9. Kralik, J., J. Sebanek, and Obhldalova. 1979. Morphological experiments on forest woody species. Priodoved Pr. Ustavu Sesk. Akad. Ved. Brne. 13:1-30.
10. Maynard, B. K. and N. L. Bassuk. 1987. Stockplant etiolation and blanching of woody plants prior to cutting propagation. J. Amer. Soc. Hort. Sci. 112:273-276.
11. Morgan, D. L. 1979. Vegetative propagation of Texas live oaks. Proc. Int. Plant Prop. Soc. 29:113-115.
12. Smith, D. J. and W. W. Schwabe. 1984. Acceleration of early growth of seedlings and rooted cuttings of *Quercus robur*. Forestry 57:143-158.
13. Van Staden, J. and A. R. Harty. 1988. Cytokinins and adventitious root formation. In: Adventitious Root Formation in Cuttings. T. D. Davis, B. E. Haissig, and N. Sankhla, Editors. pp. 185-201. Dioscorides Press, Portland, Oregon.

Proc. Fla. State Hort. Soc. 102:264-268. 1989.

IN VITRO PROPAGATION OF FLORIDA NATIVE PLANTS: *STYRAX AMERICANA* AND *PERSEA PALUSTRIS*

M. E. Kane, T. J. Sheehan, B. Dehgan, and
N. L. Philman
University of Florida, IFAS
Department of Ornamental Horticulture
Gainesville, FL 32611

Additional index words. acclimatization, micropropagation, tissue culture, woody plants.

Abstract. **Micropropagation protocols for *Styrax americana* L. (American snowbell) and *Persea palustris* (Raf.) Sarg. (Swamp bay persea) were developed and the post-transplant performances of rooted microcuttings were evaluated. In *Styrax,* maximum shoot regeneration (13-fold increase per 7 week culture cycle) from nodal explants occurred on agar-solidified Woody Plant Medium (WPM) supplemented with 1.0 mg/liter N⁶-benzyladenine (BA). In *Persea,* maximal shoot regeneration (4-fold increase per 6 week culture cycle) occurred when nodal explants were cultured in liquid Murashige & Skoog medium (MS) supplemented with 0.25 mg/liter BA and 0.5 mg/liter gibberellic acid (GA_3). *In vitro* rooting of 1.5-cm-long *Styrax* microcuttings increased 74% on agar-solidified MS medium supplemented with 1.0 mg/liter indolebutyric acid (IBA). Transplant survival of rooted *Styrax* microcuttings was 83%, but acclimatized plantlets grew slowly, increasing only 52% in length 28 days post-transplant. One hundred percent *ex vitro* rooting of 3.0 cm long *Persea* microcuttings was attained by pre-dipping microcuttings in 5)) mg/liter IBA for 15 minutes prior to direct sticking in Vergro Klay Mix A, a soilless growing medium. Although rooted *Persea* microcuttings readily acclimatized to greenhouse conditions (88% survival), post-transplant growth was slow.**

In Florida, native woody plants have emerged as desirable alternatives to exotics for use in the urban landscape and are being utilized in revegetation of disturbed sites. The market for these "new plants" is reflected by the presence of more than 50 Florida native plant nurseries. Limited information is available on the traditional propagation techniques required for successful production of many native woody species (4, 5, 6). Although *in vitro* propagation (microprogation) systems have been developed for the efficient and large-scale clonal propagation of many fruit and forest tree species (1, 2, 6, 7, 9, 10, 14), procedures are not available for the micropropagation of most native trees and shrubs possessing potential commercial value (3, 8, 10). The application of *in vitro* culture techniques to woody native plant production could be useful for production of difficult to propagate or rare and endangered species and selection of elite clonal lines exhibiting superior growth, form, enhanced disease resistance, stress tolerance, and other commercially valuable characteristics (10). The objectives of this study were to determine the feasibility of developing protocols for the *in vitro* propagation of *Styrax americana* (American snowbell) and *Persea palustris* (Swamp bay persea) and to evaluate the *ex vitro* post-transplant performance of both species.

