of plastoglobules (Fig. 3i, j).

One week later, no significant changes ($P < 0.05$) in mesophyll layers differentiation were observed (Fig. 4a; Table 1), though the difference between chloroplasts density in palisade layer versus spongy ones seemed to augment (Fig. 4a), a characteristic that remained constant in the subsequent stages of the acclimatization process. Plastids reassumed flat-convex and/or biconvex shapes

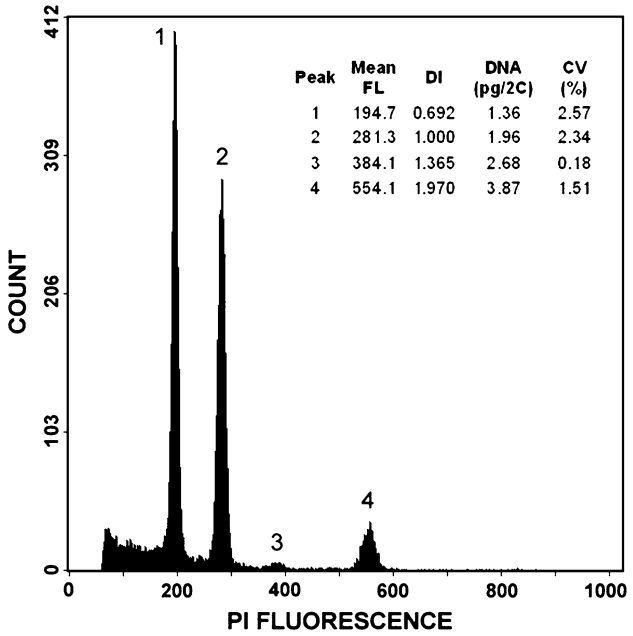

Fig. 2 Histogram of relative fluorescence intensity (PI fluorescence) obtained after simultaneous analysis of nuclei isolated from leaves emblings and *Solanum lycopersicum* cv. Stupicke ($2C = 1.96$ pg DNA, as an internal reference standard). The following four peaks were observed: *1* nuclei at G_0/G_1 phase of *E. globulus*; *2* nuclei at G_0/G_1 phase of *S. lycopersicum*; *3* nuclei at G_2 phase of *E. globulus*; *4* nuclei at G_2 phase of *S. lycopersicum* leaves. The mean fluorescence (Mean FL, channel numbers), DNA index (DI, ratio between the mean channel number of sample and reference standard), nuclear DNA content (DNA, pg/2*C*) and coefficient of variation (CV, %) of DNA peaks are given

(Fig. 4b, c), while their average size decreased significantly when compared to average size of the third day ($P < 0.05$; Table 1). Thylakoid membranes showed a higher organization on day 3, for plastoglobules richness, similar values were obtained (Fig. 4c). After 1 week of acclimatization, PAS staining did not reveal any significant carbohydrates reserves (Fig. 4a) which was confirmed by the lack, or rare presence, of starch grains in the chloroplasts (Fig. 4c, Table 1). Also, no lipid or protein reserves were detected.

By the second week, leaf thickness increased significantly ($P < 0.05$, 222.2 ± 6.0 μm) together with cuticle thickness (Fig. 4d). Palisade parenchyma was already well differentiated (this is the first stage where statistically significant differences could be observed at $P < 0.05$, Fig. 4d, Table 1). Carbohydrate reserves could be detected using the PAS reaction, mostly in palisade parenchyma where chloroplasts predominated (Table 1). Ultrastructural analyses confirmed this result by showing chloroplasts with a higher number of starch grains (0.98 ± 0.22, Fig. 4e).

One month after acclimatization, leaf thickness was similar to that found after 2 weeks, but the main vascular strand was more developed (Fig. 4f). Once again, mainly in palisade parenchyma, carbohydrate accumulation was detected by PAS (2.39 ± 0.22 grains/cell, $P < 0.05$, Table 1) and chloroplasts had one or more large starch grains while plastoglobule density decreased (Fig. 4g, Table 1).

