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Quantum Flux Density Effects on the Anatomy and Surface Morphology of in Vitro- and in Vivo-developed Sweetgum Leaves

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Abstract. **The anatomy of in vitro- and in vivo-developed leaves of sweetgum, *Liquidambar styraciflua* L., grown under three quantum fluxes (PPF), was evaluated using light and scanning electron microscopy. Leaf characteristics of both in vitro- and in vivo-developed plants were modified by light: high irradiance was associated with more compact mesophyll and larger cells than low irradiance. However, when compared to plants grown in vivo under corresponding irradiance levels, all plants grown in vitro had smaller, thinner leaves and smaller mesophyll cells lacking extensive vacuolar components. Leaves developed in vitro had larger, raised stomata regardless of light level and, except at the highest irradiance, exhibited significantly greater stomatal densities than in vivo-developed leaves.**

Tissue-cultured plantlets reportedly have a very divergent leaf anatomy and physiology, compared to noncultured plants, such as reduced mesophyll differentiation with extensive intercellular spaces, decreased cuticle and wax development, and stomata that are raised, enlarged, of increased density, and with diminished functioning (3, 7, 9, 10, 21, 29–31). As a result, cultured plants require an acclimation period during the transition between culture and field or greenhouse conditions, which usually consist of a period with a gradual decrease in humidity.

The development of tissue culture protocols often is centered around regeneration or multiplication rates. Less consideration has been given to plantlet form or integrity. We have been interested in the development of sweetgum plantlets in culture, and the effects of environment on differentiation. The purpose has been to determine how environmental factors and conditions in culture can be modified to produce plants that are more competent for field survival, with a lower mortality, and decreased acclimation requirement. Such information, if applied to culture protocols, could increase the efficiency of culture systems and allow tissue culture methods to be economically adaptable to a greater number of species.

Light intensity can have a pronounced effect on leaf development and can modify characteristics, such as leaf thickness, mesophyll differentiation, vascular development, cell division, and stomatal development (16, 27). We have reported that light intensity markedly influences photosynthesis, chlorophyll content, and chloroplast ultrastructure in evaluations of *Liquidambar* cultures, and that lack of photosynthetic capacity in this system is not a limiting characteristic in plantlet acclimatization and transplant growth (15). In this case, difficulties in transplant survival are instead most likely related to water relations adaptations, as these plants exhibit reduced cuticular development, extensive intercellular spaces in the mesophyll (30), and divergent stomatal configurations with reduced functioning (31).

Studies have reported divergences in leaves developed in vitro vs. in vivo. However, most have not compared leaf development under the same light conditions but rather have compared leaves developed in culture vs. leaves of plants grown in the greenhouse or field (3, 6, 25, 30). Similarly, the effect of differences in light during in vitro development has not been evaluated critically. Grout and Aston (11) compared cultured vs. seedling plants under similar light conditions. However, comparisons were of CO_2 fixation and photosynthesis, and were limited to a single light level. Sutter and Langhans (26) compared cultured vs. seedling leaves under similar environments, but evaluated epicuticular wax and water losses. The objective of this study is to evaluate the effects of PPF on leaf anatomy and stomatal development in leaves from plants developed in vitro and in vivo to determine if light modification in culture can produce plants with a more normal morphology.

Materials and Methods

Tissue cultures of sweetgum were rooted from adventitious shoots initiated from hypocotyl segments of 1-month-old seedlings, using methods previously described (22, 23). Plantlets were selected for uniformity and vigor and placed into a growth room maintained at 26° ± 2 °C with a 16-hr photoperiod, under one of three PPF regimes: 50 ± 5 $\mu mol \cdot s^{-1} \cdot m^{-2}$(low irradiance), 155 ± 10 $\mu mol \cdot s^{-1} \cdot m^{-2}$ (medium irradiance), and 315 ± 15 $\mu mol \cdot s^{-1} \cdot m^{-2}$ (high irradiance). Photosynthetically active radiation was measured using a LI-190S quantum sensor. Illumination was provided by Sylvania metal halide lamps suspended 1.2 m above table height. Light intensity was adjusted by use of cheesecloth. One-month-old seedlings germinated in coarse perlite were grown in the same growth room, under the same light treatments for comparison (in vivo plants), and were fed weekly with the same inorganic salts as used in the culture meduim.

