

Media effects on black walnut (*Juglans nigra* L.) shoot culture growth *in vitro*: evaluation of multiple nutrient formulations and cytokinin types

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Abstract The growth of black walnut shoot cultures was compared on media differing in nutrient formulation (MS, DKW, WPM, and 1/2X DKW), cytokinin type (ZEA, BA, and TDZ), and cytokinin concentration. On WPM and 1/2X DKW media, hyperhydricity was observed at frequencies of 60–100% compared with frequencies of 10–40% on the high-salt media (DKW and MS). All three cytokinins facilitated shoot regeneration from nodal cuttings, but recurrent elongation was only observed for BA (5–12.5 μM) and ZEA (5–25 μM) with mean shoot heights of 70–80 mm being possible after two culture periods (6–8 wk) for the fastest elongating lines. ZEA was effective across all six shoot lines with mean shoot heights of at least 35 mm over two culture periods, but two of the shoot lines were ‘nonresponsive’ to BA with mean shoot heights of <15 mm. In contrast, when shoot tip explants were used for culture multiplication, ZEA was the least effective cytokinin with proliferation frequencies of only 30–40%. The proliferation frequencies were twice as great (75–87%) for TDZ (0.05–0.1 μM), but most of the shoots regenerated were swollen or fasciated in morphology. High rates of proliferation (61–88%) were also possible using BA (12.5–25 μM), but axillary shoots did not elongate well, growing

to heights of only 5–10 mm, on average, after 4–5 wk. Since the cytokinin types and concentrations required for high-frequency (>50%) axillary proliferation had adverse effects on the morphology and growth potential of the shoots, multiplication strategies based on the use of nodal cuttings are recommended.

Keywords Hyperhydricity · Mineral nutrition · Shoot tip necrosis · Thidiazuron · Zeatin

Introduction

Black walnut (*Juglans nigra* L.) is one of the most popular and economically important North American hardwoods. Black walnut wood is hard and durable with a fine, straight grain and an unusually dark, nearly black, heartwood color. It is used primarily for solid furniture, veneer, and specialty products, such as gunstocks (Williams 1990). In the export market, black walnut logs and lumber account for over 16 million dollars annually in income for primary and secondary producers in the US (USDA-FAS 2005).

Black walnut trees with superior growth and wood quality traits have been identified (Beineke 1983), but the cloning and commercial utilization of these trees has been precluded by a lack of cost-efficient vegetative propagation techniques. Although successful softwood stem cutting protocols have been developed (Shreve 1974), black walnut appears recalcitrant for hardwood cutting propagation (Coggeshall and Beineke 1997). Grafting is possible, but the success rates are modest, 50–60% at best, and highly variable between grafting dates (Beineke 1984; Van Sambeek 1989; Coggeshall and Beineke 1997; Van Sambeek et al. 1997). In addition, grafting is more labor intensive than cutting propagation and when nonclonal

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rootstocks are employed, tree performance may be inconsistent between grafts.

Micropropagation has been evaluated as an alternative to conventional vegetative propagation by several previous research groups (Van Sambeek et al. 1997), but standardized shoot culture protocols for black walnut have not yet been developed. Successful results have been reported using at least three nutrient formulations (MS, DKW, LP), but the efficacy of these media has not been directly compared. Similarly, there is no consensus on the optimal cytokinin type. BA has traditionally been used for culture multiplication (Somers et al. 1982; Caruso 1983; Heile et al. 1984; Heile-Sudholt et al. 1986; Stefan and Millikan 1987; Stefan 1989). In more recent research, TDZ, a synthetic phenylurea-type cytokinin (Sakakibara 2006), has been shown to be superior to BA for shoot proliferation (Khan et al. 1995) but problems with shoot fasciation and elongation potential have since been reported (Van Sambeek et al. 1997).

The goals of the current research were twofold: (1) to establish optimized protocols for the *in vitro* culture of black walnut shoots based on the systematic evaluation of multiple nutrient formulations and cytokinin types and (2) to compare their growth responses with those that have been documented for English walnut (*J. regia*), the model species for tissue culture research within the genus.

Materials and Methods

Plant materials. Black walnut seedlings (1–2 yrs old) from the Indiana State Nursery at Vallonia, IN were used for culture initiation. The seedlings were maintained in cold storage in the fall and winter and transferred to a greenhouse each spring. The greenhouse room was unheated and naturally illuminated. New growth was forced by pruning or grafting using the seedlings as rootstocks. Explants were collected for culture initiation on three dates (March 2000, April 2000, and May 2001). Actively growing axillary shoots (10–15 cm long) were harvested from the seedlings and used to prepare both shoot tip and nodal cuttings.

Sterilization. After washing with liquid hand soap and rinsing, the explants were surface sterilized with ethanol (70%, 30 s) and bleach (0.8% NaOCl with 0.05% Tween-20 [polyoxyethylene sorbitan monolaurate]; 15 min). They were rinsed four times with sterile water, blotted dry on sterile paper towels, and transferred to the culture media. For the 2000 experiments, the explants were pooled across seedlings and sterilized en masse; but for the 2001 experiment, the explants were separately maintained for each seedling.

Media preparation. Three inorganic nutrient formulations (syn. basal salts) were employed: Murashige and Skoog medium (MS; Murashige and Skoog 1962), Driver and Kuniyuki walnut medium (DKW; Driver and Kuniyuki 1984), and woody plant medium (WPM; Lloyd and McCown 1981). DKW medium was tested in both undiluted and diluted form with the diluted medium containing half the mineral salt content of the original recipe (1/2X DKW). Commercially supplied basal salt mixtures were used for media preparation (JRH Biosciences, Lenexa, KS [MS] and Phytotechnology Labs, Overland Park, KS [DKW, WPM]). Vitamins (nicotinic acid, pyridoxine, and thiamine) and glycine were prepared as aqueous stocks and added per original media recommendations. The media were supplemented with adenine sulfate (20 mg l⁻¹) and sucrose (20 g l⁻¹) and adjusted to pH 5.7–5.8 before the addition of the gelling agent (2 g l⁻¹ Gelrite®; Phytotechnology Labs, Overland Park, KS).

After autoclaving (121°C, 15 psi, 20 min), the media were cooled to 65°C in a water bath, growth regulators were added, and the media were mixed and poured. Five cytokinins were utilized in total (*N*⁶-(2-isopentyl) adenine [2iP], 6-furfurylaminopurine [kinetin, KIN], 6-benzylaminopurine [BA], *N*⁶-(4-hydroxy-3-methylbut-2-enylamino)purine [zeatin, ZEA], and 1-phenyl-3-(1,2,3-thiadiazol-5-yl)urea [thidiazuron, TDZ]). A single auxin, indole-3-butyric acid (IBA), was employed as a supplement to the BA medium (5 μM BA+0.05 μM IBA). All of the growth regulators were purchased from Sigma-Aldrich in free base (cytokinin) or free acid (auxin) form, with the ZEA consisting of a mixture of isomers (91% *trans* and 9% *cis* isomer, Sigma-Aldrich Product Z1064). Growth regulator stocks (0.1–100 mM) were prepared in DMSO and stored frozen at –20°C.

