Establishing Tissue-cultured Sweetgum Plants in Soil

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ADDITIONAL INDEX WORDS. Automated plant culture system, axillary shoots, CO₂ enrichment, free-living, growth, *Liquidambar styraciflua*, micropropagation

SUMMARY. Methods to enhance sweetgum (*Liquidambar styraciflua* L.) in vitro axillary shoot formation and shoot establishment into soil are presented. Sweetgum shoots grown in an automated plant culture system (APCS) produced 400 to 500 shoots via axillary branching compared to only 40 shoots produced within Magenta vessels containing agar medium after 8 weeks of incubation. Vitrification was observed in as many as 80% of the axillary shoots produced in the APCS. A continuous carbon dioxide (CO₂)-flow-through system was tested on both vitrified and non-vitrified sweetgum shoots transferred from the APCS to soil. One- and two-cm-long vitrified shoots were grown within CO₂-flow-through system chambers and subjected to 350, 1500, 3000, 10,000, or 30,000 µL·L⁻¹ (ppm) CO₂ for 4 weeks. Administering 10,000 µL·L⁻¹ CO₂ improved culture survival and enhanced overall shoot and root growth compared to shoots grown under ambient atmosphere (i.e., 350 µL·L⁻¹ CO₂).

Sweetgum, native to southeastern U.S., is a deciduous tree grown commercially for paper pulp. Several sweetgum cultivars (e.g., *L. styraciflua* 'Rotundiloba') and related species [e.g., formosan sweetgum (*L. formosana* Mill.)] are also employed in

urban landscaping especially as park and street trees due to their rapid growth rates, size and shade canopy properties (Gilman and Watson, 1993a, 1993b). Millions of sweetgum seedlings are planted annually (Lin et al., 1995). Suitable clonal propagation methods to mass produce sweetgum for large scale field plantings are sought to improve uniformity and satisfy production demands (Lin et al., 1995; Solárová and Pospíšilová, 1997).

The ultimate goal of micropropagation is to produce free-living plants and therefore two separate cultural processes need to be addressed: mass production of shoots or plantlets in vitro and then successful propagule establishment into soil. Aside from the traditional agar tissue culture methodology, a variety of novel culture systems have also been developed to enhance micropropagation rates including: APCS, which resembles sterile hydroponics (Simonton et al., 1991, Tisserat, 1996); misting systems (Chatterjee et al., 1997); and bioreactor systems (Simonton et al., 1991). The APCS produces several times more plantlets or shoots within its chambers while requiring less maintenance compared to using traditional culture vessels (e.g., culture tubes or Magenta vessels). The increased biomass and growth responses obtained within the APCS vessels is attributed to its larger vessel size and greater media reservoir volume compared to that available using traditional culture vessels (Tisserat, 1996).

It should be noted that most micropropagation studies address in vitro shoot proliferation and shoot establishment into soil procedures individually, whereas, in reality, these procedures should be considered very closely interrelated. If an in vitro propagation technique provides numerous shoots that exhibit poor soil establishment, such a technique would be of little commercial use. Actively proliferating sweetgum shoots via axillary branching commonly exhibit vitrification. Vitrified shoots are characterized as being small, succulent (i.e., "glassy" or "wet" in appearance) and immature, but were capable of readily proliferating additional axillary shoots. Also, vitrified shoots do not transfer readily into soil well (Brand and Lineberger, 1988). Non-vitrified shoots are larger, drier, more mature, and tend to root readily. A major problem confronting