Cultures had been in their respective environments for 40 days when sampled for anatomical study. Leaf samples were obtained from the second newly developed leaves formed. For light microscopy, samples were fixed in 2% glutaraldehyde in 0.1 M cacodylate buffer for 2 hr, rinsed in buffer several times, and post-fixed with 2% osmium tetroxide in the same buffer for 2 hr. Following dehydration through an ethanol series, samples were infiltrated and embedded in Spurr's (24) low-viscosity medium. Sections (0.5 to 1 μm) were cut on a Sorval MT-2 ultramicrotome and stained with toluidine blue and basic fuchsin.

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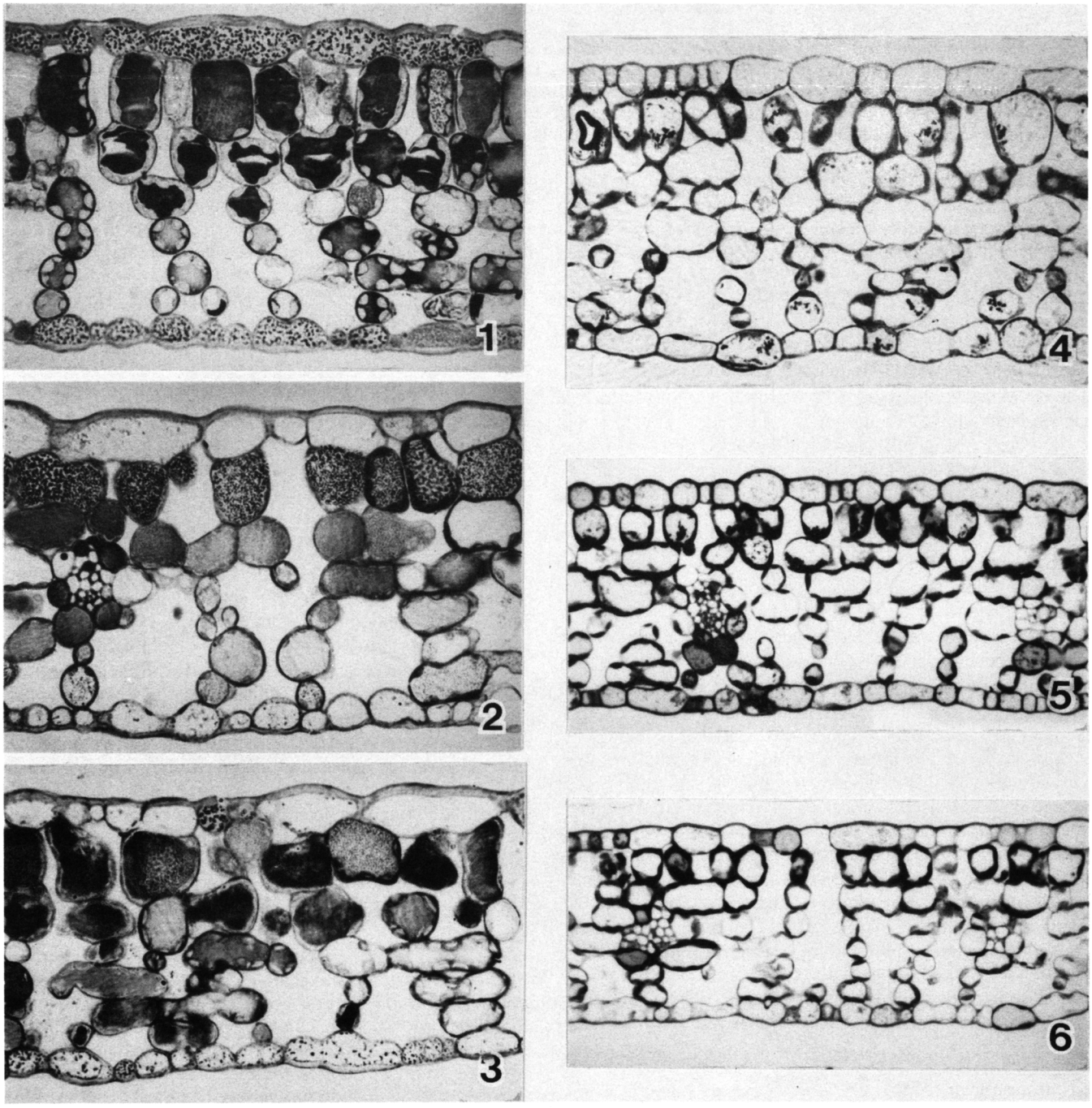
For scanning electron microscopy (SEM), samples were fixed in 2% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.2, dehydrated in an ethanol series, critical-point-dried through CO_2, mounted on stubs, and sputter-coated with gold/palladium. Samples were observed with a Cambridge Mark IIA SEM or a Phillips 505 SEM.

