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In vitro propagation of mature *Liquidambar styraciflua*

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Abstract. Mature specimens of *Liquidambar styraciflua* were propagated in vitro. Components of the nutrient medium and culture conditions were first determined for one-year-old seedling material. Mature material responded similarly to seedling material in culture, but alterations in frequency of early transfers and components of the medium were required. Explants responded best to Woody Plant Medium of Lloyd and McCown supplemented with 0.2 mg l^{-1} BA and 0.05 mg l^{-1} NAA. Root formation occurred on shoots placed on media containing 0.5–1.0 mg l^{-1} IBA. Growth in culture and percentage of rooting of mature explants were markedly affected by the individual selection, with rooting percentages varying from 33–100% among selections.

Introduction

Liquidambar styraciflua L. (American sweetgum) is grown extensively along urban streets in the United States. Sweetgum is usually propagated by seed, or budded onto seedlings rootstocks, because it is difficult to induce roots on cuttings. Despite the advantages of sweetgum that have made it a popular street tree, it seriously damages sidewalks and other street improvements [16]. Screening for genotypes that do not produce invasive roots must be done on trees in the mature phase of growth because damage to sidewalks is not done until a tree approaches its ultimate size. Clonal rootstock material from trees with non-invasive root systems to be used as rootstocks is difficult to obtain since cuttings from mature trees do not root easily. Hare [3] produced new self-rooted plants from mature trees of sweetgum by air-layering one-year-old shoots. We had no success with this technique nor with rooting leafy stem cuttings from mature trees of this species [P. Barker, unpublished].

A method that has potential for clonal propagation of trees is micropropagation. Plants regenerated in vitro form roots more easily than the cuttings taken directly from mature trees and can also be produced in large numbers [2, 5, 9, 14]. Sweetgum has been propaged in vitro from hypocotyl explants of young seedlings but the investigators did not report on propagation of mature plants in vitro, a process necessary for clonally reproducing selected superior specimens [12, 13]. A preliminary report by Sutter and

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Barker [15] indicated the feasibility of propagating sweetgum in vitro, but they obtained low rooting percentages for 3 of the 4 selections tested. The objective of this study was to see whether micropropagation could be used for the propagation of mature specimens of sweetgum.

Materials and methods

One-year-old seedlings and mature plants of *Liquidambar styraciflua* L. were used. Mature trees were seedling in origin, derived from different sources. Actively growing shoots were taken from seedlings kept in a greenhouse under lights between October and March. The terminal 5 cm of the shoots were excised and surface-sterilized by washing them for 15 min in 0.1% NaOCl, to which 0.1% Tween 20 had been added. They were rinsed 4 times with sterile distilled water. Shoot tips, 1.0–1.5 cm long, were excised for micropropagation.

Explants from mature trees were collected from February through August. Shoot tips were excised from actively growing shoots and dormant buds were excised from both dormant and actively growing shoots. Explants were washed in 1% 7X (Flow Laboratories, Dublin, VA) for 20 min followed by a 30 s dip in 5% Amphyl disinfectant (National Laboratories, Montvale, NJ). This was followed by soaking the explants in 0.1% NaOCl plus 0.1% Tween 20 for 15 min. The outer leaves of the shoot tip and outer scales of the buds were removed and the explants were soaked for an additional 5 min in a 0.1% NaOCl solution. They were rinsed 4 times in sterile, distilled water.

Explants from mature trees initially were gathered from 5 different selections. These differed phenotypically in coloration or root characteristics. Experiments were conducted on only 4 of the selections because of excessive loss of samples of 1 selection due to contamination. Eventually 8 different selections with desirable coloration or root characteristics were placed in culture. After shoots were collected from the field, they were placed indoors in a growth chamber with their bases in water. Dormant shoots were kept until active growth had started. Actively growing shoots were sampled after a new set of leaves had formed indoors. Explants consisted of shoot tips excised from the most recent flush of leaves that formed after being brought indoors.