Finally, after 3 months acclimatization, leaves showed well-differentiated palisade and spongy mesophyll with typical characteristics (e.g. predominance of chloroplasts in palisade cells) (Fig. 4h). After this period, the density of starch grains was the highest so far (2.96 ± 0.22 grains/cell, Table 1). Plastoglobules density in chloroplasts increased slightly (Fig. 4i).

Discussion

Plant transfer from in vitro to ex vitro conditions leads to substantial changes in leaf morphology and anatomy in epidermal characteristics, leaf thickness, leaf mesophyll differentiation, chloroplast number and structure (Pospisilova et al. 1999; Hazarika 2003, 2006). This paper shows that significant changes in leaf morphology and metabolism (as shown by starch grains) occur during acclimatization of in vitro plants derived from SE in *E. globulus*. In fact, improving acclimatization survival rates requires a better knowledge of the structural, histological and functional changes occurring in plants during this process.

Table 1 Dimensions (μm) of tissues and chloroplast as well as number of plastoglobules and starch grains per cell in leaves of emblings produced through SE from *E. globulus* during acclimatization (average ± standard error)

Time	Leaf thickness (μm)	Palisade parenchyma thickness (μm)	Spongy parenchyma thickness (μm)	Intercellular spaces (μm^2)	Chloroplast (μm^2)	Plastoglobuli per cell	Starch grains per cell
0	107.0 ± 6.0a	25.0 ± 2.0a	64.0 ± 3.0a	27.0 ± 4.0a	6.5 ± 0.7b	2.3 ± 0.9b	0.3 ± 0.1a
3 h	116.0 ± 5.0b	27.0 ± 1.0a	64.0 ± 3.0a	27.0 ± 5.0a	6.6 ± 1.7b	2.7 ± 1.3a	1.3 ± 0.2b
3 days	116.0 ± 5.0b	21.0 ± 1.0a	78.0 ± 5.0a	98.0 ± 15.0b	18.2 ± 1.7c	8.3 ± 1.4a	0.2 ± 0.1a
1 weeks	116.0 ± 1.0ab	25.0 ± 1.0a	66.0 ± 1.0a	67.0 ± 13.0ab	7.3 ± 1.1b	7.0 ± 1.1a	0.1 ± 0.05a
2 weeks	222.0 ± 6.0c	51.0 ± 2.0b	158.0 ± 4.0c	137.0 ± 28.0bc	5.0 ± 0.1a	3.0 ± 1.0a	1.0 ± 0.2ab
4 weeks	203.0 ± 10c	48.0 ± 3.0b	112.0 ± 5.0bc	144.0 ± 18.0c	6.8 ± 1.1b	1.3 ± 0.4b	2.4 ± 0.2c
12 weeks	165.0 ± 3.0bc	40.0 ± 1.0b	82.0 ± 3.0ab	229.0 ± 3.0c	5.6 ± 0.6b	4.5 ± 0.6a	2.9 ± 0.3c

