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Adventitious regeneration of *Juglans nigra* L. (eastern black walnut)

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Abstract. Somatic embryos and adventitious shoots were initiated from immature cotyledons 10-14 weeks after anthesis. Maximum embryogenesis occurred 12 weeks after anthesis and maximum shoot organogenesis occurred 14 weeks after anthesis. The best treatment for induction of somatic embryos and adventitious shoots from immature cotyledon explants was on agar-solidified WPM supplemented with 0.1 μM 2,4-D and 5.0 μM TDZ and incubated in light for the first four weeks. Rooting of adventitious shoots was best if they were quick-dipped in 2.5 mM IBA and 1.25 mM NAA in 1% dimethyl formamide and 3.9% ethanol (1:20 Wood's Rooting Compound: water, by volume). Plantlets from rooted adventitious shoots were acclimatized to the greenhouse.

Abbreviations. BA, Benzyladenine; 2,4-D, 2,4-dichlorophenoxyacetic acid; DKW, Driver and Kuniyuki (1984) walnut medium; IBA, indolebutyric acid; LP, Long and Preece medium described herein; NAA, naphthaleneacetic acid; PPF, photosynthetic photon flux; TDZ, thidiazuron; WPM, Woody Plant Medium of Lloyd and McCown (1980).

INTRODUCTION

Juglans nigra L. (Eastern Black Walnut) is a valuable hardwood in the eastern and central United States (Kuntz and Prey 1982). To meet the demand for high quality trees for timber production, many of the best J. nigra phenotypes have been harvested from prime walnut growing areas (Beineke 1983, Phelps 1989). Additionally, with the clearing of large forest areas in the midwestern U.S., few prime growing sites remain. This reduction of genetically superior Eastern Black Walnut has increased the need for its improvement. Biotechnology utilizing adventitious regeneration may present new opportunities for improvement of eastern black walnut. This work describes the production of somatic embryos and adventitious shoots from immature J. nigra cotyledon explants.

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MATERIALS AND METHODS

Cotyledon tissues from immature J. nigra seeds were used as explants. During the 1991 growing season, immature fruit were collected weekly from local field-grown trees. Collection began 10 weeks after anthesis on 23 July 1991 and continued until 14 weeks after anthesis on 24 August 1991. By the first collection date, seed fill had occurred and the shell had begun to harden. For a description of the seasonal changes in nut maturation for black walnut in southern Illinois, see Van Sambeek and Rink (1982).

The immature fruit were surface disinfested immediately following collection for 1 min in 70% (v/v) ethanol/water solution, followed by a 5 min immersion in 5.25% NaClO. This was followed by a 1 min rinse in sterile deionized water, 1 min in 0.01 N HCl, and three 5-min rinses in sterile deionized water. Following disinfestation a vise was used to split open the hardened endocarp. Cotyledon pieces (1 cm^2) were excised from fruit and care was taken to exclude the embryonic axis and its immediate surrounding tissue. The testa was removed from the cotyledon pieces to reduce lethal browning.

The nutrient media used in these studies were: DKW salts and organics, WPM salts and organics, and LP salts and organics (Table 1 and Preece et al. 1994). All media were supplemented with 30 g/L sucrose and the pH adjusted to 5.8 with 1 N KOH or 1 N HCl prior to the addition of either 7 g/L Sigma agar or 2 g/L Gelrite and autoclaving at 121°C and 98 kPa for 20 min.

Our purpose was to compare the major differences between the two different published procedures (Tulecke and McGranahan 1985, Neuman et al. 1992) for somatic embryogenesis of walnut. Cotyledon pieces from fruit collected 10, 11, 12 and 14 weeks after anthesis were placed in vitro onto factorial combinations of 2 media (DKW supplemented with 0.05 μM IBA, 4.4 μM BA, 9.3 μM kinetin and 250 mg/L L-glutamine (Tulecke and McGranahan 1985) or WPM supplemented with 5 μM TDZ, 0.1 μM 2,4-D and

1 g/L casein hydrolysate (Neuman 1989, Neuman et al. 1993), 2 gelling agents (2 g/L gelrite or 7 g/L Sigma agar, (Tulecke and McGranahan 1985, Neuman et al. 1992)), and 2 light treatments (continuous darkness or 16 h photoperiod provided by cool white fluorescent lamps with a PPF of 50 $\mu mol \cdot m^{-2} s^{-1}$ (Tulecke and McGranahan 1985, Neuman et al. 1992).