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the sweetgum micropropagator is to successfully establish vitrified shoots into soil. Generally a prolonged “hardening off” acclimation pre-treatment period is administered to vitrified shoots prior to their transfer to soil (Brand and Lineberger, 1988; Lin et al., 1995; Sutter and Barker, 1985). Some researchers have employed elevated concentrations of CO_2 (~1000 to 1500 $\mu\text{L}\cdot\text{L}^{-1}$ CO_2) to enhance survival and growth of sterile carnation (*Dianthus caryophyllus* L.) or grape (*Vitis vinifera* L.) shoots when transferring them to soil (Fournioux and Bessis, 1993; Lakso et al., 1986). Spearmint shoots grown in ultra-high CO_2 concentrations (≤ 3000 $\mu\text{L}\cdot\text{L}^{-1}$ CO_2) rooted faster and exhibited improved growth responses compared to shoots grown under 350 or 1500 $\mu\text{L}\cdot\text{L}^{-1}$ CO_2 (Tisserat, 2001). The influence of ultra-high CO_2 concentrations on sweetgum growth and morphogenesis is not known. In this study, improved sweetgum propagation techniques were developed. The benefit of employing a sterile hydroponics system (i.e., APCS) to increase sweetgum shooting in vitro was compared with traditional tissue culture vessels. Several elevated CO_2 concentrations were tested on a variety of sweetgum shoot sizes and types in order to improve survival and increase growth rates in soil.

Materials and methods

CULTURE EXPERIMENTS. Shoot cultures of sweetgum cultivar UCC1 were established from leaf explants on a modified woody plant medium (MWPM) consisting of the following: 278 $\text{mg}\cdot\text{L}^{-1}$ (ppm) $\text{Ca}(\text{NO}_3)_2\cdot 4\text{H}_2\text{O}$, 48 $\text{mg}\cdot\text{L}^{-1}$ $\text{CaCl}_2\cdot 2\text{H}_2\text{O}$, 6.2 $\text{mg}\cdot\text{L}^{-1}$ H_3BO_3 , 8.6 $\text{mg}\cdot\text{L}^{-1}$ $\text{ZnSO}_4\cdot 7\text{H}_2\text{O}$, 16.9 $\text{mg}\cdot\text{L}^{-1}$ $\text{MnSO}_4\cdot \text{H}_2\text{O}$, 25 $\text{mg}\cdot\text{L}^{-1}$ $\text{Na}_2\text{MoO}_4\cdot 5\text{H}_2\text{O}$, 2.5 $\text{mg}\cdot\text{L}^{-1}$ $\text{CuSO}_4\cdot 5\text{H}_2\text{O}$, 40 $\text{mg}\cdot\text{L}^{-1}$ NH_4NO_3 , 99 $\text{mg}\cdot\text{L}^{-1}$ KNO_3 , 17 $\text{mg}\cdot\text{L}^{-1}$ KH_2PO_4 , 37 $\text{mg}\cdot\text{L}^{-1}$ $\text{MgSO}_4\cdot \text{H}_2\text{O}$, 27.8 $\text{mg}\cdot\text{L}^{-1}$ $\text{FeSO}_4\cdot 7\text{H}_2\text{O}$, 37.2 $\text{mg}\cdot\text{L}^{-1}$ Na_2EDTA , 0.4 $\text{mg}\cdot\text{L}^{-1}$ thiamine HCl, 0.5 $\text{mg}\cdot\text{L}^{-1}$ nicotinic acid, 0.1 $\text{mg}\cdot\text{L}^{-1}$ pyridoxine HCl, 100 $\text{mg}\cdot\text{L}^{-1}$ myo-inositol, 20,000 $\text{mg}\cdot\text{L}^{-1}$ sucrose, 2 $\text{mg}\cdot\text{L}^{-1}$ benzyladenine (BA), 0.001 $\text{mg}\cdot\text{L}^{-1}$ naphthaleneacetic acid (NAA), and when applicable, 8000 $\text{mg}\cdot\text{L}^{-1}$ agar. The agar medium pH was adjusted to 5.7 ± 0.1 with 0.1 N HCl or NaOH. The liquid medium pH value was adjusted to 5.0. The media were dispensed in 50-mL (1.7 fl oz) aliquots into Magenta (GA-

Fig. 1. Diagrammatic representation of the automated plant culture system employed in sweetgum studies. Nutrient medium is maintained in the media reservoir and periodically pumped via the peristaltic pump into the culture chamber containing sweetgum cultures. Following a 15-min soaking period, media is then evacuated back into the media reservoir.