Culture environment. The cultures were maintained under a 16-h photoperiod on aluminum growth shelves. Lighting was provided from above by cool white fluorescent bulbs (25–30 μmol m⁻² s⁻¹), and the temperature was maintained at 23±2°C.

Culture initiation. Nearly 20 media, differing in nutrient formulation (WPM, DKW, and MS) and cytokinin type were evaluated. The cytokinin concentrations were variable, ranging from 0.01 μM for TDZ, 2.5–5 μM for KIN and 2iP, and 2.5–12.5 μM for BA and ZEA. For the first experiment (March 2000), the explants were transferred to the experimental media directly; but for subsequent experiments, the explants were precultured on hormone-free media for 12–14 d for sterility screening.

On the experimental media, the explants were transferred at 3–4 wk intervals, and culture periods (stages) were counted successively. The initial (Stage 1) media were

prepared in 25×150 mm glass culture tubes, but all subsequent media were prepared in larger containers, either glass baby food jars (230 ml) or polycarbonate GA-7 vessels (Magenta, Chicago, IL). By the end of the third culture period, subdivision of the explants (multiplication) was initiated. When axillary proliferation was present, the new shoots were cut off and individually cultured. Cultures lacking proliferation were maintained using shoot tip explants.

Despite the wide variety of media evaluated, the degree of treatment replication during culture initiation was limited as a consequence of the high rates of endogenous bacterial contamination (30–60%, with the bacteria generally not appearing until the second or third culture period), and the data from these experiments is not formally presented. In total, 14 shoot lines were maintained *in vitro* for periods of at least a year, but only six exhibited reliable growth and proliferation. Over a 2-yr period (2002–2003), explants from these shoot lines were used both (1) to confirm the nutrient formulation effects observed during culture initiation and (2) for dose–response testing for three cytokinins (ZEA, BA, and TDZ) that had promoted shoot growth during culture initiation.

Experimentation. Two types of explants from the shoot lines were employed: nodal cuttings (0.5–1 cm long and leafless with one to three buds each) and shoot tip explants (1–2 cm long). In general, the nodal cuttings were used to evaluate treatment (media) effects on shoot elongation and the shoot tip explants were used to evaluate media effects on shoot proliferation. For the experiments employing nodal cuttings, data were collected after 3–4 wk on the frequency of shoot regeneration and mean shoot heights. In addition, approximately half of the explants were maintained for a second culture period and monitored for de novo shoot elongation. Shoot tip explants, maintained for a single culture period of 4–5 wk, were evaluated for both proliferation and elongation, but with the degree of elongation being rated on an ordinal scale.

All six shoot lines were included in the experiments, but the degree of testing was variable between lines because of their different rates of growth and proliferation, and thus varying explant yields. The degree of replication was also variable between media types within shoot lines since the explants were blocked into treatments by parent shoot, but each shoot did not always supply enough explants for all of the treatments, especially for the cytokinin dose–response experiments.

Data analysis and statistical testing. Data analysis was complicated by the unbalanced experimental design with the degree of replication varying both between shoot lines and media types within lines. Although the responses of the

shoot lines to the treatments were qualitatively similar, since the degree of response (mean shoot height, etc.) varied between lines, the treatment means presented are not directly comparable. However, for several key response variables, the data is presented separately by shoot line (Table 4) and for the height data, ‘corrections’ were made during the statistical analysis to account for the differential contributions of each shoot line to the means (*see below*). SAS Version 8.02 (SAS, Research Triangle Park, NC) was used for all statistical testing.

The shoot height data were evaluated for treatment effects by *t* testing or ANOVA, depending on the number of treatments and the level of analytical complexity required. The ANOVAs were performed using a fixed effects model and type III sum of squares values for significance level determination since the data were unbalanced. Residuals from the analysis were used to verify the assumptions of normality and variance homogeneity. Treatment means were compared using the LSD protocol for one-way ANOVAs. For multiway ANOVA, pdiff arrays or orthogonal contrasts, were used to compare treatment means, provided that the treatment variable (classification factor) in question acted independently of other factors in the ANOVA model. The ‘lsmeans’ protocol, which calculates the adjusted means, termed ‘ls means’, with reference to a second classification factor, was used to ‘correct’ for differences in the number and proportion of seedlings tested on each media type.

The ordinal data (shoot elongation ratings) were evaluated by ‘nonparametric ANOVA’ (Kruskal–Wallis testing) and the nominal data, expressed as percentages in the data tables, were analyzed using contingency tables (2×*c*) and the χ^2 distribution for probability estimation. When treatment effects were detected, the treatments responses were compared using 2×2 subtables (nominal data) or Mann–Whitney *U* tests (ordinal data). Probability values for the 2×2 subtables, as well as for all 2×*c* tables with expected values <5, were calculated using the Fisher exact protocol.

Results

Nutrient formulation. During culture initiation, hyperhydricity (waterlogging; syn. vitrification) was observed at frequencies of 60–70% on WPM, as assessed at the end of the second culture period compared with frequencies of <5% on DKW and MS media (data not shown). Experiments employing nodal cuttings from the stabilized shoot lines were used to confirm these patterns, but with the addition of a fourth nutrient formulation (1/2X DKW). The diluted DKW medium treatment was included to assess whether the differences in hyperhydric

frequency were related to differences in ionic strength; 44 meq/l for WPM versus 96.2 and 94.8 meq/l for MS and DKW, respectively (George et al. 1987).

For both low-salt media (WPM and 1/2X DKW), the frequencies of hyperhydricity (85–100%) were two to three times greater than those observed on DKW or MS (23–40%), and the differences were significant at the 5% level of probability (Table 1; 12.5 μ M ZEA data). The shoots regenerated on WPM and 1/2X DKW also shared a ‘small leaf’ phenotype, i.e., with mean leaf sizes (lengths) of 0.5–2 cm compared with leaf sizes of 2–4 cm on MS (ZEA) and DKW (ZEA) media (Fig. 1A,B).

Cytokinins and shoot elongation. For all three cytokinins (BA, ZEA, and TDZ), axillary shoot development from the nodal cuttings was typically a two-step process with the shoots passing through a rosette stage before the onset of internodal elongation (Fig. 2B). In contrast, when shoot tip explants were employed, direct elongation of the axillary shoots was generally observed. The duration of the rosette stage was variable between shoot lines, and for two lines, elongated shoots were generally present by the end of the first culture period (Table 4; lines 2 and 3). For the remaining lines, this conversion was frequently delayed until the second culture period, particularly on the media with high BA concentrations (12.5–25 μ M; Table 2). Thus, the degree of height growth was generally much greater during the second culture period for the cumulative data set (Fig. 2).

ZEA and BA were superior to the TDZ for shoot elongation with mean second month shoot heights of up to 37 mm on BA (5 μ M) and 46 mm on ZEA (12.5 μ M) vs. 15–21 mm for the TDZ media (Table 2). The differences in shoot height for the 12.5- μ M ZEA and 5- μ M BA media (46 vs. 37 mm) were not statistically significant as evaluated by either *t* testing ($p=0.281$) or ANOVA using the ‘lsmeans’ protocol to correct for the differential contributions of the shoot lines to the treatment means ($p=0.092$). However, ZEA was superior to BA in being effective across all six shoot lines (Table 4). In contrast, two shoot lines (Lines 5 and 6) were recalcitrant to BA with mean second month heights of only 11–12 mm compared with the mean heights of at least 35 mm on the ZEA media (Table 4).