Florida Agricultural Experiment Station Journal Series No. N00077. This project was supported in part by a grant from the Florida Institute of Phosphate Research. We greatfully acknowledge the technical assistance of D. Wayne Porter.

Materials and Methods

Culture Protocol for Styrax americana. Shoot-tip cuttings of *Styrax americana* were collected from flowering plants growing on undisturbed sites at W. R. Grace and Company, Four Corners Mine, Hillsborough County, Florida. Single node stem explants were surface sterilized by successive immersion in 50% (v/v) ethanol for 30 seconds and 1.0% (v/v) aqueous sodium hypochlorite for 15 min, followed by three 5-min rinses in sterile deionized water. Surface sterilized nodal explants were transferred individually into 150 X 25 mm culture tubes containing 15 ml sterile establishment (Stage I) medium consisting of Murashige and Skoog [MS] inorganic salts (13), 100 mg/liter myo-inositol, 0.4 mg/liter thiamine-HCL and 3) g/liter sucrose supplemented with 0.5 mg/liter N⁶-benzyladenine (BA). The medium was solidified with 8 g/liter TC Agar (Hazelton Research Product, Inc., Lenexa, KS). All media were adjusted to pH 5.7 with 0.1 N KOH before autoclaving at 1.6 Kg cm^{-2} for 20 min at 121°C. Unless stated otherwise, all cultures were maintained at 15±2°C under a 16-hr photoperiod provided by cool-white fluorescent lights at 90 $\mu mol\ m^{-2}s^{-1}$ as measured at culture level.

Nodal explants (each 5 mm long bearing two axillary buds with attached subtending leaves) were excised from Stage I cultures and transferred into 150 x 25 mm culture tubes containing either 15 ml sterile MS or Woody Plant Medium [WPM] inorganic salts and vitamins (11) supplemented with 30 g/liter sucrose and solidified with 8 g/liter TC agar. Media were supplemented with BA at five concentrations (0, 0.1, 0.5, 1.0, and 5.0 mg/liter) alone or with 0.1 mg/liter 1-naphthaleneacetic acid [NAA]. Nodal explants were oriented horizontally onto the medium. A

culture tube inoculated with a single explant served as the experimental unit. Each treatment was replicated 10 times using a completely randomized design. Treatment effects on shoot multiplication and length were evaluated after 7 weeks.

Rooting was attempted *in vitro* by transferring 8-12 mm microcuttings into GA7 Vessels (Margenta Corp., Chicago, IL) containing 80 ml sterile agar-solifified (0-8% w/v) medium consisting of WPM supplemented with 30 g/liter sucrose and either 0, 0.05, 0.1, 0.5, or 1.0 mg/liter indolebutyric acid (IBA). The basal end of each microcutting was embedded ca. 5 mm into the medium. Each GA7 Vessel, inoculated with 10 microcuttings, served as the experimental unit. Each treatment was replicated 5 times using a completely randomized design. Treatment effects on percent rooting, root number, and length were evaluated after 4 weeks. Rooted microcuttings were then individually transferred into separate plug tray cells (60 x 40 x 55 mm cell) containing pasteurized Vergro Klay Mix A (Verlite Co., Tampa, Florida) soilless growing medium. Each tray was covered with a clear vinyl propagation dome to maintain humidity and then placed under the growing conditions described above. After 2 weeks, the propagation domes were incrementally removed over a 5-day-period to decrease humidity and acclimatize plants for transfer to greenhouse conditions. Plants were placed under shade (50% light reduction) in a greenhouse for 2 weeks before being transplanted into 4-inch plastic pots containing Vergro Klay Mix A. Plants were then transferred to 30% light reduction for 3 weeks, before shifting to full sunlight. Plants were top dressed every three months with Osmocote 14N-14P-14K (10 g/container). Percent survival, shoot growth, and branching of acclimated plants were monitored and recorded.