Cultures were initiated into 25 x 150 mm borosilicate glass culture tubes containing 15 ml medium. Each tube was capped with a clear autoclavable lid (Kaput). Transfers to fresh medium were made after 1-2 d. After 5 d, explants were transferred to 125 ml glass jars (baby food) containing 25 ml medium. These initial transfers were made to reduce the possible inhibitory effects of dark colored exudates that accumulated in the medium (Preece and Compton 1991). Subsequent transfers were performed weekly for the next 3 weeks.

After 4 weeks on the primary media at 25°C, explants were transferred to basal DKW medium without L-glutamate, casein hydrolysate, and plant growth regulators (25 ml per 100 x 15 mm petri plate). All cultures on secondary medium were incubated in continuous darkness at 25°C and were transferred to fresh medium at least twice per month.

Somatic embryos were removed individually and in clusters by breaking apart with the tips of the forceps. They were then transferred to fresh medium every 2 weeks where they underwent repetitive embryogenesis on basal DKW, unless otherwise noted. Secondary somatic embryos emerged from the surface of other somatic embryos.

Cotyledonary explants with adventitious shoots were placed into culture tubes on agar-solidified DKW with 10 μM BA until shoots began to elongate (based on Heile-Sudholt et al. 1986). Elongating shoots were then placed in liquid DKW with 10 μM BA (50 ml per Magenta GA7 vessel). These shoot cultures were incubated at 25°C and a 16 h photoperiod provided by cool white fluorescent lamps with a PPF of 50 $\mu mol \cdot m^{-2} s^{-1}$.

Histology was done on cotyledonary explants with adventitious shoots (3-5 weeks after culture initiation). The procedure described by Jensen (1962) was followed including fixation in FAA (90 ml 50% ethanol, 5 ml acetic acid, and 5 ml 37% formaldehyde), dehydration through a graded t-butanol series and embedding in paraffin. Embedded materials were sectioned (12 μm thick) on a rotary microtome and ribbons were adhered onto glass slides. The paraffin on the slides was removed by Americlear (Baxter Scientific) prior to rehydration of the tissues in a graded ethanol series and staining with 2% safranin. After rinsing to remove excess safranin, tissues were stained in 1% crystal violet, rinsed in deionized water, and then counterstained with fast green.

For rooting, shoots (2.5 cm long) were excised and cultured for 1 week on DKW with a reduced amount of nitrogen (456.2 mg/L NH_4NO_3 and 634.0 mg/L $Ca(NO_3)_2$), high sugar concentration (52.64 g/L sucrose), 1 μM IBA, 2.4 g/L gelrite, increased light (PPF 90 $\mu mol \cdot s^{-1} \cdot m^{-2}$) and supplemental bottom heat (28°C) (based on Driver and Suttle 1987). The shoots were then removed from the culture vessels and the lowest 1 cm of the stem was treated with either 3000 ppm IBA in talc (Hormodin #2), 2.5 mM IBA and 1.25 mM NAA in 50% ethanol, 5 mM IBA and 2.5 mM NAA in 7.8% ethanol and 2% dimethyl formamide (Wood's Rooting Compound diluted 1 Woods:10 water by volume), or 2.5 mM IBA and 1.25 mM NAA in 3.9% ethanol and 1% dimethyl formamide (1 Woods:20 water by volume). The shoots were then placed into 1.25 x 4 cm (5 cm^3 Techniculture) peat plugs in a 32.4 cm square styrofoam block within high humidity clear plastic flats. The flats were placed on a 28°C heating pad.