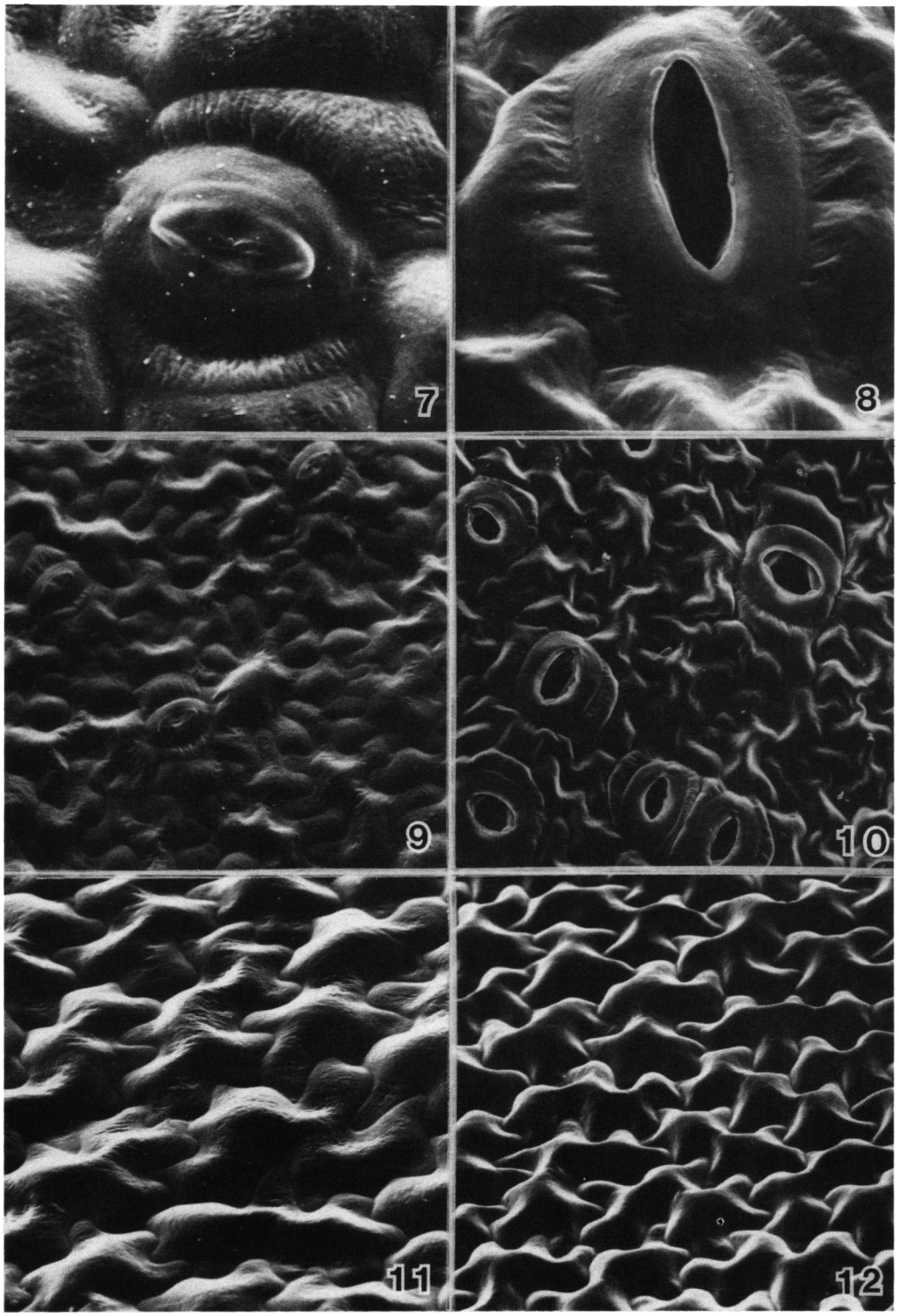
Results

Leaf anatomy. Transverse sections of leaves from both in vivo (Figs. 1–3) and in vitro developed plants (Figs. 4–6) showed that the laminae exhibited a typical dorsiventral character that varied with different light treatments. The effects of PPF on various leaf characteristics of leaves developed under in vitro or in vivo conditions is shown in Table 1.