Explants from seedlings were incubated on either Woody Plant Medium (WPM) [8] or Linsmaier-Skoog medium (LS) [6] with 0.1–2.0 mg/l benzyladenine (BA) added. The best hormone concentration for shoot elongation and multiplication was determined using BA at 0, 0.1, 0.5, 1.0, 2.0 mg l^{-1} and naphthaleneacetic acid (NAA) at 0.05, 0.2, and 0.5 mg l^{-1} in a 5 x 3 factorial design. Twelve replicates were used for each treatment. Because limited numbers of samples were available, combinations with a concentration of BA less than that of NAA were omitted. Shoots were excised from cultures on multiplication medium and rooted on WPM using 0.2–2.5 mg l^{-1}

indolebutyric acid (IBA). Twelve replicates were used in each treatment and the entire experiment was repeated twice. All media were adjusted to pH 5.2 prior to autoclaving. They were solidified with 0.7% Difco Bacto agar and autoclaved for 20 min at 121°C at 1.1 kg cm^{-2}. Cultures were incubated at 24°C under cool white fluorescent lamps at 20 $\mu Em^{-2} s^{-1}$ and a 16 hour photoperiod.

All explants from mature trees were first grown on the medium determined to be the most suitable for seedling material, WPM containing 1.0 mg l^{-1} BA and 0.05 mg l^{-1} NAA.

Results and discussion

Explants from seedlings

Preliminary studies showed that shoot tips excised from seedlings grew better on WPM than on LS medium. On LS medium shoot tips were stunted, fewer buds broke, and there was a gradual necrosis of leaves. Since WPM was superior to LS medium at all BA concentrations tested, only WPM was used in further testing.

The concentrations of hormones had significant effects on both number and height of shoots. The number of shoots produced over a 4-week period was greatest on medium containing 1.0 mg l^{-1} BA and 0.05 mg l^{-1} NAA (Table 1). The greatest shoot heights were obtained when there were no hormones added to the medium or when lower concentrations of BA (0.1, 0.5 mg l^{-1}) were combined with no or 0.05 mg l^{-1} NAA (Table 1). When

Table 1. Effect of hormone concentrations on growth of juvenile *Liquidambar styraciflua* L. shoots grown in vitro on woody plant medium after 4 week period

Hormone[a]			
BA mg l^{-1}	NAA mg l^{-1}	Final number shoots (± SEM)[b]	Final height (± SEM) (cm)
0	0	1.0 ± 0.06	1.4 ± 0.2
0.1	0	1.0 ± 0.1	1.0 ± 0.2
0.5	0	2.6 ± 1.0	1.1 ± 0.2
1.0	0	5.6 ± 1.0	0.7 ± 0.1
2.0	0	4.4 ± 1.7	0.5 ± 0.2
0.1	0.05	1.6 ± 0.4	1.2 ± 0.2
0.5	0.05	3.8 ± 1.4	1.2 ± 0.1
1.0	0.05	8.4 ± 2.0	0.6 ± 0.1
2.0	0.05	5.5 ± 1.7	0.8 ± 0.4
0.5	0.5	4.0 ± 1.5	0.6 ± 0.1
1.0	0.5	2.2 ± 0.7	0.7 ± 0.1
2.0	0.5	3.6 ± 1.1	0.7 ± 0.2
Bonferroni[c]		4.8	0.7

[a] n = 25, 2 replications
[b] Standard error of the mean
[c] Conservative correction for Least Significant Difference

shoots were grown on 2.0 mg l^{-1} BA, shoots emerged from both the axils of the leaves and from the basal mass. At lower concentrations of BA few shoots emerged from the basal mass, with most arising as axillary shoots. The number of leaves produced paralleled the number of shoots produced. From these results, the medium selected for shoot multiplication consisted of WPM inorganic and organic components with 1.0 mg l^{-1} BA and 0.05 mg l^{-1} NAA. Shoots multiplied rapidly on this medium producing 6–9 new shoots per single original shoot per month.