Rooted shoots were transplanted into an elongated pot (500 ml, 15 cm depth x 8 cm diameter) containing 2 sphagnum moss peat: 1 perlite: 1 vermiculite (by volume). Each plantlet was covered with a 200 ml (baby food) jar for increased humidity. Acclimatization was in the laboratory under the same light and temperature conditions as for shoot elongation. When new leaves began to elongate, the jars were gradually lifted by tilting them and placing a Magenta babyfood jar lid under one edge of the jar. This was done over a period of 1-2 weeks to decrease humidity and acclimatize the plants to the laboratory conditions. Acclimatized plants were then placed in a glass-covered greenhouse under 50% saran shade cloth for 1-2 weeks or until new leaves elongated.

Table 1. Components (in mg/L) of Long and Preece (LP) medium for black walnut secondary somatic embryogenesis.

Nitrates	
NH_4NO_3	908.0
$Ca(NO_3)_2 \cdot 4HOH$	1262.0
$Zn(NO_3)_2 \cdot 6HOH$	8.5
Sulfates	
K_2SO_4	1274.5
$MgSO_4 \cdot 7HOH$	555.0
$MnSO_4 \cdot HOH$	27.9
$ZnSO_4 \cdot 7HOH$	4.3
$CuSO_4 \cdot 5HOH$	0.25
Phosphate and oxides	
KH_2PO_4	217.5
H_3BO_3	5.5
$NaMoO_4 \cdot 2HOH$	0.32
Calcium	
$CaCl_2 \cdot 2HOH$	122.5
Iron	
Na_2 EDTA	41.35
$FeSO_4 \cdot 7HOH$	30.8
Organics	
Thiamine·HCl	1.5
Nicotinic Acid	0.75
Glycine	2.0
Pyridoxine·HCl	0.25
Myo-inositol	100.0
Sucrose	30000.0
Gelrite	2000.0
Casein hydrolysate[a]	500.0

[a]Casein hydrolysate can be omitted if the medium is not used for somatic embryogenesis.

The in vitro experiment was arranged as a split-plot design with media, gelling agents and light treatment in the main plot and time (weeks) in the subplot. Treatments within each run of the experiment had at least 10 replications. The General Linear Model (GLM) (SAS Institute 1989) procedure was used for statistical analysis. If more than 30% of the data were zeros, data were transformed using the following formula: $(y + 0.5)^{0.5}$.

RESULTS AND DISCUSSION

Somatic embryogenesis. There was a significant 3-way interaction among week after anthesis that fruit were harvested for cotyledon explants, nutrient medium, and whether incubation was in light or darkness for the first 4 weeks (Table 2). The highest percentage of explants producing embryos and the most somatic embryos formed on cotyledon explants from fruit collected 12 weeks after anthesis when placed on WPM containing 0.1

μM 2,4-D and 5 μM TDZ and incubated in the light for the first 4 weeks. Very few somatic embryos formed regardless of cotyledon maturity or light treatment if explants were placed on DKW with 0.5 μM IBA, 4.4μM BA, and 9.3μM kinetin. When we compared agar to gelrite as gelling agents during the first 4 weeks of culture (tabular data not shown), significantly more embryos formed using the WPM formulation with agar (7.0 somatic embryos per explant) than with gelrite (2.8 somatic embryos per explant; 5% t-test for paired comparisons = 2.9 embryos). This is consistent with the results of Neuman et al. (1993) who had best results using immature cotyledons, this WPM formulation, agar, and incubation in light for 4 weeks.

Table 2. Effects of week after anthesis, medium and light on somatic embryogenesis from immature cotyledonary explants of J. nigra (collected 10-12 and 14 weeks post anthesis), after 6 weeks culture in vitro.