BA was also inferior to ZEA to the extent that it had adverse effects on the health of the explants. For the 5- to 25- μ M BA media, the frequencies of explant necrosis (27–33%) were two to three times greater than those that were typical on ZEA, TDZ, or for the controls (Table 2), and the differences were statistically significant ($p=0.0002$, $n=314$). Although BA ‘toxicity’ was not apparent during culture initiation, this was presumably a consequence of the relatively low BA concentrations employed (primarily 2.5 μ M). Explant necrosis did not entirely preclude shoot regeneration, but for the cumulative nodal cutting data set only 5–10% of the necrotic explants produced elongated shoots compared with frequencies of >60% for the unaffected explants (data not shown).

Table 1. Nutrient formulation effects on shoot regeneration from nodal cuttings, data combined across shoot lines

ZEA	Basal salts	N	% ES ^x	Shoot characteristics			
				Ht (mm)±SE ^y	% Hyp ^z	% Tip Nec ^w	
						Stg 1	Stg 2
5 μ M	DKW	7	71	11±4	40	0	N.D.
	WPM	16	88	17±4	85	0	18
	1/2X DKW	11	90	20±5	90	11	44
12.5 μ M	DKW	43	87	16±2 ^b	33 ^b	3	19
	WPM	24	64	15±2 ^b	71 ^a	6	29
	1/2X DKW	14	71	29±3 ^a	100 ^a	17	33
	MS	21	62	15±3 ^b	23 ^b	8	53

For the 12.5- μ M ZEA data, *superscript letters* indicate treatment responses that were significantly different ($\alpha=0.05$). The height data was evaluated by ANOVA using the ‘lsmeans’ protocol of SAS to adjust for the differential contributions of the shoot lines to the treatment means. The proportion data was evaluated by contingency table testing with 2×2 subtables being used to compare treatment pairs when nutrient formulation effects were detected. The 5- μ M ZEA data was not analyzed statistically because of the more limited degree of treatment replication.

^w Percentage of shoots exhibiting tip necrosis. Explants with tip necrosis by the end of the first culture period were not subcultured.

^x Percentage of explants producing elongated shoots (>2–3 mm tall) as assessed at the end of the first 3–4 wk culture period (Stage 1)

^y Height data was collected for the subset of explants with elongated shoots (% ES). When multiple shoots were present, the tallest was measured.

^z Percentage of hyperhydric (waterlogged) shoots for the subset of explants with elongated shoots (% ES). The high hyperhydric frequencies for the DKW (ZEA) media (33–40% compared with values of 3–11% for the Table 2 data set), appeared to be an artifact of the use of nodal explants from shoots cultured on 1/2X DKW (ZEA).

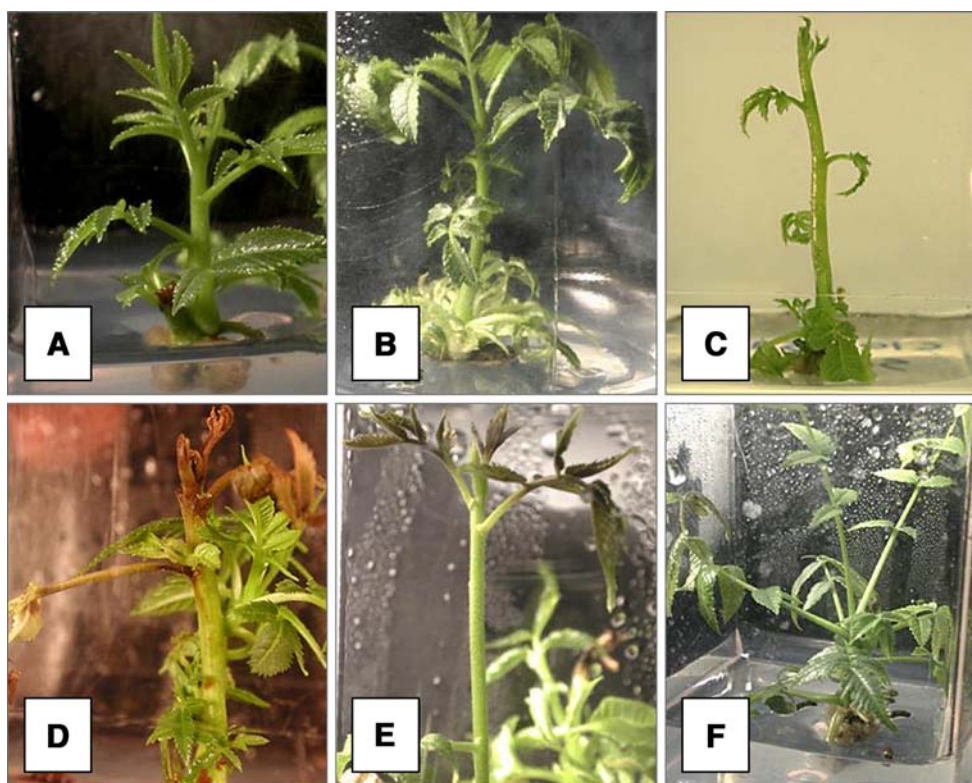


Figure 1. Shoot regeneration from nodal cuttings. (A) The bud initially developed as a rosette shoot, as indicated by the basal cluster of leaves. An elongated shoot was subsequently produced. (B) ‘Two-phase’ shoot development on 12.5 μM ZEA medium. The bud initially developed as a rosette shoot, as indicated by the basal cluster of leaves from which an elongated shoot was subsequently produced. (C) Axillary shoot development on WPM with 5 μM ZEA. The shoot is hyperhydric with small leaves, as was typical on WPM medium. (D)

Shoot tip necrosis on DKW with 25 μM ZEA. The necrosis developed as the shoot was elongating and axillary branching (regrowth) is already apparent. (E) Apical necrosis of an actively growing shoot tip on DKW medium with 5 μM ZEA. The developing leaves are affected but the shoot tip is still healthy and apical dominance has been maintained. (F) Apical quiescence on DKW medium with 2.5 μM ZEA. The axillary shoot has stopped elongating as indicated by the cluster of large leaves near the apex.

The efficacy of ZEA for shoot elongation was partly counteracted by an increased susceptibility to shoot tip necrosis (Fig. 1D). For the nodal cutting data set, shoot tip necrosis was only apparent on ZEA media (5–25 μM) with up to 50% of the explants being affected (25 μM ZEA; Table 2). Apical necrosis was also observed on the 2.5- μM ZEA and 5- μM BA media at frequencies of 18–26% (data not shown), but the necrosis only affected the developing leaves and never spread to the stem (Fig. 1E). The rates of shoot tip necrosis were also highest for ZEA for the shoot tip data set (Table 3); and for both explant types, periods of rapid elongation appeared to predispose the cultures to shoot tip necrosis (data not shown).