Culture Protocol for Persea palustris. Shoot-tip cuttings and mature fruits were collected from trees growing on undisturbed land at Occidental Chemical Company's Suwanee River Mine, Hamilton County, Florida. Preliminary experiments demonstrated that field collected shoot-tip explants were difficult to surface sterilize. Consequently, two alternative methods were examined to generate plants *in vitro* from excised embryo tissue. The first method attempted was generation of morphogenic (shoot-forming) callus from cotyledon tissue. Mature fruits (single-seeded) were surface sterilized by successive immersion in 95% (v/v) ethanol for 1 min and 2.6% (v/v) aqueous sodium hypochlorite for 15 min, followed by three 5-min rinses in sterile deionized water. Fruits were aseptically sectioned transversely cutting the embryo in half. Although the fruit walls showed no evidence of damage, about 80% of the fruits sectioned were infested internally with insect larvae that had consumed portions of the embryos (Fig. 2A). Cotyledons from non-infested embryos were cut into 2 x 2 mm explants and placed in plastic 100 X 15 mm petri plates (5 explants/plate) containing 30 ml agar-solidified MS inorganic salts and vitamins (13) supplemented with 0.1 mg/liter naphthaleneacetic acid (NAA) and one of three cytokinins: BA, 2-isopentenyladenine (2iP), or kinetin at four levels (0, 1, 5, 10 mg/liter). A petri plate inoculated with 5 explants served as the experimental unit. Each treatment was replicated 5 times using a completely randomized design. Treatment effects on callus formation were determined after 4 weeks culture at 25 ± 2°C in the dark. Cultures were transferred to light conditions for an additional 4 weeks before calli were scored for the presence of shoots.

With the second method, sterile shoot-tip explants for Stage I cultures were obtained from 4-week old *Persea* seedlings germinated *in vitro* from intact excised embryos cultured in the dark on agar-solidified MS supplemented with 1.0 mg/liter BA. Preliminary experiments indicated that, regardless of cytokinin type or level, excised seedling shoot-tips exhibited heavy callus development but no shoot multiplication when cultured on agar-solidified media. Shoot-tip explants were transferred into 125 ml Erlenmeyer flasks containing 10 ml sterile *liquid* MS supplemented with BA at five levels (0, 0.25, 0.5, 1.0, or 2.0 mg/liter) and 0.5 mg/liter gibberellic acid (GA_3). Flasks were shaken on a gyratory shaker at 100 rpm. Treatment effects on shoot number and length were evaluated and recorded after 6 weeks culture.

Effect of IBA treatment on *ex vitro* (i.e., under nonsterile conditions out of culture) rooting of 30 mm *Persea* microcuttings was examined by dipping the basal cut end of each microcutting in an aqueous IBA (500 mg/liter) solution for 0, 5, 10, 15, or 30 min prior to insertion into plug trays containing Vegro Klay Mix A. Trays were covered with clear vinyl propagation domes and placed under the aforementioned growing conditions. Treatment effects on rooting were evaluated after 4 weeks. Acclimatization procedures were similar to those described for *Styrax.*

Results and Discussion

The initial rate of shoot regeneration from *Styrax* nodal explants was very low (2 shoots/explant/4 week culture cycle). Four monthly subcultures were required to generate a sufficient number of Stage I cultures to complete Stage II shoot multiplication experiments. Shoot multiplication from *Styrax* nodal explants was significantly enhanced by the addition of BA (Table 1). Maximum shoot proliferation (13 fold increase per 7 week culture cycle; Table 1) and shoot elongation (Table 2) occurred from explants cultured on WPM supplemented with 1.0 mg/liter

Table 1. Influence of benzyladenine (BA), naphthaleneacetic acid (NAA), and gibberellic acid (GA_3) on *in vitro* shoot multiplication from nodal explants of *Styrax americana* cultured on Murashige & Skoog Medium (MS) or Woody Plant Medium (WPM) for 7 weeks.

