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Comparative Anatomy and Physiology of Microcultured, Seedling, and Greenhouse–grown Asian White Birch

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Abstract. **Anatomical and physiological comparisons were made between tissues of *Betula platyphylla* var. *szechuanica* (Schneid.) Rehd. (Asian white birch) obtained directly from microcultured shoots, from seedlings grown in a microculture-like environment, or from micropropagated plants grown in a greenhouse. The diminished stature of microcultured shoots resulted primarily from reduced cell division, although their leaves had reduced cell size as compared to shoots from greenhouse-grown plants. The area occupied by vascular tissue in midrib veins and petioles was significantly reduced in microcultured shoots as was the extent of the palisade layer, indicating that cell differentiation had also been altered. The photosynthetic capacity of leaves from microcultured shoots was less than half that of leaves from greenhouse-grown plants. However, this capacity was restored after non-in vitro rooting of the microcultured shoots. Unlike greenhouse-grown plants, photosynthetic rates of leaves from microcultured shoots were not influenced by decreasing light intensities from 1200 to 200 $\mu mol \cdot s^{-1} \cdot m^{-2}$ nor was the respiration rate strongly inhibited by cyanide. Seedlings from a high-humidity, microculture-like environment generally had characteristics intermediate between those of microcultured and greenhouse-grown tissues, indicating that the environment and not the juvenile state of the tissue was probably of overriding importance in influencing these trends.**

Plants grown as shoot cultures are usually much smaller than their greenhouse- or field-grown counterparts. The diminutive size, coupled with the unique environment within a culture vessel, can influence physiological responses of the micropropagated plant. Previous reports have noted altered leaf anatomy in vitro, including reduction of the palisade layer, increase in mesophyll air space, and reduced cuticle development (1, 7, 17). These anatomical modifications, which parallel variations between shade and sun leaves (4), may be consequences of the confinement, high humidity, and relatively low light intensity of the culture environment. Heterotrophic nutritional conditions in vitro and the observed modifications in leaf development have led to the suggestion that shoot cultures may have low or negligible photosynthetic capacity (7, 17).

This study was undertaken to quantify anatomical differences between microcultured and micropropagated greenhouse-grown birch plants, and to determine the extent to which changes in cell division, cell enlargement, and differentiation contribute to the reduced plant size in microculture. Unlike previous investigations, sections of live, nonembedded tissue were compared, permitting analysis without the complicating artifacts of fixation and allowing for observations of physiological cell responses. The influence of shoot growth in vitro on photosynthetic capacity and respiration was evaluated. Seedling tissue of the same line was analyzed further to determine if observed differences between microcultured and greenhouse-grown birch could be related to the more juvenile status of the former.

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Materials and Methods

Plant material. Actively growing shoot cultures of Asian white birch were generated as previously described (14). Each plant was originally established in vitro by excising one-node explants from rapidly growing shoots of 6-month-old stock plants, surface-sterilizing these explants, and placing them on the surface of WPM mineral salts (14) supplemented with 4 μM benzyladenine and 2% sucrose. Cultures were incubated at 25–30°C under 24-hr fluorescent lighting (20–30 $\mu mol \cdot s^{-1} \cdot m^{-2}$, 400–700 nm) in 120-ml bottles containing 30 ml of medium, and capped with Magenta B caps (Magenta Co., Chicago, Ill.). New shoots that grew from these explants were subcultured repeatedly until uniform and reproducible shoot growth and multiplication (via axillary bud growth) occurred. Only well-established shoot cultures of this type were used in the experiments. Shoot cultures were sampled and analyzed 30 to 40 days after the most recent subculture.