TDZ was ineffectual for the induction of recurrent elongation. For the nodal cutting data set on 0.05 μM TDZ, nearly half the explants that had produced elongated shoots by the end of the first culture period failed to exhibit new shoot elongation (>5 mm) after subculture compared with ‘quiescence frequencies’ from 0% to 25% for most of the BA and ZEA media (data not shown). When 0.01 μM TDZ was employed, most of the shoots continued to elongate during

the second culture period (70%), but the total degree of shoot elongation was less than on 0.05 μM TDZ (Table 2). TDZ was the only cytokinin for which the degree of shoot elongation did not increase during the second culture period (Fig. 2). The inadequacy of TDZ for recurrent shoot elongation was also apparent when shoot tip explants were employed with the mean elongation ratings of 0.18–0.19 (Table 3), indicating a lack of definite elongation.

For all three cytokinins, the lowest concentrations tested (2.5 μM BA and ZEA, 0.01 μM TDZ) were suboptimal for shoot elongation with the mean shoot heights equal only to 57–72% of the average heights for the concentrations inducing maximal elongation (Table 2). Visual signs of cytokinin depletion were also apparent for the ‘low-cytokinin’ media. Shoot elongation frequently ceased before the end of the second culture period; (Fig. 1F) and in some instances, putative terminal buds were produced, particularly on the 2.5- μM ZEA medium (data not shown).

In contrast, the highest BA concentrations (12.5, 25 μM) were supraoptimal for shoot elongation (Tables 2 and 3).

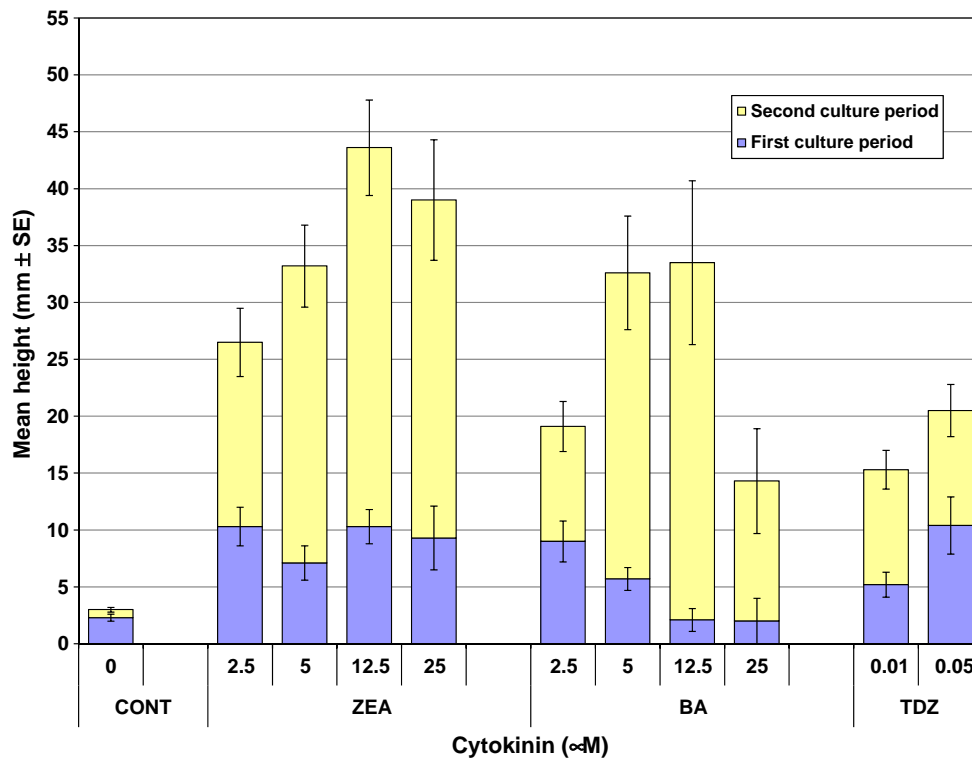


Figure 2. Time course of axillary shoot regeneration from nodal cuttings on DKW medium. The height data is pooled across shoot lines for the subset of explants that were maintained for two culture periods and had produced elongated shoots by the end of the second culture period. For some explants (33/146 for the cumulative data set), shoot elongation was not apparent at the end of the first culture period and a zero was assigned for the first culture period height values.

Thus, the first culture period height values reported are not directly comparable with those reported in Table 2, which are higher because the averages were calculated for the subset of explants with elongated shoots. For most treatments, the means incorporate data for all six shoot lines: however, for the 12.5- and 25- μM BA media, the two lines were classified as BA ‘non-responsive’ (Lines 5 and 6, Table 4) failed to produce any elongated shoots and thus are excluded.

For the nodal cutting data set, the mean final shoot height on 12.5 μM BA medium was only 16% lower than the mean for 5 μM BA (31 vs. 37 mm; Table 2), but the averages are misleading as the second month height data for the 12.5- μM BA medium was only available for the most elongation-competent lines (lines 1, 2, and 4; Table 4), and the differences in shoot height were statistically significant when the corrections for the differential contributions of each shoot line to the averages were made during the analysis (ANOVA, lsmeans protocol). For the 25- μM BA treatment, the shoots were less than half as tall as those regenerated on 5 or 12.5 μM BA (14 vs. 31–37 mm; Table 2) and only 18% of the explants had produced elongated shoots by the end of the first culture period.

For ZEA, the mean shoot heights were statistically equivalent on 12.5 and 25 μM media for the nodal cutting data set (Table 2). However, for two of five lines where replicated data was available for both ZEA concentrations ($n=5-10$ explants per concentration), the mean final shoot heights were distinctly lower on the 25- μM ZEA (Lines 1 and 6), and for Line 6, the differences in shoot height (48.8

vs. 25.3 mm) were statistically significant. In addition, for the shoot tip data set, 12.5 μM ZEA was clearly superior to 25 μM ZEA with the mean elongation ratings of 2.62 vs 1.90 (Table 3).

The effects of IBA (0.05 μM) inclusion on shoot elongation were evaluated using 5 μM BA medium. For nodal cutting data set, IBA inclusion facilitated shoot elongation for one of the shoot lines (Line 5) with the mean shoot heights being twice as great when IBA was present (data not shown); but similar positive effects were not observed for any of the other shoot lines, and for the cumulative data set, the mean second month shoot heights for both media (37 vs. 38 mm; Table 2) were statistically equivalent as assessed by *t* testing ($p=0.460$).