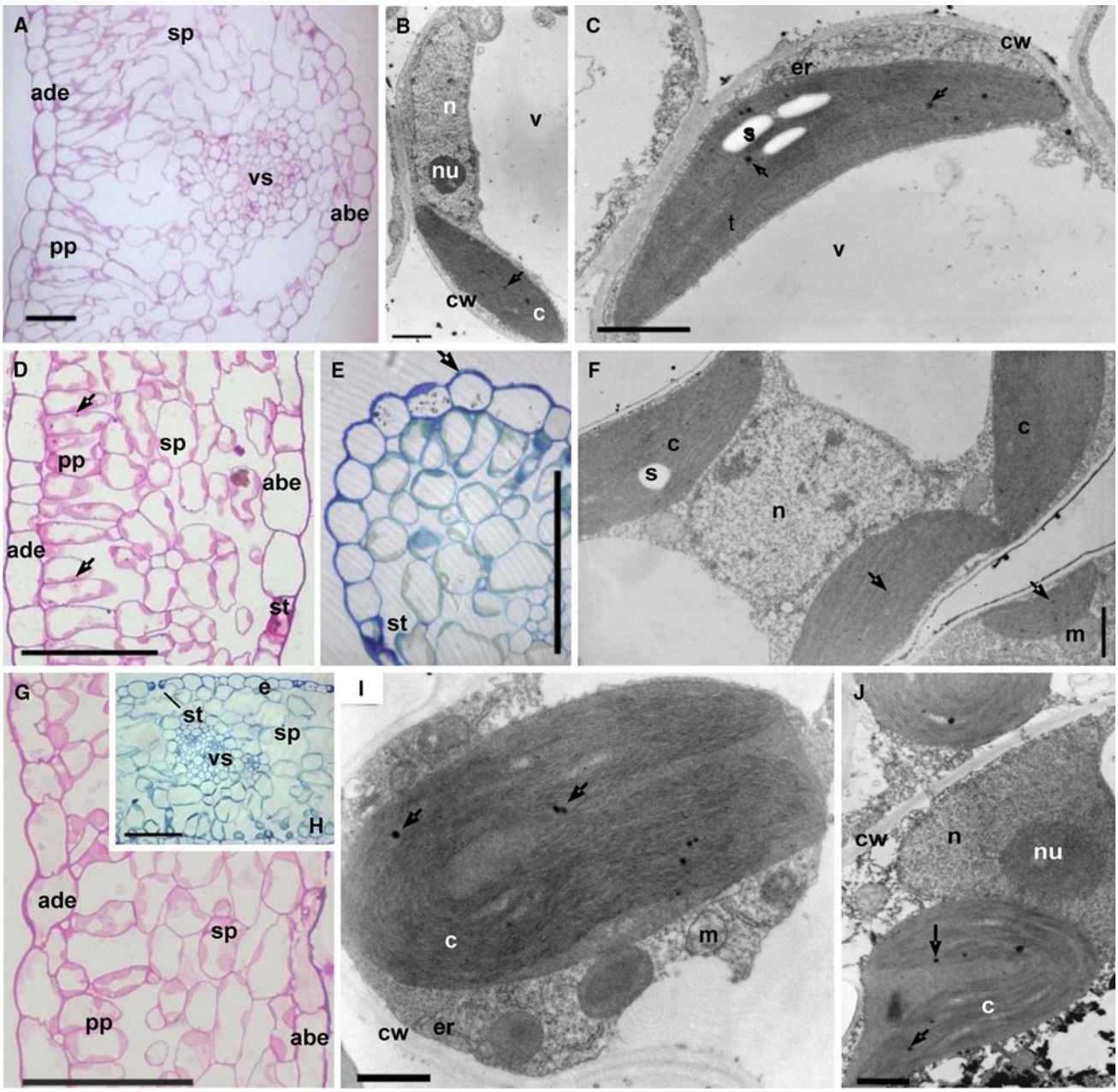

Fig. 3 **a–c** In vitro leaf: **a** light microphotographs of transversal leaf sections, stained with PAS showing poorly differentiated mesophyll (*bars* 50 μm). **b** and **c** TEM microphotographs showing vacuolated mesophyll cells with chloroplasts, starch grains and plastoglobules (*arrow*) (*bars* 1 μm). **d–f** Leaf sections, 3 h after transfer to ex vitro conditions: **d** and **e** light microphotograph of transversal leaf sections, stained with PAS (**d**, *arrows*: starch) and toluidine blue 0.1% (*E*) showing poorly differentiated mesophyll, closed stomata and epicuticular wax at the epidermal cells (*E*) (*bars* 50 μm). **f** TEM microphotograph showing vacuolated mesophyll cells with chloroplasts, starch grains and plastoglobules (*arrow*) (*bars* 1 μm). **g–j** leaf sections, 3 days after transfer to ex vitro conditions: **g–h** light microphotograph of transversal leaf sections, stained with PAS (**g**, no carbohydrates stained) and with toluidine blue (**h**) showing poorly differentiated mesophyll, partially opened stomata (*bars* 50 μm). **i–j** TEM microphotographs showing vacuolated mesophyll cells with chloroplasts with high irregular shapes and internal thylakoidal arrangements, and with plastoglobules (*arrows*) (*bars* 1 μm). *ade* adaxial epidermis, *abe* abaxial epidermis, *pp* palisade parenchyma, *sp* spongy parenchyma, *st* stomata, *vs* vascular strand, *cw* cell wall, *c* chloroplast, *er* endoplasmic reticulum, *m* mitochondria, *s* starch granule, *n* nucleus, *nu* nucleolus, *pl* plasmodesmata, *v* vacuole