Greenhouse specimens originated from microcuttings that were harvested from culture (6 weeks after the most recent subculture), placed in Techniculture plugs (Castle and Cooke Techniculture, Watsenville, Calif.), and rooted in a high humidity chamber under light and temperature conditions identical to the shoot cultures. After 2 weeks under these conditions, the cuttings were acclimated by gradually decreasing humidity over a 2 to 3 week period. Rooted cuttings were then transplanted into a soil/peat/perlite medium and placed into the greenhouse, where the new growth assumed the normal leaf, stem, and petiole sizes characteristic of the species. Greenhouse-grown plants were sampled after 2 to 3 months of active growth.

Seeds (provided courtesy of Evergreen Nursery Company, Inc., Sturgeon Bay, Wis.) were distributed on a 7.62-cm bed of moist vermiculite and covered with a thin layer of vermiculite. The seeds were germinated in a humidity chamber under light and temperature conditions identical to the shoot cultures, as described above. Seedlings were transplanted into a greenhouse 30 days after germination and sampled 10 days later. All of the sampled tissue from seedlings developed fully in the humidity chamber, but, because of extreme succulence, could

not be sectioned for analysis until briefly hardened in the greenhouse.

In a separate experiment, leaves from shoot cultures were harvested at 2, 4, 6, and 8 weeks after the most recent subculture and tested for photosynthetic/respiratory capacity. Cuttings from additional 6-week-old shoot cultures also were harvested and rooted in Techniculture plugs in a high-humidity chamber as described previously. After 2 weeks in the rooting chamber, leaves from these rooted cuttings (that had been previously developed fully in vitro) were similarily subjected to photosynthetic/respiratory measurements.

Anatomical comparisons. The 3rd fully expanded leaf below the apex was harvested from sample plants and used for anatomical comparisons. Leaf and petiole cross-sections were made, perpendicular to the midrib, with an Oxford vibratome (Model G). The vibratome prepares 80–100 μm-thick sections of live plant material without embedding, allowing microscopic observations of intact cells (12). Similar cross-sections were also obtained from stem segments between the 2nd and 3rd nodes. Sections were photographed using a Bausch and Lomb light microscope fitted with an automatic camera system (Model AX-1). Developed black and white slides of photomicrographs of sections were then projected onto a large screen to make measurements on leaf thickness, relative contributions of various cell types to leaf thickness, palisade cell size, and vascular tissue dimensions. Palisade cell diameter was estimated by counting the number of palisade cells present in a given linear dimension (parallel to the leaf surface).

For making measurements of the cross-sectional area of upper and lower epidermal cells, the photomicrographs of epidermal peels were obtained with a light microscope in the same way as cross-sections. In some instances it was also possible to observe leaf surfaces directly under the microscope. The average cell cross-sectional area of epidermal cells was determined from the projections of the photomicrographs by counting the total number of cells per unit area. Leaf areas were measured with a LI-COR area meter. By dividing the total leaf area by the average cross-sectional area, the total number of epidermal cells per leaf was estimated.

Photosynthetic capacity and respiration. Photosynthetic oxygen evolution and respiratory oxygen uptake were determined by a technique similar to that of Ishi et al. (9) that uses a Clark-type oxygen monitor (Model 53, Yellow Springs Instruments, Yellow Springs, Ohio). Plexiglass water barriers (2.5-cm thick) were used to reduce heat accumulation. Two 150-W spot lamps (General Electric) served as the light source, giving a full light intensity of 1127 $\mu mol \cdot s^{-1} \cdot m^{-2}$. Illumination intensity was reduced to 587 $\mu mol \cdot s^{-1} \cdot m^{-2}$ or 239 $\mu mol \cdot s^{-1} \cdot m^{-2}$ in some experiments using neutral density gradient screens. Leaf tissue slices (about 2 × 5 mm) were submerged in a reaction medium (3.0 ml total volume) containing 50 mM Tris·HCl at pH 7.8–7.85, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 100 mM sorbitol, 2.5 mM KH_2PO_4, 0.1% PVP-40, and 20 mM $NaHCO_3$. The medium was purged with air prior to the assay. A constant temperature circulating-bath (Lauda-Brinkmann, Model K4-R/D) was used to maintain the temperature of the reaction mixture at 25.5°C. The measured rates of oxygen evolution over the course of the assay was constant, demonstrating that CO_2 was not limiting during photosynthetic measurements. Respiration was monitored on the same apparatus by blocking out light around the reaction mixture. To assess cyanide-insensitive respiration, 50 μl of a 40 mM KCN solution were added to the assay medium. Chlorophyll was extracted and read in 96% ethanol with a Beckman Acta III Spectrophotometer, and quantified according to Wintermans and DeMots (18).