Leaves from in vitro-cultured plantlets (Figs. 4–6) developed under different light intensities were modified by light level. Leaves developed under high irradiance (Fig. 4) were thicker, had a more differentiated mesophyll, and larger cells than leaves developed under low irradiance (Fig. 6). Leaves developed under intermediate irradiance had characteristics intermediate to high and low levels. Leaves developed in vitro under high ir-



Figs. 1–6. Light micrographs of transverse sections of *Liquidambar* leaves developed under different PPF, ×1200. (**1–3**) Leaves developed in vivo. (**1**) High irradiance, (**2**) medium irradiance, (**3**) low irradiance. (**4–6**) Leaves developed in vitro. (**4**) high irradiance, (**5**) medium irradiance, (**6**) low irradiance.



Figs. 7–12. Scanning electron micrographs of leaf surfaces. (**7**) Leaf developed in vivo showing depressed stomate with ellipsoid guard cells. ×1900. (**8**) Leaf developed in vitro with enlarged elevated stomate. ×1900. (**9**) Abaxial surface of in vivo-developed leaf. Note stomatal density and closed stomata. ×600. (**10**) Abaxial surface of leaf developed in vitro. Stomata are numerous and open. ×600. (**11**) Abaxial surface of leaf developed in vivo with large epidermal cells with sinuous anticlinal walls. ×576. (**12**) Abaxial surface of leaf developed in vitro with smaller, raised epidermal cells. ×576.

radiance had thicknesses similar to those of in vivo leaves. Leaf area was not affected by light level (Table 1).

In comparison, characteristics of in vivo-differentiated leaves (Figs. 1–3) were modified with light intensity to a lesser extent. Under high irradiance, leaves had a relatively compact and elongated mesophyll. Leaves developed under high irradiance (Fig. 1) had one to two layers of palisade mesophyll cells. Whereas leaves developed under low irradiance (Fig. 3) had only one layer of partially differentiated palisade cells. Leaves developed under low irradiance had slightly fewer cell layers than leaves

Table 1. Leaf characteristics of in vitro and in vivo sweetgum leaves developed under 315 (high), 155 (medium), and 50 (low) $\mu mol \cdot s^{-1} \cdot m^{-2}$.

Leaf characteristic	Irradiance		
	High	Medium	Low
Thickness (μm)			
In vitro	114 a[z]	107 ab	101 b
In vivo	130 a	124 a	123 a
Significance[y]	**	*	**
Leaf area (cm^2)			
In vitro	5.0 a	4.7 a	3.9 a
In vivo	14.9 a	16.4 a	20.2 a
Significance	**	**	**
Stomatal density ($no./mm^2$)			
In vitro	370 a	353 ab	305 b
In vivo	330 a	148 b	127 b
Significance	NS	**	**

[z]Means separated in rows by Duncan's multiple range test, $p = 5\%$.
[y]Significance within leaf characteristic and column by F-test.
**,*,ns Significant at the 1% and 5% levels and not significant, respectively.

developed under high irradiance. Neither leaf area nor total leaf thickness were significantly affected by light level (Table 1).

Differences in leaf characteristics were observed in comparisons between leaves initiated in vitro vs. in vivo, even when developed under corresponding light levels, i.e., high irradiance (Fig. 1 vs. Fig. 4), medium irradiance (Fig. 2 vs. Fig. 5), or low irradiance (Fig. 3 vs. Fig. 6). Leaves of plantlets developed in vitro were smaller, thinner, and had smaller mesophyll cells than leaves developed in vivo under corresponding light intensities (Table 1). In vivo-developed leaves had an abundance of dark osmiophilic staining deposits (Figs. 1–3), which were generally absent in cultured plantlet leaves.

Surface morphology. Light intensity had little effect on leaf surface morphology within in vivo or in vitro groups. However, distinct differences were evident in comparisons between the two groups. Stomata of leaves developed in vivo (Figs. 7 and 9) were depressed and had ellipsoid guard cells, whereas in leaves of plants grown in vitro, stomata were larger, more elevated, rounded, and with raised subsidiary cells (Figs. 8 and 10). Stomata of in vivo and in vitro leaves had average lengths of 20 and 28 μm, respectively. Stomata in all instances were confined to abaxial surfaces. Stomata of leaves developed in vitro were observed to be consistently open, whereas those of in vivo leaves were frequently closed. Adaxial epidermal cells of in vivo- (Fig. 11) and in vitro-grown (Fig. 12) leaves had sinuous anticlinal walls. In vitro-grown leaf epidermal cells were smaller than seedling epidermal cells.