7) polycarbonate containers [70 mm long $\times$ 70 mm wide $\times$ 100 mm high (2.76 $\times$ 2.76 $\times$ 3.94 inches), 365- cm^3 (22.3 inch^3) capacity] and capped with polypropylene lids (Magenta Corp., Chicago). The APCS employed a 1-L (0.26 gal) glass bottle reservoir containing 1 L MWPM. All media were sterilized for 15 min at 103.42 kPa (15.0 psi) and 121 $^\circ\text{C}$ (249.8 $^\circ\text{F}$).

The APCS has been described elsewhere (Fig. 1) (Tisserat, 1996). The culture chamber was constructed of a 2.4-L (0.63 gal) polycarbonate bottle [32.5 cm high $\times$ 26.5 cm diameter (12.80 $\times$ 10.43 inches)] and a polypropylene closure. Closures were drilled and tapped with three 1/8-27 NPT (national pipe thread) threads to accommodate miniature polypropylene barbed fittings (Ark-Plas Products, Flippin, Ark.). A polypropylene spigot was attached to one fitting (Fig. 1). Two 0.45- μm air vent filters (Gelman Sciences, Ann Arbor, Mich.) were attached via silicone tubing to the other fittings. A layer of glass gravel, 15 mm (0.59 inch) deep, was placed in the bottom of the chamber. Sweetgum cultures were given one, two, or four immersions per day for 15 min with MPWM and then evacuated back to the reservoir via peristaltic pumps. Two immersions per day were spaced 12 h apart; four immersions/day were spaced 6 h apart. Media pumping was controlled using a digital program timer (Intermatic, Spring Grove, Ill.). One 3-cm-long (1.2 inch) shoot tip was cultured in each system. Twenty

replicates per treatment for Magenta experiments and five replicates per treatment for APCS experiments were planted and experiments were repeated twice. Following 8 weeks of incubation, data on fresh weight and leaf number, shoot length, and shoot number were recorded. Cultures were incubated at a constant 26 ± 1 $^\circ\text{C}$ (78.8 $\pm$ 1.8 $^\circ\text{F}$) under a 16-h daily exposure to a total photosynthetic photon flux (PPF) of 100 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ at the vessel periphery.

CO_2 -FLOW-THROUGH SYSTEM. The CO_2 -flow-through system chambers were constructed using a 17.5-L (4.62 gal) transparent polycarbonate boxes (Consolidated Plastics, Twinsburg, Ohio) [32.5 cm long $\times$ 26.3 cm wide $\times$ 30 cm high (12.80 $\times$ 10.35 $\times$ 11.8 inches)] capped with a transparent lid (Tisserat, 2001). A silicone tape gasket [112 cm long $\times$ 6.3 mm wide $\times$ 3.2 mm thick (44.09 $\times$ 0.25 $\times$ 0.13 inches)] (Furon, New Haven, Conn.) was attached to the lid. The boxes were modified by mounting three polypropylene barbed fittings to allow for the inflow and evacuation of gases. Three air vent filters were attached via silicone tubing to the fittings. The box and lid were clamped with 10 equally spaced stationary binder clips [50 mm long (2.0 inches)]. Carbon dioxide from a gas cylinder (BOC, Peoria, Ill.) rated 99.8% pure and was mixed with room air flow produced by an aquarium pump (Whisper no. 1000; Carolina Biological Supply Co., Burlington, N.C.). Carbon dioxide was regulated with a

flow meter (Cole Parmer Instrument Co., Niles, Ill.) to provide 350, 1500, 3000, 10,000, or 30,000 $\mu\text{L}\cdot\text{L}^{-1}$ CO_2 . A LIRA infrared gas analyzer (model no. 3000; Mine Safety Appliances Co., Pittsburgh) was employed to adjust CO_2 ranges $\geq 10,000 \mu\text{L}\cdot\text{L}^{-1}$ CO_2 ; and a Li-Cor $\text{CO}_2/\text{H}_2\text{O}$ infrared gas analyzer (model no. LI-6262; Li-Cor, Lincoln, Nebr.) was employed to adjust CO_2 ranges $\leq 3,000 \mu\text{L}\cdot\text{L}^{-1}$. The CO_2 and air streams were administered at 1500 $\text{mL}\cdot\text{min}^{-1}$ during the 16-h photoperiod only. No CO_2 applications were administered during the 8-h dark periods. Control cultures were provided with a stream of room air generated by the aquarium pump.