Cytokinins and shoot proliferation. For the shoot tip explant data set, proliferation was observed at frequencies of >50% for all of the TDZ media (0.01–0.1 μM ; Table 3), and at 0.05–0.1 μM concentration, multiple ‘generations’ of shoots were routinely produced during the same culture period (Fig. 3A). However, the axillary shoots regenerated

Table 2. Cytokinin effects on shoot regeneration from nodal cuttings, DKW medium, data combined across shoot lines

Cytokinin ^v	μM	N ^w	First culture period				Second culture period		
			Response ^u		Elongated shoots		Elongated shoots		
			% Nec	% ES	Ht (mm)±SE ^x	% Hyp ^y	Ht (mm)±SE ^x	% Tip Nec	% Prolif ^z
Control	0	44 (14)	11	52	3±1	0	3±1	0	0
ZEA	2.5	21 (10)	0	71	14±3	20	26±4 ^c	0 ^b	0 ^b
	5.0	41 (26)	5	71	15±2	3	33±4 ^{bc}	15 ^b	8 ^b (0)
	12.5	43 (25)	12	65	15±2	11	46±4 ^a	19 ^{ab}	16 ^b (11)
	25.0	26 (12)	12	54	14±3	29	38±6 ^{ab}	46 ^a	46 ^a (14)
BA	2.5	21 (9)	10	76 ^a	12±2	6	24±5 ^c	0	0
	5.0	46 (23)	33	52 ^b	10±1	0	37±5 ^a	0	17 (17)
	12.5	29 (9)	28	45 ^{bc}	10±1	8	31±7 ^{bc}	0	10 (10)
	25.0	11 (5)	27	18 ^c	6±2	0	14±6 ^{cd}	0	33 (33)
BA+IBA	5.0	28 (9)	18	61	14±3	12	38±7	0	33 (33)
TDZ	0.01	34 (20)	12	59	7±1 ^b	5 ^b	15±2 ^b	0	0 ^b
	0.05	39 (8)	8	72	12±1 ^a	25 ^a	21±2 ^a	0	100 ^a (100)

The response data were analyzed for dose effects separately for each cytokinin type. Superscript letters indicate treatment responses that were significantly different ($\alpha=0.05$). The height data was evaluated by ANOVA using the 'lsmeans' protocol of SAS to correct for the differential contributions of the shoot lines. The proportion data was evaluated by contingency table testing with 2×2 subtables being used to compare individual treatment pairs, once dose effects were detected.

^u % Nec is the percentage of explants with necrosis (>50% brown coloration). % ES is the percentage of explants with elongated shoots (>2–3 mm). For several explants elongated shoots were not present at the end of the first culture period but developed during later culture periods.

^v BA (5 μM) was tested both alone and in combination with IBA (0.05 μM).

^w The numbers in parentheses indicate the number of explants that were kept on the experimental media for a second 3–4 wk culture period.

^x Height data was collected for the subset of explants with elongated shoots (% ES). When multiple shoots were present, the tallest was measured.

^y Percentage of hyperhydric (waterlogged) shoots for the subset of explants with elongated shoots (% ES).

^z Percentage of explants with 'second generation' axillary shoots. The percentage of explants with spontaneous proliferation, i.e., in the absence of shoot tip necrosis (% Tip Nec), is indicated in parentheses. Only the total proliferation data was evaluated statistically.

on the 'high-TDZ' media (0.05–0.1 μM) were typically hypertrophic with stem diameters of up to 5–7 mm compared to more typical diameters of 1.5–2 mm and were light green in color with epinastic leaves (Fig. 3A–C). Additionally, for five of six lines, shoot fasciation (Fig. 3B) was observed at frequencies on 5–10%. The shoots also generally appeared determinate in height, even after transfer to media with optimal BA (5 μM) or ZEA (12.5 μM) concentrations for elongation (data not shown); and in some cases, tissue differentiation was observed at the shoot apex (Fig. 3C). Excluding fasciation, similar morphological defects were observed for the nodal cutting data set, but the phenotypes were generally less severe.

For both explant types, the frequency and severity of morphological aberrations was reduced by lowering the TDZ concentration (0.01 μM), but proliferation rates were adversely affected (Tables 2 and 3). However, when MS medium (MS 0.5 μM TDZ) was used in place of DKW, a

partial amelioration of the morphological defects was possible (Fig. 3D) without any obvious adverse effects on the rate of proliferation (data not shown).

BA also induced axillary proliferation at high frequencies (39–88%; Table 3). However, unlike TDZ, BA supported simultaneous shoot elongation and proliferation, especially at the 5- and 12.5-μM concentrations (Table 3). In addition, axillary proliferation was frequently apparent both at the shoot base, as was typical for shoots cultured on TDZ media, and from more distal regions of the parent shoot (Fig. 3E). However, the degree of axillary shoot growth was generally limited, i.e., the shoots were typically <10–15 mm tall at the point of data collection (4–5 wk) and somewhat compressed and 'shrubby' in appearance (Fig. 3F). In addition, for some shoot lines, on 12.5–25 μM BA media, the shoots exhibited some of the same aberrant phenotypes as documented on TDZ media, including hypertrophy and leaf epinasty. For the shoot

Table 3. Shoot tip explant growth responses for the subset of cytokinin treatments that induced axillary proliferation at mean frequencies of 25% or greater, DKW medium, data combined across shoot lines

Cytokinin ^y	μM	N	Elongation		% Proliferation ^x	
			Rating ^z	% Tip Nec	Total	Spont.
ZEA	12.5	181	2.62 ^a	21	30 ^b	17 ^b
	25	91	1.90 ^b	23	43 ^a	30 ^a
BA	5	79	2.00 ^a	9	39 ^c	38 ^c
	12.5	41	1.53 ^{ab}	7	61 ^b	61 ^b
	25	25	1.00 ^b	16	88 ^a	86 ^a
BA+IBA	5	47	2.29	6	32	29
TDZ	0.01	27	0.18	7	56 ^b	56 ^b
	0.05	54	0.19	6	87 ^a	86 ^a
	0.1	12	0.18	0	75 ^a	75 ^a

Data were collected after 4–5 wk of culture. The data were analyzed for dose effects, separately for each cytokinin. Superscript letters indicate treatment responses that were significantly different ($\alpha=0.05$). The proportion data was evaluated by contingency table testing with 2×2 subtables being used to compare individual pairs of treatments when dose effects were detected. The ordinal data (elongation rating) was evaluated by nonparametric ANOVA (Kruskal–Wallis testing) with the Mann–Whitney test being used to compare individual treatment pairs.

^x Spontaneous proliferation (Spont.) was defined as the production of axillary shoots in the absence of shoot tip necrosis (% Tip Nec) of the parent shoot.

^y BA (5 μM) was also tested in combination with IBA (0.05 μM).

^z Shoot elongation was rated on a 0–5 scale based on the degree of de novo elongation as visually assessed; 0: no obvious elongation, 1: <10 mm, 2: 10–20 mm, 3: 20–30 mm, 4: 30–50 mm, and 5: >50 mm. Average values are presented.

Table 4. Contrasting phenotypes of the six shoot lines used for media testing

Line ^y	Regeneration			Nodal cuttings					Shoot tip explants		
	% Nec ^v		% ES ^w	Elongation (mm±SE) ^u					% Proliferation ^x		
	BA	ZEA		First culture period		Second culture period		% Tip Nec ^z	ZEA	BA	TDZ
			BA	ZEA	BA	ZEA					
1	36	25	50	9±1	11±3 ^b	41±11 ^{bc}	53±7	33	10 ^{cd}	23	83 ^{ab}
2	0	0	100	10±2	24±14 ^a	69±10 ^{ab}	63±12	50	27 ^{bc}	55	100 ^a
3	33	0	100	15±4	25±5 ^a	88 (–) ^a	59±7	67	17 ^{bcd}	50	100 ^a
4	22	0	71	11±2	18±5 ^{ab}	43±7 ^b	43±8	60	52 ^a	39	83 ^{ab}
5	25	7	67	7±1	11±2 ^b	11±2 ^c	35±6	9	29 ^b	44	80 ^{ab}
6	71	13	44	11 (–)	9±1 ^b	12 (–) ^c	41±6	17	4 ^d	0	50 ^b

The responses of the shoot lines are compared on BA (DKW 5 μM BA), ZEA (DKW, 12.5 and 25 μM ZEA), and ZEA (DKW 0.05 μM TDZ) media. *Superscript letters* indicate averages that differed significantly between the shoot lines ($\alpha=0.05$). The height data was evaluated for treatment effects by ANOVA using the LSD means separation protocol to compare treatment means. The proportion data was evaluated by contingency table testing using 2×2 subtables to compare individual treatment pairs.