Stomatal densities were affected by PPF (Table 1). Greater stomatal densities were associated with higher light levels. However, except at the highest irradiance, in vitro-grown leaves had significantly greater stomatal frequencies under corresponding levels.

Discussion

The anatomy of both in vitro- and in vivo-developed leaves was altered by light intensity. Sweetgum leaves developed in vitro exhibited foliar modifications similar to those reported in other deciduous tree species. Generally, leaves differentiated under low light levels are thinner than those differentiated at high light levels, have a less differentiated mesophyll, and a higher proportion of intercellular spaces (e.g., refs. 12, 13, 17, 33). Observations of leaf expansion in *Helianthus annuus* (5) under different light intensities indicated a phenotypic response to lowered light intensity, primarily by a reduction in cell division resulting in reduced leaf area, and secondarily by modification of cell expansion in a plane perpendicular to paradermal, resulting in thinner, shaded leaves. The enhancement of final cell number and cell size under high irradiance also has been reported by Milthorpe and Newton (18). Although in vivo leaves exhibited some modifications in palisade elongation, leaf thickness was not responsive to light changes.

Increasing the PPF in culture results in thicker, more compact leaves and may be a means of obtaining more "normal", functional leaves in vitro. Leaves developed under high irradiance were thicker than those developed under low irradiance and exhibited a leaf anatomy more like seedling leaves. However, in vitro-grown leaves consistently were thinner and had smaller cells than leaves developed in vivo, under similar light levels. This observation is in accord with results of Smith et al. (21). In comparisons of in vitro and seedling leaves, they found that in vitro leaves were thinner than in vivo leaves and had less palisade development, although both were grown under a similar light condition (20 to 30 $\mu mol \cdot s^{-1} \cdot m^{-2}$). Our study shows this relation to occur, even under much higher light levels.

Stomatal configuration was little affected by quantum flux. However, conditions of in vitro vs. in vivo environments had a pronounced effect on stomatal topography. Leaves of plantlets in culture exhibited raised guard cell pairs and gaping, open stomata, regardless of light condition. Stomata in sweetgum (31) and other cultured plantlets (1, 2, 28, 29) usually have been observed to be open, whereas those of greenhouse-grown seedlings have been closed.

A delayed stomatal-closure response was observed in in vivo vs. greenhouse-grown *Malus domestica* (2). Stomata of leaves of tissue-cultured apple plantlets did not close in dark treatments, under low osmotic pressures, with exogenous abscisic acid (ABA), or in high atmospheric CO_2. In contrast, greenhouse-acclimatized plantlet leaf stomata closed under each of these treatments. In vitro *Brassica* plantlet leaf stomata did not respond to polyvinyl resin or ABA (28). Brainerd and Fuchigami (2) concluded that the development of functioning mature stomata involves the development of a closure mechanism, and that lack of closure causes the major water loss during transfer of in vitro plantlets to a low-humidity environment. Stomata from in vitro-developed sweetgum leaves were consistently open, suggesting that they may lack a stomatal closure mechanism; leaves to which ABA has been applied exogenously have stomata that are nonresponsive and remain open (30).

A tendency for increasing stomatal densities was observed under increasing light levels in both in vitro and in vivo conditions. Leaves developed under high light intensity also have been reported by others to have greater stomatal numbers than leaves under low light levels (8, 14, 32). However, except at the highest light level, in vitro leaves exhibited significantly greater stomatal densities than in vivo leaves. In addition to irradiance, stomatal differentiation can be affected by factors such as CO_2 concentration, water relations, and hormone levels (4, 19, 20).

This study shows that differences in quantum flux can modify leaf development in vitro. Increased light levels produced thicker leaves in culture, with a more differentiated palisade mesophyll. The leaf anatomy of these plantlets appeared more seedling-like than in vitro leaves grown under low irradiance. However, nei-

ther in vitro nor in vivo leaves developed under the highest light levels expressed the degree of sun leaf characteristics previously seen in field leaves of sweetgum (30). High light levels in this study were limited to $\approx 315\ \mu mol \cdot s^{-1} \cdot m^{-2}$. Preliminary studies showed that higher light levels adversely affected cultures, resulting in leaf chlorosis.

Increasing light irradiance in culture thus can be a means of improving the anatomy of leaves differentiating in culture. Thick leaves, with large differentiated mesophyll cells, can be induced. However, water deficit problems, may be associated with greater stomatal numbers and raised, nonfunctional stomata, are not reversed by culture development at high light levels. Factors unique to culture conditions other than light are contributing to these leaf modifications and are areas for further evaluation.

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