Results

Comparative cellular anatomy. Although the surface area of greenhouse leaves was about 83 times greater than microcultured leaves, the epidermal cells on both surfaces of the former were only about 40% larger (Table 1). Seedling leaves were about 3 times the size of microcultured leaves, yet seedling epidermal cell sizes were only about 40% larger than cells in shoot culture leaves (Table 1). The seedling and greenhouse leaf epidermal cells were quite similar in size (Table 1).

In contrast to some previous studies on cultured plants (7, 17), the palisade cell layer of microcultured birch was always visible (Fig. 1A), although less prominent than in seedling (Fig. 1B) and greenhouse leaves (Fig. 1C). Microcultured leaves had one palisade layer comprised of short, rounded, loosely packed cells (Fig. 1A). The tightly packed palisade cells of both seedling and greenhouse leaves were arranged in 2 layers of slender, elongated cells [more distinct in greenhouse leaves (Fig. 1C)]. Palisade cells from microcultured leaves had a slightly larger average diameter than palisade cells from greenhouse leaves (Table 2). Palisade cells accounted for about 38% of the overall leaf thickness in greenhouse and seedling samples, but only 21% in microculture samples (Table 2).

Microcultured leaf midribs had only 13% of the cross sectional area devoted to xylem and phloem cells (see Table 2 and Fig. 1D). In contrast, leaves of greenhouse-grown plants had midrib veins with a well-developed vascular system comprising about 27% of the cross-sectional area (see Table 2 and Fig. 1F). The vascular system of seedling midrib veins was intermediate, comprising about 17% of the total cross-sectional area (Table 2). However, the vascular cells in seedling samples were as well-delineated as for greenhouse samples (Fig. 1 E and F). Side venation was also more prominent in greenhouse and seedling leaves than in microcultured leaves.

Petiolar vascular tissue paralleled the trend noted for midrib veins. Vascular connections were small and poorly structured in microcultured shoots, larger and more prominent in seedlings, and very well-developed in greenhouse samples (Table 3). In stems, however, similar cross-sectional areas were devoted to vascular systems in plants from all 3 sources (Table 3).

Photosynthesis and respiration analyses. At maximal light intensities (1127 $\mu mol \cdot s^{-1} \cdot m^{-2}$), greenhouse and seedling leaf tissues exhibited similar high rates of net photosynthesis based on chlorophyll content (Fig. 2). Leaves from microcultured shoots had only about one-third the rate of greenhouse leaves. When light intensities were reduced to 587 $\mu mol \cdot s^{-1} \cdot m-2$ and 239 $\mu mol \cdot s^{-1} \cdot m^{-2}$, microcultured tissue exhibited no drop in photosynthetic rate (Fig. 3). The photosynthetic rate for the greenhouse tissue, however, dropped almost linearly when light intensity was cut to about 20% of maximal levels.

The photosynthetic capacity of leaves from microcultured birch was partially dependent on the shoot age (Fig. 4). Shoots 2 and 4 weeks after subculturing had somewhat higher photosynthetic rates than those maintained for longer periods in culture. When microcultured shoots were excised after 6 weeks in culture and rooted, however, the photosynthetic activity of the leaves increased dramatically (Fig. 4) and approached the rates of seedling birch grown in the same environment. Chlorophyll content does not account for these differences, as the levels of pigment remained at 2.7 ± 0.6 mg $chlorophyll \cdot g^{-1}$ fresh weight of

Table 1. Surface areas, epidermal cell sizes, and total numbers of epidermal cells for leaves of microcultured, seedling, and greenhouse-grown Asian white birch.