Preliminary studies indicated that lower concentrations of $CaCl_2$ were beneficial for growth of shoots [15]. However, after 6 months on such a medium, shoots became necrotic and the original WPM formulation was adopted for all future work.

Individual shoots were excised from cultures on multiplication medium and were placed on a series of different IBA concentrations to induce roots. The effect of full strength and half strength inorganic salt concentrations in the medium was also tested (Table 2). Roots were first seen at 3 weeks with IBA. By 6 weeks some shoots in all treatments had rooted but there were significant differences among the treatments (Table 2). The most effective root-promoting treatments were the addition of 0.2, 0.5 and 1.0 mg l^{-1} IBA. One mg l^{-1} IBA was unacceptable because of the formation of callus at the base of the shoot cuttings and 2.5 mg l^{-1} IBA produced necrosis of the leaves.

Explants from mature trees

Shoot tips, taken from mature trees in March, April, May and June responded better in culture than those taken during July, August, and September.

Table 2. Effect of IBA and salt concentrations on rooting of shoots of juvenile *Liquidambar styraciflua* L. regenerated in vitro

IBA in medium[a] mg l^{-1}	Other treatments	Percentage of plants forming roots			
		Time after treatment started			
		3 weeks	4 weeks	5 weeks	6 weeks
0.0		0	0	0	0c[b]
0.2		31	54	62	62a
0.5		22	44	78	83a
1.0		40	60	60	69a
2.5		0	0	13	19b
0.0	Half-strength salts	0	10	20	11b
0.0	Pretreat with 10.0 mg l^{-1} IBA dip	0	0	0	11b
	Humidity tent	0	0	0	25b

[a] Grown on Woody Plant Medium
[b] Differences between means calculated at 6 weeks only, by Z score for p
[c] n = 30, 3 replications

Contamination rates averaged 5–10% in spring and 55–60% during late summer. Of the explants that were not contaminated, 93% of those taken during the spring survived, but only 10% of those taken during late summer survived. Such seasonal responses have been noted in other plants such as *Carica papaya* L. [7].

Shoot tips from mature plants became chlorotic and eventually died if they were not transferred frequently to fresh medium during the first month in culture. All explants were transferred every 2–3 days during the first 2 weeks and weekly for the next 2 weeks. After the first month in culture, shoots were transferred every 4 weeks. Slower growing selections responded better to frequent transfers than did the more vigorous selections. Why explants from mature trees required frequent transfers is not known, but may be related to changes in metabolism that occur as plants mature [10].

Dormant buds from mature trees grew slowly; leaf expansion and emergence occurred after 3–4 weeks. An additional 2–3 weeks were necessary for buds to start active growth. A lower percentage of dormant buds survived than did actively growing shoot tips.

During the first 4 months in culture there were striking differences in growth among selections. Selection 793A was stunted, producing little growth in either height or numbers of leaves, averaging only a 40% increase in height each month. Selections 33C and 335D grew moderately well, with 70 and 80% increases in height at monthly intervals respectively. Cultured shoots of 1929E grew the most vigorously, producing 160% increase in height during each subculture. After 8 months in culture, all selections were growing actively, including those that grew slowly when originally placed in culture. Multiplication rates averaged 7–9 new shoots from a single initial shoot per month (Figure 1).

Single shoots, 1 cm long excised from proliferating cultures derived from mature trees were placed either horizontally or vertically with basipetal end in the medium to test the effect of orientation of shoots on multiplication rate. When grown vertically, shoots of 33C, 335D, and 1929E had a significantly greater increase in height and equal to or greater mean number of shoots compared to shoots placed horizontally (Table 3). We had an insufficient number of shoots of 793A to compare the effects of orientation on shoot multiplication.

Shoot cuttings excised from cultures derived from mature plants grown in vitro were placed on medium containing 0.5 mg l^{-1} IBA for root production. The number of roots was recorded after 6 weeks. Shoots were taken the first, second, and third month after the original explant was placed in culture. The percentage of shoots that formed roots during the first 3 months remained fairly constant but varied markedly among genotypes (Table 4).