Eucalyptus globulus in vitro leaves presented a very thin cuticle that increased with acclimatization time. Acclimatization studies with other species also showed that, in general, cuticle deposition increases as this process advances (Pospisilova et al. 1999). Poor deposition of cuticle on in vitro leaves, allowing high rates of non-stomata transpiration, has been regarded as a critical factor responsible for excessive water loss during acclimatization, which

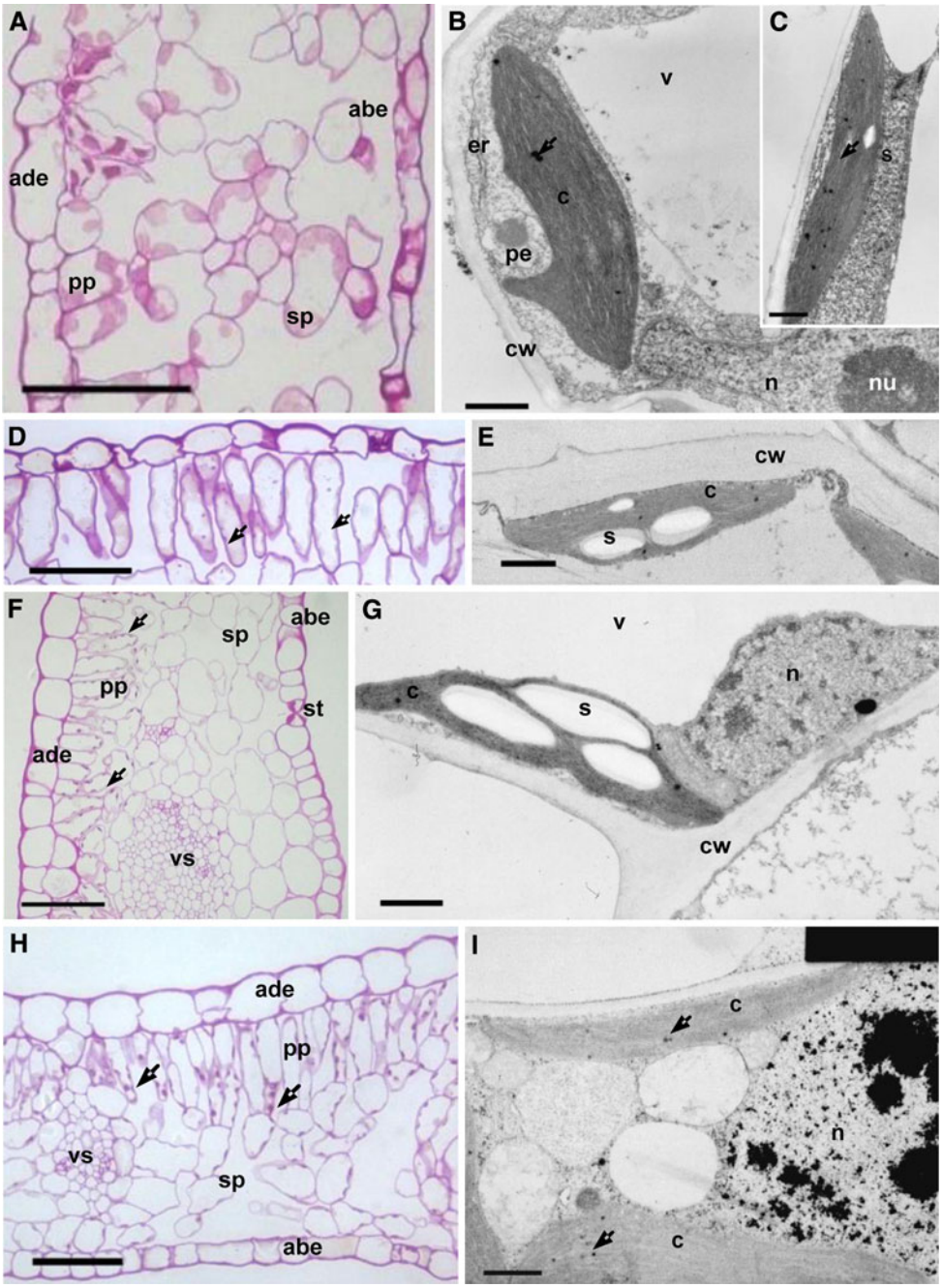

Fig. 4 **a–c** Leaf sections, 1 week after plant transfer to ex vitro conditions: **a** light microphotograph of transversal leaf sections stained with PAS showing poorly differentiated mesophyll (*bars* 50 μm). **b–c** TEM microphotographs showing vacuolated mesophyll cells with regular-shaped chloroplasts, with thylakoidal arrangements and with plastoglobules (*arrows*) (*bars* 1 μm). **d**, **e** Leaf sections, 2 weeks after aclimatization conditions: **d** light microphotograph of a transversal leaf section stained with PAS (*arrows*: starch) showing differentiated mesophyll (*bar* 50 μm). **e** TEM microphotograph showing vacuolated mesophyll cells with regular shaped chloroplasts with thylakoidal arrangements starch grains (*bars* 1 μm). **f**, **g** Leaf sections, 1 month after plant transfer to ex vitro conditions: **f** light microphotograph of a transversal leaf section stained with PAS (*arrows*: carbohydrate staining) showing well differentiated mesophyll and the main vascular strand (*bar* 50 μm). **g** TEM microphotograph showing vacuolated mesophyll cells with regularly shaped chloroplasts rich in starch grains (*bars* 1 μm). **h**, **i** Leaf sections, 3 months after plant transfer to ex vitro conditions:**h** light microphotograph of a transversal leaf section stained with PAS (*arrows*: carbohydrate staining, *bars* 50 μm). **i** TEM microphotographs showing mesophyll cells with regularly shaped chloroplasts (*bar* 1 μm). *ade* adaxial epidermis, *abe* abaxial epidermis, *pp* palisade parenchyma, *sp* spongy parenchyma, *st* stomata, *vs* vascular strand, *cw* cell wall, *c* chloroplast, *er* endoplasmic reticulum, *n* mitochondria, *s* starch granule, *n* nucleus, *nu* nucleolus, *v* vacuole