^u Height data was collected for the subset of explants with elongated shoots (% ES). When multiple shoots were present, the tallest was measured.

^v Percentage of explants with necrosis (>50% brown coloration). Most (>90%) failed to produce elongated shoots even when maintained for a second culture period.

^w Percentage of stem cuttings that produced elongated shoots (>2–3 mm tall) on DKW (ZEA) medium, combined across 12.5 and 25 μM ZEA concentrations, as assessed at the end of the first culture period.

^x Percentage of shoot tip explants with spontaneous axillary proliferation, i.e., for the subset of explants lacking tip necrosis (% Tip Nec).

^y Lines 2 and 3 are derived from different explants from the same seedling. The other four lines presumably represent distinct genotypes, but since they are from the Year 2000 Experiments, when the explants were pooled across seedlings, this is not certain.

^z Percentage of explants on DKW medium with 12.5 or 25 μM ZEA exhibiting shoot tip necrosis for the subset with elongated axillary shoots. Data collected at the end of the second culture period.

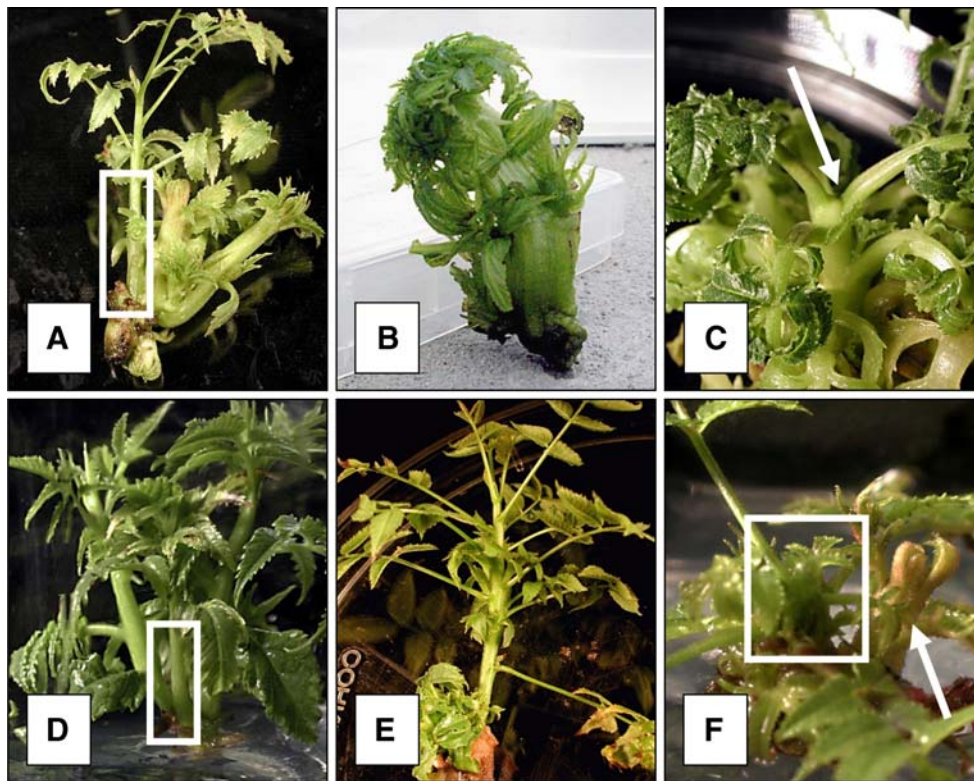


Figure 3. Cytokinin-induced proliferation of shoot tip explants. (A) Axillary proliferation on DKW medium with 0.05 μM TDZ. The original shoot (*framed*) failed to elongate after subculture, but two axillary shoots have been produced from a basal node. Each of the axillary shoots is highly swollen (hypertrophic) and has already started to produce second generation axillary shoots. (B) A fasciated axillary shoot regenerated on DKW medium with 0.1 μM TDZ. (C) Differentiation of the shoot apical meristem on DKW medium with 0.05 μM TDZ. The shoot lacks a viable apical meristem as evidenced by the lack of developing leaves. (D) Axillary proliferation on MS

medium with 0.05 μM TDZ. The original shoot, indicated with a framing box, has elongated to only a minimal degree and is overtopped by two vigorous axillary shoots. (E) Concurrent elongation and proliferation (25 μM BA). A cluster of small axillary shoots, 5–7 mm tall on average, has developed from the base of the explant. Shoot regeneration is also apparent from the upper nodes. (F) Axillary proliferation in the absence of elongation of the parent shoot (25 μM BA). The shoot tip explant is placed in a framing box and the axillary shoot, indicated with the *arrow*, is distinctly hypertrophic (swollen).

tip explants, IBA inclusion (5 μM BA+0.05 μM IBA) had a negative, but statistically insignificant, effect on proliferation (Table 3). In contrast, the rates of proliferation were twice as high with IBA supplementation for the nodal cutting data set (Table 2), and the differences were statistically significant as evaluated by *t* testing ($p=0.0284$).

Proliferation rates of up to 43–47% were possible on ZEA media (Tables 2 and 3), but the proliferation was typically preceded by shoot tip necrosis. The proliferation frequencies for the subset of cultures lack tip necrosis, termed as ‘spontaneous’ proliferation, were lower, ranging from 11% to 17% on 12.5 μM ZEA medium and 18% to 30% on 25 μM ZEA medium (Table 4). Shoot tip necrosis was also apparent on BA and TDZ media for the shoot tip data set (0–16%, Table 3). However, this necrosis was generally not a consequence of rapid shoot growth, as on ZEA media, but was indicative of shoot senescence and was not followed by axillary proliferation.

Discussion

Nutrient formulation. MS and DKW were both suitable for long-term culture maintenance, but the hyperhydricity frequencies were unacceptably high (70–100%) for the WPM and 1/2X DKW nutrient formulations. The preference of walnut shoot cultures for nutrient-rich (high-salt) media, such as MS and DKW, has been well-documented using culture growth indices (Cossio and Minolta 1983; Lee et al. 1986; Heile-Sudholt et al. 1986; Saadat and Hennerty 2002). However, we are not aware of any previous reports of mineral nutrient effects on hyperhydricity within the genus. Even so, our results are somewhat surprising since MS medium has generally been shown to predispose shoot cultures to hyperhydricity (Gaspar 1991; Ziv 1991; George 1996). In contrast, hyperhydricity has been rarely reported on low-salt media (Gaspar 1991) and MS dilution alleviates hyperhydricity in both carnation and cucumber (Ziv and Gadasi 1986; Ziv et al. 1987).