EX VITRO EXPERIMENTS. Vitrified and non-vitrified sweetgum shoot clumps were removed from APCS chambers and soaked in distilled water for 10 min to remove sucrose residue. Shoot clumps were then cut into individual 1-, 2-, or 3-cm-long (0.4, 0.8, or 1.2 inches) shoots with a razor blade and sprayed with a 0.5% solution of chlorothalonil tetrachloroisophthalonitrile (Daconil; Monsanto, San Ramon, Calif.). Cut shoot ends were dipped into a Rootone Brand F powder mixture (Green Light Co., San Antonio, Texas) and then transferred to a 50 peat:50 vermiculite mixture amended with 4.7 $\text{g}\cdot\text{kg}^{-1}$ (0.075 oz/lb) Micromax (Scotts Co., Marysville, Ohio) and 18.5 $\text{g}\cdot\text{kg}^{-1}$ (0.30 oz/lb) Osmocote 14-14-14 (10N-4.4P-8.3K; Scotts Co.). Shoots were grown in white foam trays [36.3 cm long $\times$ 22.5 cm wide $\times$ 6.0 cm high (14.29 $\times$ 8.86 $\times$ 2.36 inches)] (Gardener's Supply, Burlington, Vt.) containing 40 equally spaced openings covered with transparent polystyrene tents. Trays were placed into CO_2 -flow-through chambers. Initially, 2- and 3-cm-long non-vitrified shoots were tested under 350 or 10,000 $\mu\text{L}\cdot\text{L}^{-1}$ CO_2 for survival and growth responses. Subsequent experiments were conducted with 1- and 2-cm-long vitrified shoots grown under 350, 1500, 3000, 10,000, or 30,000 $\mu\text{L}\cdot\text{L}^{-1}$ CO_2 . A photoperiod of 16 h was supplied by a combination of cool-white fluorescent tubes, metal-halide and incandescent lamps to provide a PPF of 200 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ at the chamber periphery. Shoots were watered three times per week and sprayed with the Daconil fungicide once per week.

For soil- CO_2 experiments, 24 replicates were planted originally and

Fig. 2. Responses of sweetgum grown in various culture systems. Culture systems tested include: Agar = Magenta on agar modified woody plant medium (MWPM); Liquid = Magenta with liquid MWPM; APCS-1x = automated plant culture system (APCS) given one immersion/day for 15 min with liquid MWPM; APCS-2x = APCS given two immersions/day with liquid MPWM; and APCS-4x = APCS given four immersions/day with liquid MWPM. Data were averages of two tests consisting of five replications/treatments for the APCS and 20 replications/treatments for the Magentas. Mean separation was obtained by Duncan's multiple range test ($\alpha = 0.05$). Columns with the same letter on top were not significantly different.

experiments were repeated at least twice. Data was collected after 4 weeks of incubation and included culture survival, shoot height, leaf number, leaf length, and root number.

STATISTICAL ANALYSIS. Tissue culture data was analyzed by analysis of variance and multiple comparisons of means were accomplished with the Duncan's multiple range test ($\alpha = 0.05$). For soil testing regression analyses predicting growth responses from CO_2 concentrations was performed using PROC REG from SAS for PC Windows version 8.2 software (SAS Inc., Cary, N.C.).

