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MICROPROPAGATION

Rapid *in vitro* shoot multiplication of the recalcitrant species *Juglans nigra* L.

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Abstract

Black walnut (*Juglans nigra* L.) has long been prized for its timber, leading to commercial cultivation and significant breeding efforts for improving marketable traits. Vegetative and *in vitro* black walnut propagation techniques, however, are variable and highly genotype dependent. Optimizing plant growth regulator type and concentration are integral for developing a successful micropropagation protocol. The addition of meta-topolin (MT) combined with the novel use of a liquid medium has led to the development of a rapid shoot multiplication system. The objective of this research was to develop a reproducible and dependable micropropagation protocol for elite black walnut genotypes. *In vitro* shoot cultures were established from nodal explants cultured on semi-solid Driver and Kuniyuki walnut (DKW) medium supplemented with 8.9 μM benzyladenine, 0.005 μM indole-3-butyric acid (IBA), 200 mg L^{-1} casein hydrolysate, 50 mg L^{-1} adenine hemisulfate, 2 mL L^{-1} Plant Preservative Mixture™, and 4.1 μM MT. Long-term survival and proliferation of microshoots was achieved when nodal segments of *in vitro* grown shoots were cultured in liquid initiation medium in 3-L polycarbonate Fernbach-style flasks on a rotary shaker (100 rpm) under a 16-h photoperiod at 25°C. Elongated microshoots (5–7 cm in length) were rooted in a slurry-like medium composed of half-strength DKW medium with 0.11% (*w/v*) Phytagel™ and coarse vermiculite (2:1, *v/v*) supplemented with 50 μM IBA for 5 wk. Rooted shoots were acclimatized to ambient culture room conditions, but plantlets did not survive once transferred to the greenhouse.

Keywords Benzyladenine · Black walnut · Liquid culture · Meta-topolin · Micropropagation

Introduction

Black walnut (*Juglans nigra* L.) has long been a highly valued hardwood species in the central hardwood region of the USA. Owing to its unique wood qualities, black walnut has historically been a premier timber species for veneer, cabinetry, gunstocks, and furniture (Cassens 2004; Michler *et al.* 2007). The value of black walnut has merited its cultivation and genetic improvement for over a century. Although initially conventional breeding efforts sought superior nut producing genotypes, work over the past 40 yr has produced high-valued timber varieties (Beineke 1983; Victory *et al.* 2004). The development of genetically superior black walnut has led to increased commercial demand for clonal material necessitating a routine clonal propagation system. Methods such as grafting or budding are possible, but economically and physiologically are not ideal (Beineke 1984; Coggeshall and Beineke 1997; Hasey *et al.* 2001; Lopez 2001).

As an alternative, micropropagation can be used to rapidly multiply superior genotypes, circumvent problems associated with variable seed production, aid in maintaining genetic diversity, help accelerate genetic improvement programs for forestry species, and act as a safe guard against extirpation (Macdonald 1986; Timmis *et al.* 1987; Merkle *et al.* 2007; Hartmann *et al.* 2011). Specifically, micropropagation methods have been employed to significantly improve reproducibility of progeny tests, and increase commercial production of superior clones of English walnut (*Juglans regia* L.) (Leslie and McGranahan 2014). Developed by Driver and Kuniyuki (1984), the walnut specific nutrient tissue culture medium permitted more routine micropropagation of *J. regia*, the model species within the genus. Previous attempts to propagate *Juglans* species *via* tissue culture had variable

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results owing to a variety of endogenous and exogenous factors (Driver and Kuniyuki 1984; Gruselle *et al.* 1987; McGranahan *et al.* 1988; Rodriguez *et al.* 1989; Jay-Allemand *et al.* 1993; McCown 2000; Navatel and Bourrain 2001; Lopez 2004; Bosela and Michler 2008; Sharifian *et al.* 2009; Vahdati *et al.* 2009). Licea-Moreno *et al.* (2015) reported significant advances in micropropagating *J. major* (Arizona black walnut) 209 × *J. regia* hybrids, and successfully field-planting over 5800 clonal walnuts. A major issue associated with walnut micropropagation, however, is the interaction between genotype and both plant growth regulator type and concentration in *in vitro* culture conditions. These factors have led to the inability of tissue culture advances with *J. regia* and its hybrids to be applied interspecifically to *J. nigra*. Highlighting these difficulties, Scaltsoyiannes *et al.* (1997) reported significant differences in shoot elongation between half-sibling *J. regia* genotypes exposed to the same concentrations of plant growth regulators.