Tissue source	Leaf area[z]	Cell cross-sectional area (μm^2)[y]		No. of cells/ leaf ($\times 10^4$)[x]	
		Upper epidermis	Lower epidermis	Upper epidermis	Lower epidermis
Shoots directly from the microculture vessel	0.64 ± 0.02	1025 ± 44	888 ± 27	6.2	7.2
Seedling shoots grown in a microculture-like environment	1.93 ± 0.19	1402 ± 60	1216 ± 53	13.7	15.9
Micropropagated plants grown in a greenhouse	52.83 ± 0.35	1467 ± 93	1100 ± 46	360.2	430.3

[z]Averages of 15 replicates ± SD.
[y]Mean values calculated from 10 projected leaf-surface photographs per treatment.
[x]Calculated from the above 2 sets of data.

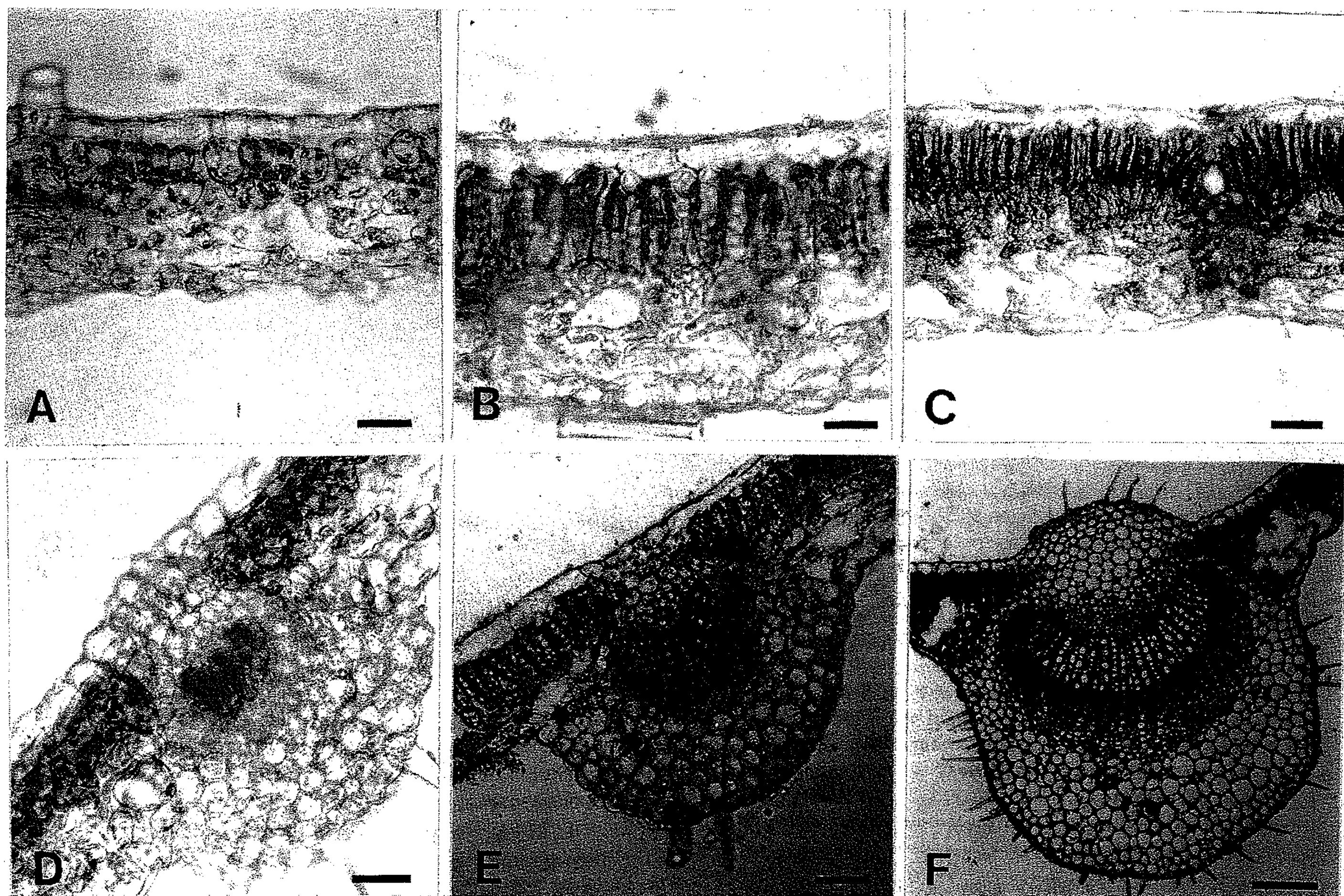

Fig. 1. Anatomical comparisons between shoots from microculture, seedlings grown in a microculture-like environment, and micropropagated (but greenhouse-grown) Asian white birch. **A–C**, leaf cross sections: **A** microculture; **B**, seedling; and **C**, greenhouse. **D–F**, midrid vein cross sections: **D**, microculture; **E**, seedling; and **F** greenhouse. (**A**, **B**, and **D**—bar = 20 μm; **C** and **E**—bar = 40 μm; and **f**—bar = 200 μm).

tissue and did not vary with shoot culture age or presence of roots.

Respiration rates for all 3 tissue sources were similar (Table 4). However, when KCN was introduced into the reaction mixture, marked rate differences became apparent. Respiration rates for greenhouse and seedling tissue dropped about 46% and 60%, respectively. Rates for microcultured tissue were altered only 13%.