BA (mg/liter)	NAA (mg/liter)	Shoots/Culture $-GA_3$ MS	Shoots/Culture $-GA_3$ WPM	Shoots/Culture $+0.5\ GA_3$[y] MS	Shoots/Culture $+0.5\ GA_3$[y] WPM
0	0	1.4 ± 0.2[z]	1.6 ± 0.2	—	—
0.1	0	2.6 ± 0.8	2.2 ± 0.3	—	—
0.5	0	9.2 ± 1.9	8.0 ± 2.3	—	—
1.0	0	7.1 ± 1.3	13.8 ± 4.1	—	—
5.0	0	5.4 ± 0.7	6.8 ± 1.0	—	—
0	0.1	1.1 ± 0.1	1.7 ± 0.6	1.0 ± 0	1.1 ± 0.1
0.1	0.1	1.2 ± 0.2	2.0 ± 0.4	2.8 ± 0.5	1.3 ± 0.3
0.5	0.1	7.0 ± 0.9	6.6 ± 2.3	7.6 ± 1.0	4.1 ± 1.2
1.0	0.1	8.9 ± 1.6	7.6 ± 0.8	7.3 ± 0.8	7.8 ± 2.2
5.0	0.1	3.8 ± 0.5	6.4 ± 0.8	2.5 ± 0.8	5.2 ± 0.8

[z]Each value represents the mean response (± SE) of 10 replicate cultures.
[y]GA_3 concentration in mg/liter.

Table 2. Influence of benzyladenine (BA), naphthaleneacetic acid (NAA), and gibberellic acid (GA_3) on length of shoots generated from nodal explants of *Styrax americana* cultured on Murashige & Skoog (MS) or Woody Plant Medium (WPM) for 7 weeks.

BA	NAA	Shoot Length (mm)			
		$-GA_3$		$+0.5\ GA_3$[y]	
(mg/liter)		MS	WPM	MS	WPM
0	0	3.6 ± 0.9[z]	1.6 ± 0.1	—	—
0.1	0	4.1 ± 0.9	3.9 ± 1.0	—	—
0.5	0	13.0 ± 1.9	9.4 ± 1.8	—	—
1.0	0	9.4 ± 1.2	12.7 ± 1.2	—	—
5.0	0	6.7 ± 0.4	5.9 ± 0.7	—	—
0	0.1	3.6 ± 1.0	2.0 ± 0.4	2.2 ± 0.4	2.2 ± 0.5
0.1	0.1	5.1 ± 1.5	2.3 ± 0.2	3.3 ± 0.6	1.5 ± 0.2
0.5	0.1	7.0 ± 1.1	8.4 ± 1.6	11.6 ± 1.4	8.6 ± 1.1
1.0	0.1	8.1 ± 0.9	11.2 ± 1.4	9.7 ± 1.1	9.7 ± 1.2
5.0	0.1	4.7 ± 0.3	7.8 ± 0.9	6.1 ± 0.9	5.8 ± 0.6

[z]Each value represents the mean response (± SE) of 10 replicate cultures.
[y]GA_3 concentration in mg/liter.

BA alone (Fig. 1B). Effects of various levels of BA on shoot proliferation and length are shown in Figure 1A. Brand and Lineberger (2) have similarly reported WPM supplemented with 1.0 mg/liter BA alone to be the optimal Stage II medium for shoot proliferation in the native shrub *Halesia carolina* L., also a member of the Storax family (Styracaceae).

Shoot clusters produced on Stage II medium consisted of stems that were very thin and brittle (Fig. 1B). Therefore, no attempts were made to root microcuttings *ex vitro*. *In vitro* rooting was promoted in the presence of IBA (Table 3). Maximum rooting (74%) was achieved with microcuttings cultured on WPM supplemented with 1.0 mg/liter IBA (Table 3; Fig. 1C). Transplant survival of microcuttings rooted in this medium was 83% (Fig. 1D). However, rooted plantlets grew slowly, increasing only 52% (mean height: 15.2 mm) in length 28 days post-transplant (Fig. 1D).

Based on the rate of shoot proliferation, percent rooting, and post-transplant survival, 1.8 x 10^8 rooted plants