			Mean Number of Somatic Embryos				
			All Explants				
Week	Medium	Light	%[a]	explants	n	with embryos	n
10	WPM	Light	30	6.8	10	22.7	3
		Dark	33	2.5	12	7.8	4
	DKW	Light	21	1.6	14	7.3	3
		Dark	0	0.0	9	-	0
11	WPM	Light	57	4.0	7	7.0	4
		Dark	8	0.7	12	8.0	1
	DKW	Light	0	0.0	12	-	0
		Dark	30	1.3	10	4.3	3
12	WPM	Light	65	14.4	17	22.2	11
		Dark	31	2.6	16	8.4	5
	DKW	Light	0	0.0	12	-	0
		Dark	19	0.2	16	1.0	3
14	WPM	Light	50	4.1	20	8.1	10
		Dark	42	3.8	14	8.8	6
	DKW	Light	13	0.1	15	1.0	2
		Dark	18	0.2	17	1.0	3
Significance				*		NS	
5% t-test				5.3			
1% t-test				5.7			

[a]%Percent of explants producing somatic embryos.
*, NS, Significant 3-way interaction at the 5% level or non-significant according to F-test with 3 and 120 df. Data were transformed for analysis using $(y + 0.5)^{0.5}$, untransformed data are presented. t-tests for paired comparisons are presented.

Repetitive Somatic Embryogenesis. To multiply somatic embryos, they were placed on medium individually and in clusters where they produced secondary somatic embryos over their surfaces. These were repeatedly subdivided resulting in continued repetitive somatic embryogenesis. After 3 months of repetitive embryogenesis using DKW with no plant growth regulators, most new embryos failed to develop beyond the globular stage. All embryos and embryo clusters were then placed on WPM with 1 $g\,l^{-1}$ casein hydrolysate, based on results reported by Neuman (1989) and Neuman et al. (1993). Some somatic embryos developed beyond the globular stage with this medium, but after 6 months many of the new somatic embryos were in an arrested globular stage. Therefore, all embryos and clusters were moved to a new medium that was a combination of half-strength WPM and half-strength DKW salts and organics (Long and Preece (LP) medium, Table 1). Within 3 months after placing the explants on LP medium, many more embryos were produced that matured beyond the cotyledonary stage than with any previous medium. After more than one year on LP medium, cultures continued to produce a large number of well developed somatic embryos. This LP medium has also resulted in good growth of adult J. nigra shoot explants (unpublished results of Khan and Preece).

Adventitious Shoots. An unexpected observation was the production of adventitious shoots from the cotyledonary explants placed on WPM containing 2,4-D and TDZ (Fig. 1). No shoots formed from explants placed on the DKW medium with IBA, BA, and kinetin; therefore, explants cultured on WPM were analyzed separately.

The main effect of week after anthesis was significant with adventitious shoot number increasing in a quadratic manner as the number of weeks after anthesis increased (Table 3). The highest percentage of cotyledonary explants producing adventitious shoots and highest number of shoots per explant were from fruit collected 14 weeks after anthesis. According to Christianson and Warnick (1987) shoot organogenesis follows three physiological stages: acquisition of competence for induction (ability to respond to the inducer), induction (changing the fate of the tissue), and morphological differentiation and growth. Developing tissues, such as the immature cotyledon segments used in this study, may not be competent for induction of adventitious shoots until later in seed development. This could account for the increase in number of adventitious shoots produced with increasing weeks after anthesis.

There was a significant interaction between gelling agent and light treatment for adventitious shoot production (tabular data not shown). The greatest number of adventitious shoots (28.9

shoots per explant with 62% of the explants organogenic) formed when explants were cultured on medium gelled with agar and incubated for 4 weeks in light, and the fewest shoots formed (11 shoots per explant; 5% t-test for paired comparisons = 10.2 shoots; 42% organogenesis) when gelrite was used, regardless of whether the explants were in light or darkness. The best treatment for shoot organogenesis was also the best treatment for somatic embryogenesis.