Discussion

Microcultured plants have found widespread application for both experimental manipulation and commercial production (5,

Table 2. Cell dimensions from leaf cross-sections of Asian white birch from 3 sources.[z]

Variables	Tissue source: Shoots directly from the microculture vessel	Seedling shoots grown in a microculture-like environment	Micropropagated plants grown in a greenhouse
Cross-sectional thickness (μm)	89.3 a	138.9 b	237.8 c
Depth of cell layers (μm)			
Palisade	19.2 a	56.8 b	90.1 c
Spongy	47.4 a	55.6 a	114.6 b
Upper epidermal	14.5 a	17.6 a	18.0 a
Palisade			
Length (μm) adaxial cell layer	19.2 a	35.7 b	59.5 c
Diameter (μm)	14.9 b	14.1 b	11.6 a
Volume ($\mu m^3 \times 10^3$)	3.44 a	5.76 b	6.24 b
Midrib vein			
Xylem (%)	6.8 a	8.8 b	14.1 c
Phloem (%)	6.4 a	8.7 b	13.1 c

[z]Mean separation in rows by LSD, 1% level. Each mean was calculated from 10 replicates.

Table 3. Percentage of cross sectional area occupied by vascular system in petioles and stems of Asian white birch from 3 sources.[z]

Plant part	Tissue source: Shoots directly from the microculture vessel	Seedling shoots grown in a microculture-like environment	Micropropagated plants grown ina greenhouse
Petiole	12.5 ± 3.0	17.0 ± 1.2	25.5 ± 4.5
Stem	32.0 ± 4.1	28.1 ± 4.2	34.0 ± 5.0

[z]Each mean was calculated from 5 replicates (± SD).