Within *Juglans*, sub- or supra-optimal concentrations of traditionally used cytokinins such as benzyladenine, kinetin, and zeatin used during the multiplication phase have led to many deleterious morphological effects. Abnormalities such as stunted growth, severe phenolic exudation, shoot hyperhydricity, fasciation, shoot-tip necrosis, and chlorosis negatively impacted microculture production (Heile-Sudholt *et al.* 1986; Revilla *et al.* 1989; Van Sambeek *et al.* 1997; Bosela and Michler 2008). These results, along with the inability of current protocols to be applied across genotypes, underscore the need for an improved *J. nigra* micropropagation system. The goals of this study were to evaluate whether manipulating plant growth regulator and the culture environment by using novel techniques could further improve black walnut *in vitro* shoot proliferation, independent of genotype, and generate plantlets from *in vitro* grown shoots. Development of an optimized *in vitro* propagation system will benefit private landowners and commercial forest industries wanting to improve production of elite black walnut genotypes.

Materials and Methods

Plant material Juvenile plant tissues were obtained from 4- to 6-wk-old half-sibling seedlings grown from mature black walnut (*J. nigra*) seeds of elite genotypes, designated number 55 and number 189, collected at the Martell Forest (40.4322° N, 87.0389° W, West Lafayette, IN). Mature seeds were dehusked, cleaned, and stratified in moist peat moss at 5°C in the dark for 120 d. Nuts were then germinated and grown in seedling trays (Polyflat 40 cm × 40 cm × 12.7 cm deep; Anderson Die and Mfg. Co., Portland, OR) in a potting mix (1:1:1 (*v/v/v*), peat moss:perlite:vermiculite) under ambient greenhouse conditions (22 ± 2°C).

Mature plant tissues were collected from 1- to 3-yr-old grafted black walnut stock plants of the same genotypes grown in the greenhouse. Scion wood was originally collected from the canopy of mature elite black walnut genotypes at the Martell Forest (West Lafayette, IN) in March of 2012 and 2014, and grafted onto wild-type black walnut seedling rootstock following a modified established protocol (Beineke 1984). Successfully grafted plants were overwintered for 1 yr in cold storage (3–4 °C) prior to being used as stock plants in the greenhouse.

Physiologically juvenile or mature nodal sections were collected from shoots 30–35 cm in length. Stems were defoliated and cut into individual nodal sections, surface disinfested in 70% (*v/v*) ethanol for 30 s, washed in 15% (*v/v*) bleach solution (5.25% sodium hypochlorite) with 0.01% (*v/v*) Tween® 20 for 20 min, and rinsed in sterile water three times. There were a total of three biological replications of 12 nodal sections per genotype per treatment used. All cultures were maintained under a 16-h photoperiod provided by cool-white fluorescent lamps (Philips Lighting, Somerset, NJ) (80 μmol m^{-2} s^{-1}) at 24 ± 2°C. Unless noted otherwise, all chemicals were obtained from Sigma-Aldrich®, St. Louis, MO.

Shoot initiation and proliferation All nodal sections were initiated on a semi-solid basal Driver and Kuniyuki walnut (DKW) medium (D190, PhytoTechnology Laboratories, Shawnee Mission, KS; Driver and Kuniyuki 1984) supplemented with 0.005 μM indole-3-butyric acid (IBA), 200 mg L^{-1} casein hydrolysate, 50 mg L^{-1} adenine hemisulfate, 2 mL L^{-1} Plant Preservative Mixture™ (Caisson Laboratories, North Logan, UT), 3% (*w/v*) sucrose, and solidified with 0.22% (*w/v*) Phytagel™ in Magenta™ GA-7 vessels (Magenta® Corp., Chicago, IL) at 50 mL medium per vessel, and the pH was adjusted to 5.7 with 1 N NaOH prior to autoclaving at 120°C for 20 min. To measure the influence of cytokinin either 0.0 or 8.9 μM benzyladenine (BA) in combination with 0.0, 4.1, 6.2, 8.3, or 10.4 μM metatopolin (MT), or 4.1 μM MT in combination with 2.5, .05, or 12.5 μM zeatin was added to the basal medium. Treatments 0.0 μM BA in combination with 4.1, 6.2, 8.3, or 10.4 μM MT, and 8.9 μM BA in combination with 0.0 or 2.1 μM MT were not used for mature nodal cuttings because these treatments resulted in the poorest performance with juvenile material. Shoot length, number of nodes per shoot, percentage of necrosis, microbial contamination and leaf set, and growth cessation were recorded 8 wk after culture initiation, with transfer to fresh medium of the same composition at 4-wk intervals.