Results and discussion

Cultures grown in stationary liquid medium within Magenta vessels produced the least fresh weight and shoot number compared to other treatments (Fig. 2). In the liquid medium, sweetgum shoots often browned and died after 6 weeks following planting. Sweetgum cultures grown in agar medium remained green and proliferated numerous axillary shoots during the entire 8-week culture period. Sweetgum cultures maintained on liquid

medium only produced 1.6 g (0.06 oz) fresh weight and 17 shoots while sweetgum on agar doubled their fresh weight [3.4 g (0.12 oz)] and shoot numbers (40.3). Sweetgum cultures produce greater numbers of axillary shoots and fresh weight when grown within the APCS (Figs. 2 and 3). Best growth and shooting responses occurred employing the four immersions/day treatment (Fig. 2). Increasing the number of immersions from one to four per day doubled the culture fresh weights from 13.9 g (0.49 oz) to 29.3 g (1.03 oz). A 9.3-fold increase in shoots and 7.4-fold increase in fresh weight were obtained from sweetgum cultures grown in the APCS given four immersions/day compared to the responses of shoots grown in agar medium (Figs. 2 and 3).

Cultured sweetgum leaf explants readily produce shoots in vitro with as many as 57 meristem shoots obtained from a single leaf explant (Brand and Lineberger, 1991; Sutter and Barker, 1985). Brand and Lineberger (1988) obtained about 20 shoots per explant and from 30 to 100 shoots per 125- cm^3 (7.63 inch³) jar vessel containing

Fig. 3. Responses of 8-week-old sweetgum cultures grown on agar modified woody plant medium (MWPM) in Magenta vessel (left) and with liquid MWPM in automated plant culture system vessels (right); bar = 20 mm (0.79 inch).

25 mL (0.84 fl oz) medium with five explants per vessel. In this study, sweetgum shoots were derived exclusively from axillary budding only and up to 500 shoots were obtained within a single APCS vessel. Axillary-derived shoots were considerably larger and more mature than meristem-derived shoots produced from leaf explants. Two dissimilar shoot types occurred

within the APCS (i.e., the ~70% to 80% vitrified and ~20% to 30% non-vitrified shoots). Non-vitrified shoots produced fewer axillary shoots compared to the vitrified shoots. After 8 weeks of culture, large shooting clumps were derived within the APCS vessels which can be subdivided into smaller clumps to serve as the source for more shoots to be employed in the multiplication process. The numerous vitrified shoots generated in the APCS can be either proliferated further via shoot clumps or rooted into soil to give rise to free-living plantlets.

Lower survival rates were exhibited by 1- to 2-cm-long vitrified shoots (i.e., 40% to 60%) when transferred

to soil compared to non-vitrified shoots (i.e., 60% to 90%) (Brand and Lineberger, 1988). Lin et al. (1995), studying rooting of sweetgum shoots generated *in vitro*, found that the taller 2-cm shoots had inherently higher rooting rates than the shorter 1-cm shoots. In our study, non-vitrified shoots of 2- and 3-cm in length grown in either 350 or 10,000 $\mu\text{L}\cdot\text{L}^{-1}$ CO_2 concentrations showed no difference in survival rates (~80% to 90%). However, both types of shoots gave rise to larger leaves and more roots when grown under the elevated CO_2 concentrations (data not shown). These results suggested merit in the further testing of various CO_2 concentrations on the rooting of smaller vitrified shoots. Vitrified shoots, 1- and 2-cm long, exhibited enhanced survival rates and growth responses under 10,000 $\mu\text{L}\cdot\text{L}^{-1}$ CO_2 when compared to survival rates and growth responses obtained from ambient (350 $\mu\text{L}\cdot\text{L}^{-1}$ CO_2) or other CO_2 concentrations (Tables 1 and 2). Similar CO_2 dose-growth relationships have been reported in other plants (Solárová and Pospíšilová, 1997; Tisserat, 2001). High CO_2 concentrations enhance photosynthesis resulting in higher growth rates (Lasko et al., 1986; Solárová and Pospíšilová, 1997; Tisserat, 2001). It should be noted that the CO_2 -flow-through chambers maintained a high humidity (~100%) during testing that prevented shoot desiccation.