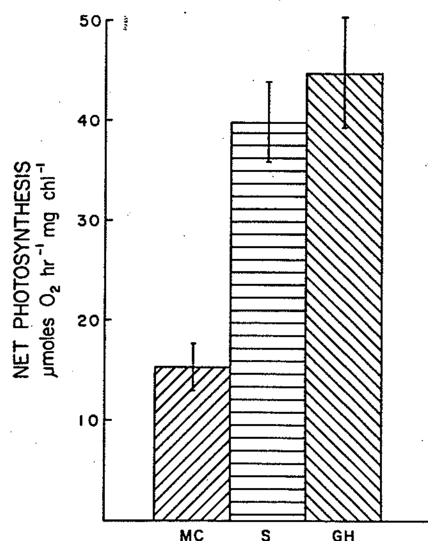

Fig. 2. Photosynthetic capacities of microcultured (MC), seedling (S), and greenhouse-grown (GH) Asian white birch leaves expressed on a chlorophyll content basis. The light intensity was 1127 $\mu mol \cdot s^{-1} \cdot m^{-2}$. Values are the mean of 9 replicate assays. Brackets show ± SD.

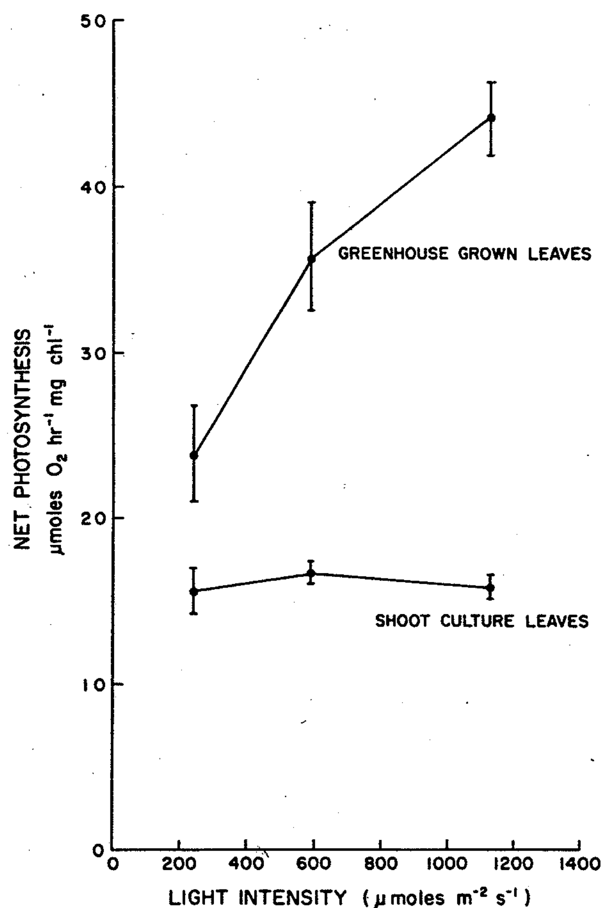

Fig. 3. Photosynthetic capacities of microcultured and greenhouse-grown Asian white birch leaves as a function of light intensity. Mean data points for full light intensity based on 9 replicate assays; lower light intensities based on 5 replicate asays. Brackets indicate ± SE.

8, 15); thus, it is important to define the extent to which these plants have been altered from their counterparts not grown in vitro. The diminutive stature of microcultured birch resulted more from decreased cell division than from reduced cell size, since cell sizes in vitro were not small enough to account for the large overall reduction in plant size. Individual leaf cell dimensions for plants from all 3 sources, for example, did not parallel the vast differences in overall leaf size (Tables 1 and 2). Consistent alterations in cell differentiation established that