may decrease plant survival (Hazarika 2006). The stomata of *E. globulus* emblings showed an amphistomatous distribution both in vitro and during acclimatization, supporting previous data for this species under field conditions (Pereira et al. 1987). Louro et al. (1999) described a similar distribution in in vitro plants of *Eucalyptus* hybrids, which was then replaced by a hypostomatous organization after acclimatization (Louro et al. 2003). Curiously, the amphistomatous distribution in *E. globulus* was reported to be a characteristic of adult-type leaves while it has a hypostomatous distribution in juvenile leaves (James et al. 1999). 1 week after transfer to ex vitro conditions, stomata morphology changed from circular (characteristic of the in vitro cultured plants) to elliptical. Stomata behaviour also changed along the process of plantlet hardening. In vitro leaves showed full opened stomata while a full closure was observed during the first hours of acclimatization. This suggests that these plants possess a short-term mechanism of stomata regulation that may rapidly evolve from one status to another and thus controlling aperture and water losses in recently acclimatized plants.

The mesophyll of *E. globulus* in vitro leaves was weakly differentiated, as has already been reported for other micropropagated plants like *Liquidambar stryraciflua* (Wetzstein and Sommer 1982) and *Eucalyptus* hybrids (Louro et al. 1999). In general, leaves of in vitro-grown plants lacked a differentiated palisade parenchyma and presented a spongy parenchyma interspersed with large air spaces when compared to greenhouse-grown/acclimatized plants (Pospisilova et al. 1999; Hazarika 2003, 2006). *E. globulus* in vitro leaves showed dorsiventral characteristics identical to those of ex vitro young leaves (James et al. 1999). Similarly, in *Eucalyptus* hybrids transferred from in vitro to ex vitro conditions, an increase in the size and number of mesophyll cells occurred, thus providing a better distinction between palisade and spongy parenchyma (Louro et al. 2003).