Morphologically, it was relatively easy to distinguish between somatic embryos and adventitious shoots. The adventitious shoots were reddish, and the young leaf tips were visible and pointed (Fig. 1). When placed on DKW medium with 10 μM BA and incubated in the light, these structures turned green and elongated. Because cotyledons comprised much of the mass of the somatic embryos, they had a rounded appearance and were white to translucent or yellow in color. When these structures were placed in the light with 10 μM BA, they did not elongate or turn green. When subcultured, somatic embryos produced secondary embryos (repetitive embryogenesis). Subculture of shoots resulted in axillary shoot proliferation, but not secondary somatic embryogenesis.

Histologically, adventitious buds were unipolar structures that formed directly from immature cotyledonary explants without an intervening callus stage (Fig. 2). Obeidy and Smith (1993), showed similar adventitious buds arising from callus of mature Carya illinoinensis embryonic tissue (another member of the Juglandaceae). Their shoots were regenerated from explants placed on MS medium with 25 μM TDZ.

Organogenesis has been previously reported in J. nigra by Cornu (1988) where he described somatic embryogenesis but not organogenesis. Neuman et al. (1993) did not report shoot organogenesis in their experiments in which they placed immature cotyledonary explants on WPM with 2,4-D and TDZ from 8 to 14 weeks after anthesis. Preece, however, did observe shoot organogenesis when J. nigra cotyledon explants from southern Illinois were placed on WPM with 2,4-D and TDZ about 14 weeks after anthesis (unpublished results).

Adventitious Shoot Elongation. A problem with multiplication of walnut by somatic embryogenesis is the poor conversion rate into plantlets (Preece et al. 1994). Adventitious shoots are readily multiplied through axillary shoot proliferation; however, subsequent rooting is necessary. Adventitious shoots from cotyledonary explants turned green and elongated when incubated in the light on agar solidified DKW with 10 μM BA. After 5 d, explants were transferred to Magenta GA7 vessels containing liquid DKW with 10 μM BA. Fasciation of some shoots was observed, possibly due to the presence of TDZ in the induction medium (Huetteman and Preece 1993). Two months after placing explants on liquid media, many shoots were of sufficient length (>2 cm long) to be harvested (Fig. 3). Following harvest, the proliferating mass of short shoots was placed back on the elongation medium and shoots continued to elongate for 9 months, at which time this study was terminated.

Table 3. Effect of week after anthesis on adventitious shoot organogenesis from immature cotyledonary explants of J. nigra (collected 10-14 weeks post anthesis) and cultured on WPM only (excluding DKW treatments), after 6 weeks in vitro.

	Adventitious shoots		
Week	%[a]	Number[b]	n
10	14	2.8	22
11	11	0.9	19
12	56	15.5	33
14	76	34.5	34
Significance		**	
5% t-test		9.6	
1% t-test		13.4	
Contrasts			
Linear		**	
Quadratic		**	

** Significant at the 1% level (**) according to F-test with 3 (weeks) or 1 (linear or quadratic contrasts) and 120 df.
[a]% Percent of explants producing adventitious shoots.
[b]Number of adventitious shoots across all cultures, including those that did not produce shoots. These data were transformed for analysis using $(y+0.5)^{0.5}$ nontransformed data are presented.

Rooting of Adventitious Shoots. Following the pretreatment of harvested shoots with reduced amount of nitrogen, increased sucrose, and light, shoots treated with 3000 ppm IBA in talc (Hormodin #2) or 2.5 mM IBA and 1.25 mM NAA in 50% ethanol, or 5 mM IBA and 2.5 mM NAA in dimethyl formamide (Wood's Rooting Compound at a dilution rate of 1:10) did not root. However, treatment of adventitious shoots with 2.5 mM IBA and 1.25 mM NAA in dimethyl formamide (Wood's Rooting Compound at a dilution rate of 1:20) resulted in an overall average of 40% (19 rooted plantlets out of 47 shoots). Rooted shoots

were acclimatized in the laboratory and then in the greenhouse where leaves elongated and stems grew to 5-7 cm long before going dormant.