For some plant taxa, a major causative factor appears to be the high ammonium ion content of the MS medium (Beauchesne 1981; Vieitez et al. 1985; Daguin and Letouze 1986; Leonhardt and Kandeler 1987). However, since DKW and MS media contain more ammonium (17.7 and 20.6 mM, respectively) than WPM (5.0 mM; George et al. 1987), a similar relationship does not appear to apply in this study. In fact, MS medium has been successfully used for *J. regia* micropropagation by several previous research groups (Chalupa 1981; Cossio and Minolta 1983; Revilla et al. 1989; Gruselle and Boxus 1990; Fatima et al. 2006). In addition, *J. regia* shoot culture growth was improved by doubling the ammonium concentration of the DKW medium, apparently without adverse effects on shoot hyperhydricity (Zamani and Vahdati 2001).

Our literature review revealed only two prior reports of shoot hyperhydricity in the walnut genus. In the first study, hyperhydricity in shoot cultures of *J. regia* was observed on media with supraoptimal BA concentrations (2–5 mg l⁻¹), but the hyperhydricity developed slowly over the course of weeks or months and data is not provided on the frequency of hyperhydricity (Revilla et al. 1989). In the second study, also employing *J. regia* shoot cultures, hyperhydricity was triggered by carbohydrate dilution, from 3% to 1.5% (Gruselle et al. 1995). In contrast, the hyperhydricity observed in this study was presumably a consequence of mineral nutrient deficiencies.

Deficiencies of calcium and magnesium have been correlated with hyperhydric differentiation in many previous studies (Kreutmeier et al. 1984; Kevers and Gaspar 1986; Orlikowska 1987; Ziv et al. 1987; Yadov et al. 2003). However, aside from nitrogen, potassium is the only plant macronutrient present at distinctly lower levels in the WPM medium (12.6 mM) than in MS (20.1 mM) or DKW (19.7 mM). In apple, potassium dilution from 20.1 to 8.1 mM resulted in twofold to threefold increases in hyperhydric frequency for shoots cultured on MS medium with Gelrite® (Pasqualetto et al. 1988). However, since Gelrite® is prepared as a potassium salt and thus contains a higher potassium content (5- to 30-fold) than agar (Pasqualetto et al. 1988; Scherer et al. 1988; Barbas et al. 1993b), this hypothesis is not entirely compelling.

Alternatively, the problem may have been primarily one of nutrient loss from the explants, rather than insufficient supply. Mineral nutrient loss from plants to the culture medium has been demonstrated for up to 2 wk after subculture during shoot micropropagation (Williams 1993); and if the loss is partly diffusion-mediated, the amount of nutrient depletion may be greater for low-salt media, such as WPM and 1/2X DKW, than for more concentrated media. Another possibility is that the hyperhydricity was mediated by changes in osmotic potential, since media dilution would result in higher (less negative) osmotic

potentials and greater water availability. However, this explanation is unlikely since hyperhydricity frequencies were not increased when the osmotic potential of the medium was raised by changing the sucrose content (20 to 10 g l⁻¹) or Gelrite® concentration (2 to 1.5 g l⁻¹), as evaluated in supplemental experiments (data not shown).

Nutrient formulation effects on shoot growth were also noted in our study with shoot elongation being promoted by DKW media dilution (Table 1). In contrast, for *J. regia* media dilution has been observed to have adverse effects on shoot growth (Cossio and Minolta 1983; Gruselle et al. 1987; Amiri 2006; Fatima et al. 2006). Although most of the shoots regenerated on 1/2X DKW medium were hyperhydric (90–100%; Table 1), these problems may be unique to nodal cutting explants. In preliminary tests with shoot tip explants, the frequency of hyperhydricity on 1/2X DKW 12.5 µM ZEA medium was only 50% (data not shown). In addition, 1/2X DKW medium has been used for black walnut micropropagation by previous research groups (Stefan and Millikan 1987; Stefan 1989). However, the media used by these researchers contained more sucrose (30 g l⁻¹) and was gelled with agar; differences that may have decreased the susceptibility of the cultures to hyperhydricity (Ziv 1991; Schloupf et al. 1995).

Explant type and culture multiplication. Culture maintenance was possible using both explant types (nodal cuttings and shoot tip explants), but higher cytokinin concentrations were required to effect proliferation from the shoot tip explants; and the axillary shoots regenerated were often morphologically aberrant (hypertrophic or fasciated), particularly on TDZ media (0.05–0.1 µM). In addition, the elongation potential of the axillary shoots appeared limited, especially for those forced using BA or TDZ, and the morphological defects appeared to have adverse effects on proliferation (data not shown).

Similar types of ‘high-cytokinin’ effects have also been reported by previous *Juglans* researchers. For example, when WPM with 8.8–44 µM BA (2–10 mg l⁻¹) was used for culture multiplication in black walnut, the shoots regenerated were short (5–10 mm tall) and swollen (hypertrophic), and etiolation was required to produce shoots of sufficient height for root induction (Heile-Sudholt et al. 1986). On DKW media with TDZ (0.3 µM), the shoots were taller (to nearly 20 mm) but were frequently fasciated and were generally nonresponsive when separated and individually subcultured (Van Sambeek et al. 1997). For *J. regia*, BA, at concentrations of 2–5 mg l⁻¹, caused “morphological modifications” of the leaves and shoots (Revilla et al. 1989).

In this study, the use of nodal cuttings for culture multiplication appeared to buffer against several of the adverse effects of high-cytokinin exposure. On TDZ media,

for example, the frequency and severity of aberrant shoot phenotypes was less for axillary shoots of nodal cutting origin than for those ‘forced’ from shoot tip explants (data not shown). Similarly, the concentrations of ZEA that were supraoptimal for shoot elongation were higher for the nodal cutting data set than was typical when shoot tip explants were employed (Tables 2 and 3).

Because of the more limited risk of cytokinin carryover from the micropropagation medium, shoots of nodal cutting origin may be more competent for rooting than would be typical for shoots forced from shoot tip explants using high-cytokinin concentrations. The taller stature of these shoots would also be expected to facilitate *ex vitro* establishment (Sanchez-Olate et al. 1997; Compton et al. 2004), presumably by limiting the amount of auxin movement from the shoot base, the site of application, to the shoot tip where it could inhibit shoot elongation after rooting.

The main drawback we noted when using nodal cuttings for culture multiplication was the lag time between axillary bud ‘break’ and shoot elongation. In exceptional cases, shoot elongation was not apparent until the third culture period after explant isolation (9–12 wk total). However, the speed with which the conversion from rosette to elongated growth form occurred varied between shoot lines and media types, and for two of the six shoot lines evaluated, this conversion almost always occurred during the initial culture period (Table 4, Lines 2 and 3).