In vitro elongated shoots (three to four) were then defoliated and partially submerged to a depth of 1–3 cm in a liquid DKW medium containing 0.005 μM IBA, 200 mg L^{-1} casein hydrolysate, 50 mg L^{-1} adenine hemisulfate, 2 mL L^{-1} Plant Preservative Mixture™, 8.9 μM BA, and 4.1 μM MT in 3 L

polycarbonate Fernbach-style flasks on a rotary shaker (100 rpm) for shoot proliferation. Flasks were modified (Fig. 1) to create an opening that was 12 cm in diameter, covered with a 14-cm glass Petri dish, and sealed with Parafilm® M (Bemis® Co. Inc., Oshkosh, WI). Liquid culture medium was changed weekly and the shoots were subcultured every 4 wk.

Rooting and acclimatization of microshoots Elongated microshoots (5–7 cm in length) were induced to root in a slurry-like medium composed of half-strength DKW medium, 3% (*w/v*) sucrose, with 0.11% (*w/v*) Phytagel™ and coarse vermiculite (2:1, *v/v*) supplemented with 30 μM or 50 μM IBA in combination with 0.0, 50, or 100 μM indole-3-acetic acid (IAA). Two technical replicates of 20 explants per treatment were performed. Five weeks after root initiation, percent root formation, number of roots per shoot, length of roots, and the number of lateral roots were evaluated for each microshoot. Plantlets were gently removed from the rooting medium and potted in 12.5 cm × 8 cm plastic pots containing a moist autoclaved potting mix (1:1:1 (*v/v/v*), peat moss:perlite:vermiculite) and grown under a 16-h photoperiod (Philips Lighting, Somerset, NJ (80 μmol m^{-2} s^{-1}). Root systems were rinsed with water prior to transplanting to remove residual rooting medium. Pots were placed in perforated 3.8-L ziplock plastic bags to provide a high relative humidity and air exchange. Bags were gradually opened over a period of 2–3 wk allowing plants to acclimatize to ambient conditions. Plants were watered every 4–5 d until the bags were fully opened, and then watered as needed.

Statistical analysis Data were analyzed with an analysis of variance (ANOVA) performed with R statistical software (R Development Core Team 2006). Post hoc Tukey's honestly significant difference (HSD) comparison tests at the 5% level of probability were run on means shown to be significant by ANOVA.

Figure 1. Three-liter polycarbonate Fernbach-style flasks on a rotary shaker that have been modified to create a larger opening to accommodate *Juglans nigra* L. shoot cultures.

Results

Shoot initiation and multiplication *In vitro* shoot cultures were successfully established for elite genotypes number 55 and number189 using nodal sections as the initial explant. Elongated microshoots appeared healthy and were free of morphological abnormalities. Exogenous cytokinins were necessary for microshoot growth. Shoots from juvenile material cultured without cytokinin were significantly shorter with fewer nodes than the optimum treatment, often failing to elongate (Fig. 2*a*; Table 1). Cytokinin type and concentration had a significant effect on juvenile material shoot growth and number of nodes per microshoot. Shoots cultured with 8.9 μM BA plus 4.1 μM MT produced shoots (1.7 and 1.3 cm) significantly ($P \leq 0.05$) longer than all other treatments and with more nodes (6.4 and 5.1) for juvenile material independent of genotype (Table 1; Fig. 2*d*). The cytokinin type and concentration significantly influenced the mean number of shoots per explant. In particular, a synergistic effect of combining BA and MT was observed. While shoots exposed to 8.9 μM BA (Fig. 2*b*) or 4.1 μM MT (Fig. 2*c*) elongated normally, the combination of the two cytokinins resulted in longer shoots with more nodes per shoot.