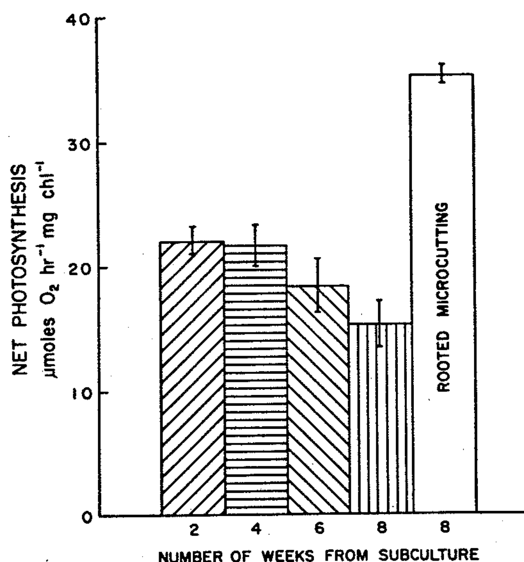

Fig. 4. Photosynthetic capacities of leaves of Asian white birch obtained from microcultured shoots at 2, 4, 6, and 8 weeks following subculture; and of leaves developed in culture and taken from microcultured shoots harvested at 6 weeks and rooted for an additonal 2 weeks prior to sampling. The light intensity was 1127 $\mu mol \cdot s^{-1} \cdot m^{-2}$. Values are the mean of 3 replicate assays. Brackets show ± SE.

Table 4. Respiratory oxygen uptake of Asian white birch leaves from three sources in the presence and absence of cyanide.[z]

Tissue source	Respiration ($\mu mol\ O_2 \cdot hr^{-1} \cdot g^{-1}$)		
	Without KCN	With KCN	Reduction (%)
Shoots directly from the microculture vessel	29.2 ± 5.5	25.4 ± 5.4	13.1
Seedling shoots grown in a microculture-like environment	33.4 ± 5.0	13.3 ± 1.7	60.5
Micropropagated plants grown in a greenhouse	39.8 ± 7.0	21.0 ± 5.1	45.7

[z]Means calculated from 9 replicate runs (± SD). Weights refer to a fresh-weight basis.

not only a small—but an anatomically altered plant—was produced in vitro. The increased susceptibility of microculture shoots to water stress has been documented (6, 7, 17) and the poor vascular system development as we have shown may contribute to this phenomenon (Tables 2 and 3).

The juvenility of microcultures, manifested in their rapid growth rates and ease of rootability (10, 11), was not reflected in anatomical similarity to juvenile seedling material, other than in overall size.

Low photosynthetic capacity characterized shoot cultures, and light saturation apparently was achieved at very low light levels. This low-light saturation level for photosynthesis probably results in part from the low ambient light levels (20–50 $\mu mol \cdot s^{-1} \cdot m^{-2}$) under which shoot cultures are grown. The weak palisade of microcultured birch might result in low photosynthetic capacity on a fresh weight basis, but the similarly low rates, as expressed on a chlorophyll basis, indicated that additional differences (probably involving chloroplast functioning) are also involved.

The reduced photosynthetic capacity of older microcultures may relate to the comparative superiority of leaves from young, freshly subcultured microcultures as a source of metabolically active protoplasts (14). Interestingly, much of the photosynthetic capacity of microcultured leaves is restored after rooting of cultured shoots, although the sampled leaves were grown in vitro. This restoration indicates that the environment of the plant may be more important than its anatomy in regards to the altered physiological behavior in vitro. The marked increase in photosynthetic capacity that follows rooting of a microculture shoot may be caused by the altered gaseous atmosphere of the high-humidity rooting chamber (identical to the chamber used for seedling germination), the components of the tissue culture medium, or to the presence of roots. In the latter case, phytohormones synthesized in the roots may be contributing to enhanced leaf photosynthetic capacity (16).

The comparative respiration efficiency of microcultured tissue in the presence of KCN may be linked to shifts in hormonal balance that occur in the culture environment. Cyanide-insensitive respiration is a phenomenon commonly related to hormonal imbalance, including increases in ethylene or auxin deprivation (2, 13). Similar hormonal alterations have been found in the microculture environment (3) and could contribute to the development of an alternate respiratory pathway.

Plants grown in microculture are altered significantly, both anatomically and physiologically, from their noncultured counterparts. However, their differences appear to be primarily the result of the microculture environment itself, and, thus, normal development and metabolism is quickly restored upon removal from microculture.

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