Cytokinins and shoot elongation. ZEA was superior to BA for shoot elongation. For the nodal cutting data set, the mean shoot heights, as assessed during at the end of the second culture period, were greater on ZEA (12.5 μM) than on BA (5 μM) (Table 2) and the differences were significant the 10% level of probability ($p=0.092$). ZEA was effective across all six shoot lines (Table 4), over a greater concentration range (2.5–25 μM), and did not have adverse effects on explant health. However, the incidence of shoot tip necrosis was higher on ZEA than on BA medium with mean shoot tip necrosis frequencies of 23–47% for the highest ZEA concentration (25 μM ; Tables 2 and 3).

For both cytokinin types (BA and ZEA), positive associations were noted between the elongation rate and the frequency of ‘apical necrosis’; however, on BA medium, the necrosis was generally limited to the developing leaves and did not spread to the shoot apical meristem, even for shoot lines that elongated at more or less equivalent rates on BA and ZEA (Table 4, Lines 2–4). Shoot tip necrosis facilitated culture multiplication since the injury generally induced axillary branching. However, for protocols requiring the elongation of individual shoots (prerooting, etc.), the process of shoot dieback would be detrimental.

Shoot tip necrosis is a stereotypical calcium deficiency symptom both *ex vitro* and in tissue culture (Sha et al. 1985; Debergh 1988; Vieitez et al. 1989; George 1993; Barghchi and Alderson 1996; Piagnani et al. 1996). However, in this study, the frequencies of shoot tip necrosis were statistically equivalent across all four nutrient formulations (Table 1) despite threefold differences in calcium concentration (3 mM for MS and WPM, 9.3 mM for DKW, and 4.6 mM for 1/2X DKW; George et al. 1987). Thus, if the necrosis was truly calcium-mediated the limitation may have occurred at the point of calcium uptake. Since calcium is transported only in the xylem of plants, it is not recycled between organs (leaves) and must be continuously absorbed and transported to the growing shoot tip. Thus, transpiration is more important for calcium nutrition than for most other mineral nutrients. However, in the absence of root pressure-generated water flow, the degree of transpiration *in vitro* may be limited as a consequence of water vapor accumulation in the head space of traditional (nonventilated) culture vessels, such as those employed in this study (Sha et al. 1985; Geerts et al. 1987).

Transpirational limitations to calcium uptake may have been especially pronounced in this study since overhead lighting was employed and the fluorescent lamps used for illumination were hung from the shelves holding the cultures. Because of this arrangement the cultures would have been subjected to ‘bottom heating’, a condition that facilitates evaporation from the media into the head space of the culture vessels. The strong shoot growth responses that we observed, with 75–95 mm of *de novo* elongation being possible during a single culture period for the fastest elongating lines (data not shown), may have also contributed to the high rates of shoot tip necrosis. If the upper regions of culture vessels are more humid than the lower regions, as would be expected during periods of bottom heating, the driving force for transpiration (i.e., the water potential greater between the leaves and the culture atmosphere), would decrease with shoot height. In fact, for shoot cultures of some plant species, there appear to be very specific size thresholds above which calcium transport becomes limiting and tip necrosis is induced (Amin and Jaiswal 1988).

Summary. This study involved a comprehensive assessment of mineral nutrition and cytokinin effects on the growth of black walnut shoot cultures. In addition, our evaluation of the utility of ZEA for culture multiplication appears to be a first for the genus. Although ZEA is more expensive than BA, it was superior for shoot elongation, being effective across all six shoot lines, and is recommended for culture initiation from valuable genotypes (elite selections, genetic mutants, etc.). ZEA

may also be useful for specific culture stages, such as 'prerooting' where rapid shoot elongation is desirable.

TDZ had strong positive effects on the rate of axillary proliferation, confirming the results of previous research groups (Khan et al. 1995; Van Sambeek et al. 1997), but it was effective only over a relatively narrow concentration range and the regenerated shoots exhibited morphological defects at high frequencies. Similar problems were reported by Van Sambeek et al. (1997). In contrast, KIN and 2iP appeared wholly ineffective for shoot culture growth, but were only tested during culture initiation and at relatively low concentrations (2.5–5 μM).

BA was the most versatile cytokinin evaluated. For four of six shoot lines, the rates of shoot elongation were equivalent on BA and ZEA media. However, BA was also effective for the induction of shoot proliferation with proliferation frequencies of up to 88% being possible for shoot tip explants. BA has generally been used in combination with an auxin, typically IBA, for walnut micropropagation (Rodriguez et al. 1989); and for *J. regia* taxa, IBA supplementation has been demonstrated to have positive effects on shoot culture growth (Rodriguez 1982; Driver and Kuniyuki 1984; Gruselle et al. 1987; Saadat and Hennerty 2002). In the current study, IBA (0.05 μM) facilitated both elongation and proliferation, depending on the explant type, but the improvements were generally not statistically significant.

The primary negative consequence associated with the use of BA was the higher frequencies of explant browning for the nodal cutting data set compared with the other media (ZEA, TDZ) or controls. These results suggest that the hypersensitivity that has been so widely reported as an obstacle to walnut shoot culture initiation (Rodriguez et al. 1989; Leslie and McGranahan 1992; Van Sambeek et al. 1997) may be partly cytokinin-mediated. Similar observations have been reported by Khan et al. (1996); i.e., the degree of phenolic exudation from black walnut shoot cultures was heightened by BA inclusion in the culture medium. In addition, both BA and KIN, the aromatic cytokinins (Strand 1997), have been suggested to contribute to hypersensitivity during micropropagation more generally (Preece and Compton 1991).

In a separate series of experiments that will be discussed more fully in a subsequent paper, axillary shoots regenerated from nodal cuttings cultured on BA or ZEA media were tested for rooting ability. Using both quick dip and pulse (3–5 d) auxin treatments (Leslie and McGranahan 1992; Jay-Allemand et al. 1992; Long et al. 1995) rooting was achieved at mean frequencies of 20–50%, depending on the shoot line, and the rooted shoots have been established *ex vitro* (Bosela and Michler, in preparation).

Compared with previous walnut micropropagation research, the strong shoot elongation responses docu-

mented in this study were unusual. Whereas height growth increments of 10–20 mm per 4–6 wk are typical for the genus (Heile-Sudholt et al. 1986; Revilla et al. 1989; Barbas et al. 1993a; Sanchez-Olate et al. 1997; Saadat and Hennerty 2002), the elongation rates averaged 25–35 mm per culture period (3–4 wk) for several of the media evaluated in this study (Fig. 2, second culture period). In addition, on a per shoot basis, growth spurts of 75–95 mm per culture period were possible for the most responsive lines (data not shown). However, the rapid elongation predisposed the cultures to shoot tip necrosis on ZEA media.

A variable that undoubtedly contributed to vigorous shoot elongation was the use of Gelrite® (gellan gum) instead of agar. In previous studies comparing the responses of shoot cultures of *J. regia* taxa on media gelled with Gelrite® vs. agar, the rates of growth were two to threefold greater for the gellan gum media, as assessed using both height and weight indices (Barbas et al. 1993b; Saadat and Hennerty 2002). However, since other research groups that have used gellan gum for *Juglans* micropropagation have not reported equivalent elongation rates (Jay-Allemand et al. 1993; Saadat and Hennerty 2002), other facts may have also contributed, such as media supplementation with adenine (20 mg l⁻¹ adenine sulfate), a cytokinin biosynthesis precursor (Sakakibara 2006).

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