As expected, the carbohydrate reserves decreased in the first period of acclimatization. This response is probably due to the adaptation period of leaves to new environmental conditions, because in the first days of acclimatization the stomata were kept continuously closed, thus compromising photosynthetic efficiency. This response suggests that during the first days, plants are mostly mixotrophic, with the transition for a fully autotrophic regime starting thereafter. This transition is revealed by an increase of the starch content after longer periods of acclimatization, which is in agreement with previous results for other species (e.g. Van Huylenbroeck and Debergh 1996). The development of photoautotrophy in micropropagated plants represents one of the most important turnovers during the transition from in vitro to greenhouse conditions (Piqueras et al. 1998). It has also been demonstrated that an increase of exogenous carbohydrate reserves (sucrose) leads to higher starch and sucrose contents in micropropagated plants, which improves the success of acclimatization and speeds up physiological adaptations (e.g. Piqueras et al. 1998). Such an increase may also advantageously decrease the medium osmotic potential to which the in vitro plants must adapt before acclimatization.

During *E. globulus* acclimatization, the most marked changes occurred in the chloroplasts. A few hours after transfer to ex vitro conditions, these plastids acquired unexpected forms with the loosely organised internal membranes bending in several shapes. Simultaneously, starch grain density decreased, which suggested a strong pressure in plastid turnover during the first days of acclimatization. Besides starch content, thylakoidal organization and plastoglobules density suffered the most visible change during the early acclimatization period. As lipid and protein reservoirs, plastoglobules are very dynamic particles that accumulate in young leaves (Kessler et al. 1999) or during thylakoid disassembly in senescing chloroplasts (Ytterberg et al. 2006). These structures may play a role in the removal of protein catabolites as part of thylakoid turnover (Smith et al. 2000). In our emblings the transient plastoglobule accumulation during the plastid transition (decreasing after 1 and 3 months of acclimatization), may represent a lipid reservoir that allows the rapid formation of thylakoids and support the structural plastid polymorphism during the critical first stages of acclimatization.

There are still some plant quality problems in somatic embryo derived plants, namely, the fragility of the root system that hampers plant isolation from clusters. Studies carried out on other species demonstrated that plantlets propagated by organogenesis frequently had deficient vascular connections between the root system and the stem and that roots formed in vitro were often non-functional (Grout and Aston 1977; Debergh and Maene 1981; Zimmerman 1981). Root elongation in vitro was insufficient in the primary SE process of *E. globulus*, where only a few plantlets were obtained (Pinto et al. 2002a. This constraint in root formation and/or elongation is common within the *Eucalyptus* genus (Eldridge et al. 1993). To overcome this problem, it was decided to improve the in vitro rooting elongation of emblings, before transferring them to ex vitro conditions. This strategy was efficient and appropriate. A similar strategy was used with success in the SE process of *Castanea sativa* (Corredoira et al. 2003).

Since the emblings were obtained from clusters maintained in vitro for 3 years, all plantlets were screened for morphological and ploidy abnormalities before starting acclimatization. This screening revealed no morphological variations and a homogeneous nuclear DNA content. This shows that plants obtained by repetitive SE are "true to

type" propagation method. This is in accordance to what was previously reported by Pinto et al. (2004). The 2C nuclear DNA content for these emblings (1.38 pg/2*C*) was similar to the values reported for somatic embryos (1.39 pg/2*C*) and leaves of field plants (1.40 pg/2*C*) (Pinto et al. 2004).This ploidy stability demonstrates that our SE-protocol allows long term in vitro culture. However, there are still technical limitations that need to be corrected before the process can be used in large-scale tree improvement programmes. So far as we know, this is the first report about of *E. globulus* emblings acclimatization. Our results represent the first step towards a better histocytological understanding of SE-derived plants, prior and during ex vitro acclimatization.

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