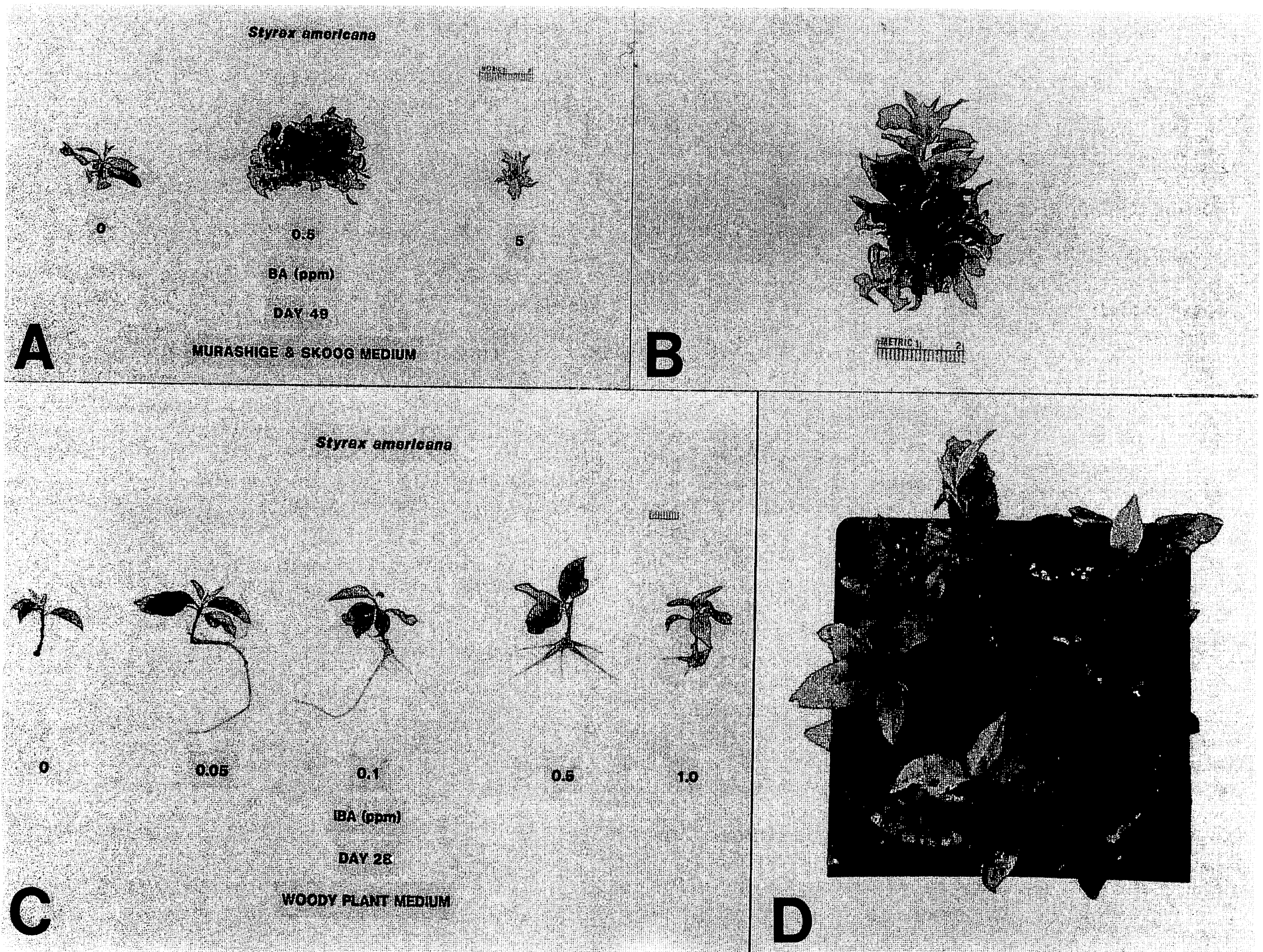

Fig. 1. Micropropagation of *Styrax americana*. A. Effect of benzyladenine (BA) level on shoot proliferation and length from nodal explants. B. Maximum shoot proliferation from a nodal explant after 49 days culture on Woody Plant Medium supplemented with 1.0 mg/liter BA. C. Influence of indolebutyric acid (IBA) on *in vitro* rooting of microcuttings after 28 days. D. Rooted plantlets 28 days post-transplant in Vergro Klay Mix A soilless growing medium.

of *Styrax americana* could be conservatively produced per year from a single nodal explant. The ability to regenerate *Styrax in vitro* from nodal explants taken from mature plants of known phenotype demonstrates that selection of elite clonal lines is possible.