Sutter and Barker (1985) used a rooting medium containing 0.5 $\text{mg}\cdot\text{L}^{-1}$ indole-3-butyric acid for 5 d to initi-

Table 1. Influence of various concentrations of carbon dioxide (CO_2) on survival of 1- and 2-cm-long vitrified sweetgum shoots *ex vitro*. Shoots were grown in soil for 4 weeks before data were taken. Data were averages from two tests consisting of survival percentage based on 24 replications/treatment. Predicted mean values within a column followed by the same letters are not significantly different based on overlap of the 95% confidence intervals from their respective regression equations.

CO_2 concn [$\mu\text{L}\cdot\text{L}^{-1}$ (ppm)]	Explant length (cm) ^z	
	1 ^y	2 ^x
	-----Survival (%) ---	
350	48.6 a	61.2 a
1500	56.5 a	64.1 a
3000	65.7 a	69.2 b
10,000	93.1 b	93.9 c
30,000	67.1 ab	64.3 ab
r^2	0.95	0.99
P	0.0269	0.0024

^z1 cm = 0.39 inch.

^ySurvival percentage (Y) = $0.0000004639 \times (\text{CO}_2)^{1.5} + 0.0000131 \times (\text{CO}_2) \times \text{Ln}(\text{CO}_2)$.

^xSurvival percentage (Y) = $0.60778 + 0.00000067665 \times (\text{CO}_2)^{1.5} - 0.0000000003751 \times (\text{CO}_2)^2 \times \text{Ln}(\text{CO}_2)$.

Table 2. Influence of carbon dioxide (CO_2) on the morphogenesis responses from 1-cm-long vitrified sweetgum explants after 4 weeks of incubation. Data were averages from two tests consisting of 12 replications/treatment. Predicted mean values within a column followed by the same letters are not significantly different based on overlap of the 95% confidence intervals from their respective regression equations.

CO_2 concn [$\mu\text{L}\cdot\text{L}^{-1}$ (ppm)]	Leaves (no.) ^z	Roots (no.) ^y	Leaf length (mm) ^x	Shoot length (mm) ^w
350	4.17 a	5.35 a	6.83 a	15.08 a
1500	5.38 a	8.58 b	6.17 b	16.84 a
3000	5.85 a	9.19 b	9.21 c	20.25 a
10,000	6.14 a	9.66 b	9.71 d	19.75 a
30,000	4.83 a	9.82 b	8.08 e	17.42 a
r^2	0.83	0.94	1.00	0.73
P	0.085	0.004	0.002	0.133

^zLeaves (Y) = $-1.203 - 0.00012 \times (\text{CO}_2) + 0.924 \times \text{Ln}(\text{CO}_2)$.

^yRoots (Y) = $9.914 - 26722.944 \times \text{Ln}(\text{CO}_2) / (\text{CO}_2)$.

^x10 mm = 0.39 inch; leaf length (Y) = $18.742 - 0.099 \times \text{Ln}(\text{CO}_2)^2 - 765876 / (\text{CO}_2)^{1.5} + 13285719 / (\text{CO}_2)^2$.

^wShoot length (Y) = $1.025 - 0.21 \times \text{Ln}(\text{CO}_2) + 0.012 \times \text{Ln}(\text{CO}_2)^2$.

ate roots from shoots prior to their transfer to nutrient medium without hormones. After 6 weeks of incubation, shoots were then transferred to soil. Similarly, Brand and Lineberger (1988) employed several transfers to different media over a 9-week period prior to transferring shoots to soil. Lin et al. (1995) employed a liquid medium containing rockwool and auxins to promote rooting from sweetgum shoots prior to transfer to soil. In contrast, vitrified shoots can be successfully rooted directly into soil by employing an ultra-high CO₂ atmosphere (i.e., 10,000 µL·L⁻¹ CO₂) coupled with high humidity.

Vitrification is common to rapidly shooting sweetgum cultures. The APCS beneficially exploits this phenomenon, allowing for the production of shoot numbers that exceed those possible by using traditional culture vessels. High rooting percentages were then achieved from vitrified shoots obtained from the APCS by employing ultra-high CO₂ atmospheres. Coupling these two procedures together will minimize the time and labor involved in sweetgum micropropagation. The procedures presented here can be readily adapted to the micropropagation of other woody and non-woody plants.

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