Addition of dimethyl formamide may account for the increased rooting of J. nigra associated with the use of Wood's Rooting compound. The same concentrations of growth regulators in ethanol resulted in no rooting. The dimethyl formamide may have aided the absorption of the growth regulators into the plant tissue.

Shoot organogenesis is an alternate adventitious regeneration route for black walnut. Its advantages over somatic embryogenesis are the later time during the season when more cotyledonary explants are responsive than with somatic embryogenesis, and the comparative ease of production of whole plants.

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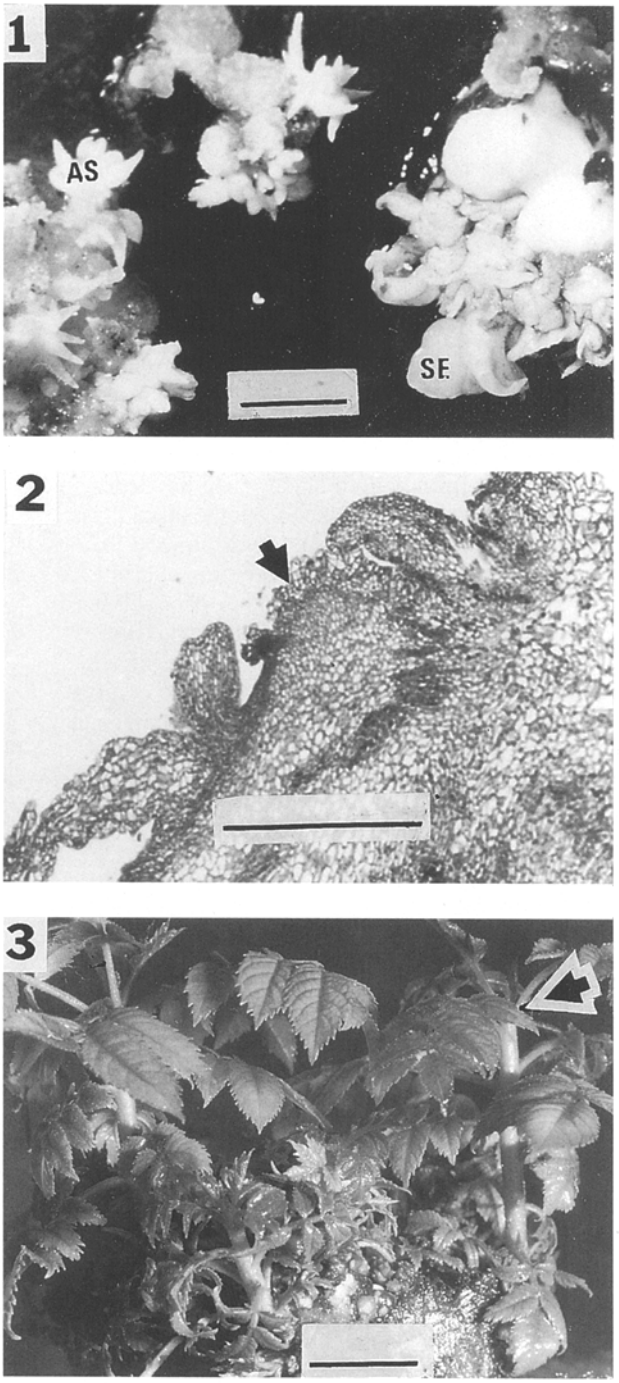

Fig. 1-3. (1) Formation of adventitious shoots (AS) and somatic embryos (SE) from J. nigra cotyledon explants after 8 weeks culture in vitro. Scale bar = 0.5 cm. (2) Adventitious shoot forming on the surface of an immature J. nigra cotyledon explant. Arrow points to apical meristem. Scale bar = 1 mm. (3) Elongating adventitious shoots (arrow) that are sufficiently long for rooting. Scale bar = 1 cm.