Excised *Persea* cotyledon explants produced maximum callus when cultured on MS supplemented with 10 mg/liter 2iP and 0.1 mg/liter NAA (Fig. 2B). However, regardless of cytokinin type or level, all calli produced were non-morphogenic (Fig. 2B). Thus, shoot organogenesis of *P. palustris* indirectly through a callus stage was not feasible. This limitation has also been reported for other *Persea* species (15).

Maximum axillary shoot proliferation from *Persea* shoot-tips occurred when explants were shake cultured (Fig. 2c) in liquid MS supplemented with 0.25 mg/liter BA

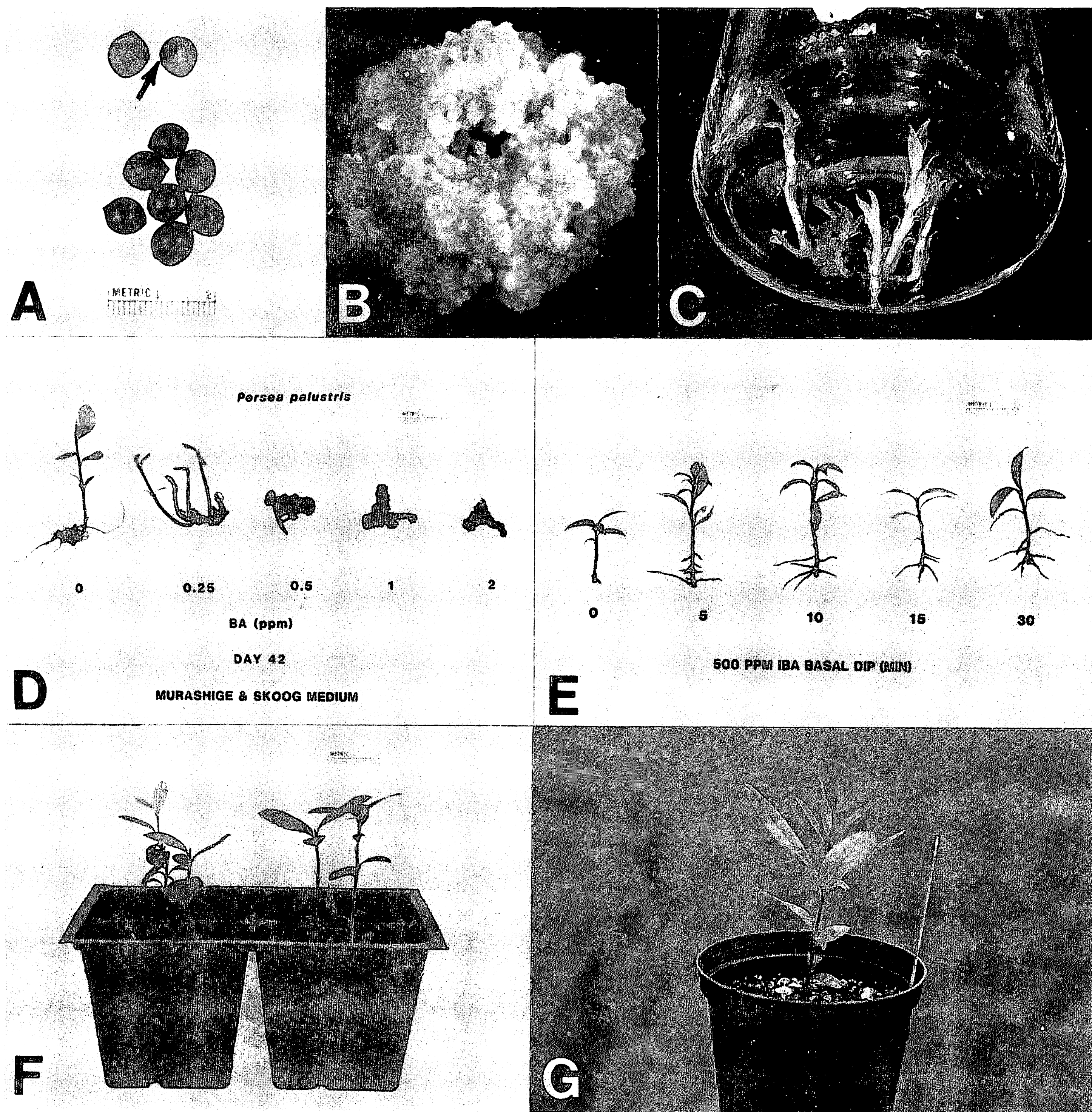

Fig. 2. Micropropagation of *Persea palustris*. A. Sectioned single-seeded fruit showing insect damage (arrow). B. production of non-shoot forming callus from cotyledon tissue after 28 days culture. C. Shoot proliferation from shoot-tip explants after 42 days in liquid shake culture. D. Typical cytokinin effect on shoot proliferation. E. Effect of IBA on *ex vitro* rooting. F. 4-week-old rooted microcuttings acclimatized to 30% shade. G. 8-week-old plant acclimatized to full sunlight.

Table 3. Influence of indolebutyric acid (IBA) on *in vitro* rooting of *Styrax americana* microcuttings after 4 weeks culture on Woody Plant Medium.

IBA (mg/liter)	Rooting[z] (%)	Root Number[y]	Root Length[x] (mm)
0	8	3.7±0.4	58.3±20.2
0.05	22	1.5±0.3	33.9± 5.3
0.1	34	1.5±0.2	34.4± 3.3
0.5	72	3.0±0.4	22.5± 1.7
1.0	74	2.5±0.2	12.2± 0.9

[z]Mean rooting response of 50 replicate microcuttings per treatment.
[y]Mean number (±SE) of roots produced per rooted microcutting.
[x]Mean length (±SE) of longest root per microcutting.

Table 4. Influence of benzyladenine (BA) and gibberellic acid (GA_3) on *in vitro* shoot multiplication from shoot-tip explants of *Persea palustris* cultured in liquid Murashige & Skoog Medium for 6 weeks.

BA (mg/liter)	Shoots/Culture		