Several treatments to improve rooting of genotypes 923A, 33C, and 335D were tried the third month after shoots were originally placed in culture. Increasing the concentration of IBA to 1.0 mg l^{-1}, placing the shoots in the

Figure 1. Mass of shoots of *Liquidambar styraciflua* L., derived from mature tree, grown in vitro on Woody Plant Medium with 1.0 mg l^{-1} BA and 0.05 mg l^{-1} NAA.

dark during the first 5 days on rooting medium, and a 5-day pretreatment with IBA (1.0 mg l^{-1}) followed by transfer to medium without IBA did not increase the percentage of rooting. In all treatments, 1929E rooted at 100%, 923A less than 30%, and 33C and 335D at approximately 50%. Shoot cuttings of 335D were placed on medium containing 1.0 mg l^{-1} IBA for 1 month during which time they did not root. When transferred to medium lacking hormones the shoots started to form roots within 3 weeks and after one month 80% of the shoots had rooted (Figure 2). After 1 year in culture, 80–90% of shoots of all selections formed roots when placed on 1.0 mg l^{-1} IBA for 1 month.

Table 3. Effect of orientation on growth in vitro of *Liquidambar styraciflua* L. shoots derived from mature trees

Selection	Treatment	Mean increase in height of shoots (cm ± SEM)	Mean number of shoots (± SEM)[c]
33C	Horizontal[a]	0.49 ± 0.00	9.0 ± 0.20
	Vertical[b]	0.67 ± 0.08*	9.0 ± 0.19
335D	Horizontal	0.51 ± 0.05	5.3 ± 0.06
	Vertical	0.85 ± 0.04*	9.4 ± 0.04*
1929E	Horizontal	0.56 ± 0.04	5.0 ± 0.10
	Vertical	0.88 ± 0.09*	9.2 ± 0.25*

* Significantly different within each genotype ($p = 0.05$) by Student's t-test
[a] $n = 38$, 2 replications
[b] $n = 30$, 2 replications
[c] Standard error of the mean

Table 4. Root formation of shoots derived from mature trees of *Liquidambar styraciflua* L. regenerated in vitro after repeated subcultures

Selection	Percentage of plants forming roots[a]			
	Time after initial incubation in vitro[b]			
	1 month	2 months	3 months	12 months
923A	0	25	8	85
33C	50	20	25	88
335D	50	33	42	89
1929E	67	100	100	88
Mean of all genotypes	44	50	44	88

[a] n = 20; scored after 4 weeks on rooting medium
[b] Subcultured every month

Figure 2. Regenerated plant of *Liquidambar styraciflua* L. derived from mature tree.

The plants obtained from rooted cuttings were transferred to a mixture of equal parts by volume of peat, perlite, and vermiculite, drenched with Benlate and placed in a humidity tent. They were transferred to the greenhouse after 2 weeks. Percentage of survival of shoots in spring and summer was approximately 90%. During the winter as many as 50% of the rooted cuttings died when they were potted up and transferred to the greenhouse.

Although differences existed between the growth of seedling and mature material in culture, the use of seedling material for determining the proper conditions for culture of mature material has certain advantages and is recommended when one is first trying to propagate a woody species in vitro. Ease of growing seedlings in a greenhouse, tolerance to a wider range of nutrient media and growth conditions, and large numbers that are available are advantages of using seedlings. The mature material, which is usually less plentiful, can be saved for critical determinations of media and other culture conditions.

To date all selections of sweetgum that we have placed in culture have responded well, producing 5–8 shoots per month from an original shoot. From 85–90% rooting has been obtained depending on the selection. Multiplication by axillary buds was ensured by using concentrations of BA less than 2.0 mg l^{-1} and by avoiding bud formation on basal callus-like tissue, which was promoted by higher concentrations of BA. Although fewer plants were produced using lower concentrations of BA, there is greater assurance of genetic uniformity than when higher concentrations of BA are used [11]. Preliminary results indicate that plants derived from micropropagation are phenotypically similar to the parent plants.

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