	$-GA_3$	$+0.5\ GA_3$	$+1.0\ GA_3$[y]
0	21±0.4[z]	1.2±0.4	2.0±0.5
0.25	2.1±0.3	3.7±0.9	2.9±0.6
0.5	0.4±0.5	2.5±0.3	2.3±0.3
1.0	0.9±0.3	1.2±0.3	1.7±0.3
2.0	0.6±0.4	2.8±0.8	0.3±0.2

[z]Each value represents the mean response (±SE) of 10 replicate cultures.
[y]GA_3 concentrations in mg/liter.

Table 5. Influence of benzyladenine (BA) and gibberellic acid (GA_3) on length of shoots produced *in vitro* from shoot-tip explants of *Persea palustris* cultured in liquid Murashige & Skoog (MS) for 6 weeks.

BA (mg/liter)	Shoot Length (mm)		
	$-GA_3$	$+0.5\ GA_3$	$+1.0\ GA_3$[y]
0	21.1±3.7[z]	14.7±3.0	26.4±9.9
0.25	19.2±2.7	21.8±4.8	35.7±7.7
0.5	12.3±4.7	23.2±2.8	24.2±5.1
1.0	7.7±0.6	17.8±3.8	20.4±2.5
2.0	8.2±2.3	14.6±2.5	10.0±7.1

[z]Each value represents the mean response (±SE) of 10 replicate cultures.
[y]GA_3 concentrations in mg/liter.

Table 6. Rooting response of 30 mm microcuttings of *Persea palustris* in Vergro Klay Mix A 4 weeks following timed exposure to 500 mg/liter indolebutyric acid (PBA).

IBA Exposure (min)	Rooting[z] (%)	Root Number[y]	Root Length[x] (mm)
0	87	3.2±0.5	9.4±1.2
5	87	4.8±0.8	9.5±0.8
10	93	4.8±1.0	10.7±0.9
15	100	4.8±0.7	10.8±0.6
30	87	4.9±0.7	11.2±0.9

[z]Each value represents the mean response (±SE) of 15 microcuttings per treatment.
[y]Mean number (±SE) per rooted microcutting.
[x]Mean length (±SE) of longest root per microcutting.

and 0.5 mg/liter GA_3 (Table 4; Fig. 2D). Both shoot multiplication and length (Table 5) were reduced in MS containing greater than 0.25 mg/liter BA. Microcuttings pre-dipped in 500 mg/liter IBA were effectively rooted (100%) *ex vitro* (Table 6; Fig. 2E). Post-transplant survival of rooted *Persea* microcuttings was high (88%). Relative to other native tree species produced *in vitro* (10), post-transplant growth (Fig. 2F) of *Persea palustris* was slow (230% increase 8 weeks post-transplant).

Using the micropropagation protocol described above, approximately 7.7 x 10^4 rooted *Persea* could be produced per year. However, requirements for costly gyratory shakers and liquid culture combined with relatively low regeneration rates, make the protocol too expensive and impractical for large-scale commercial production. A recent report of the induction of indirect somatic embryogenesis in callus from immature embryos of *Persea americana* (12), suggests that micropropagation of *P. palustris* via somatic embryogenesis may be a more efficient method to mass-produce plants.

Literature Cited

1. Bajaj, Y. P. S. 1986. Biotechnology of tree improvement for rapid propagation and biomass energy production, p. 1-23. *In*: Y. P. S. Bajaj (ed.) Biotechnology in agriculture and forestry 1: Trees I. Springer-Verlag, New York.
2. Brand, M. H. and R. D. Lineberger. 1986. *In vitro* propagation of *Halesia carolina* L. and the influence of explantation timing on initial shoot proliferation. Plant Cell Tissue Organ Culture 7: 101-113.
3. Briggs, B. A. and S. M. McCulloch. 1983. Progress in micropropagation of woody plants in the United States and western Canada. Comb. Proc. Inter. Plant Prop. Soc. 33:239-248.
4. Dehgan, B., F. Almira, M. Gooch, and M. Kane. 1988. Vegetative propagation of Florida native plants: I. Hollies (*Ilex* spp.). Proc. Fla. State Hort. Soc. 101:291-293.
5. Dehgan, B., F. Almira, M. Gooch, and B. Poole. 1988. Vegetative propagation of Florida native plants: II. *Acer rubrum*, *Gordonia lasianthus*, *Magnolia virginiana*, and *Styrax americana*. Proc. Fla. State Hort. Soc. 101:293-296.
6. Dirr, M. A. and C. W. Heuser, Jr. 1987. The reference manual of woody plant propagation: from seed to tissue culture. Varsity Press, Inc. Athens, Georgia.
7. Dodds, J. H. 1983. Tissue culture of hardwoods, p. 22-28. *In*: J. H. Dodds (ed.). Tissue culture of trees. AVI Publishing, Westport.
8. Garton, S. and M. Moses. 1985. Production of native plants in tissue culture. Comb. Proc. Inter. Plant Prop. Soc. 35:306-317.
9. Kane, M. E., T. J. Sheehan, and N. L. Philman. 1987. A micropropagation protocol using Fraser photinia for mutation induction and new cultivar selection. Proc. Fla. State Hort. Soc. 100:334-337.
10. Kane, M. E., T. J. Sheehan, B. Dehgan, and N. L. Philman. 1988. *In vitro* propagation and post-transplant performance of black tupelo. Proc. Fla. State Hort. Soc. 101:301-304.
11. Lloyd, G. and B. McCown. 1980. Commercially-feasible micropropagation of mountain laurel, *Kalmia latifolia* by use of shoot-tip culture. Proc. Inter. Plant Prop. Soc. 30:421-427.
12. Mooney, P. A. and J. Van Staden. 1987. Induction of embryogenesis in callus from immature embryos of *Persea americana*. Can. J. Bot. 65:622-635.
13. Murashige, T. and F. Skoog. 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant. 15:473-497.
14. Sommer, H. E. and H. Y. Wetzstein. 1984. Hardwoods, p. 511-540. *In*: P. V. Ammirato, D. A. Evans, W. R. Sharp, and Y. Yamada (eds.). Handbook of plant cell culture. Vol. 3. Crop species. Macmillan Publishing Company, New York.
15. Young, M. J. 1983. Avocado callus and bud culture. Proc. Fla. State Hort. Soc. 96:181-182.