Physiological age of the explants essentially determined if microshoots elongated and survived. Regardless of cytokinin type or concentration, shoots from mature material explants often failed to elongate. The optimum plant growth regulator combination and concentration for material from mature genotype 55 was 8.9 μM BA plus 6.2 μM MT, which resulted in an average microshoot length of 0.4 cm and 2.8 nodes per shoot (Table 2). Genotype number 189, however, had no clear optimum shoot initiation treatment. The greatest microshoot length was 0.2 cm (Table 2). Independent of genotype, all explants from mature material responded poorly to culture conditions. Nodal sections from mature material experienced an increased incidence of necrosis, endogenous microbial exudation, and long leaf set across all treatments. Microshoot elongation from mature material ranged from 5.5 to 36.1% and had necrosis rates of 41.6–100% (Table 3). Microshoot necrosis was a severely limiting factor on culture initiation of explants derived from a mature source.

Explants from juvenile material were highly responsive to culture conditions with 83–100% of shoots elongating depending on the treatment with minimal necrosis (Table 3). Exogenous cytokinins, however, seemed to influence the prevalence (visualization) of endogenous microbial exudation and leaf set. Microbial contamination occurred at low frequencies (2.8–12.5%) for BA and MT treatments, but shoots from juvenile material cultured with zeatin had an increased rate of microbial contamination ranging from 18 to 65% (Table 3). Microbial contamination often led to the collapse of cultures, and repeated sub-culture to fresh medium could not rescue nodal explants, eventually leading to prolific phenolic

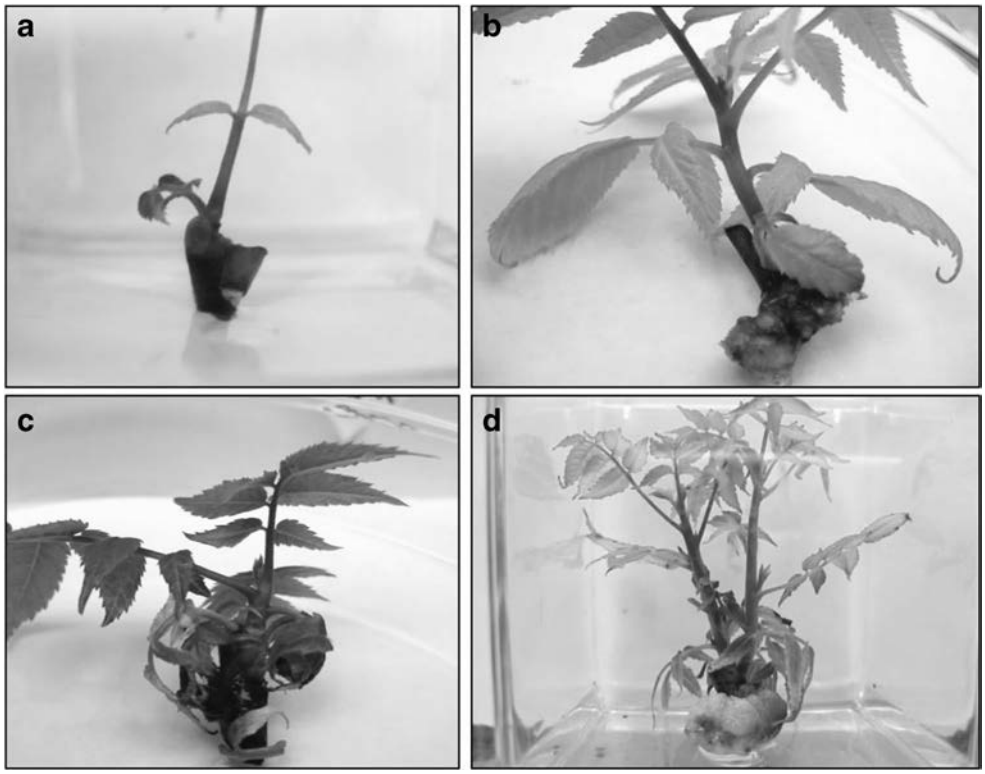

Figure 2. Shoot elongation of juvenile, elite *Juglans nigra* L. nodal explants after 8 wk exposure to varying concentrations of benzyladenine and meta-topolin. (*a*) Control shoot on DKW medium with 0.0 μM benzyladenine or meta-topolin showing limited elongation and cessation of growth, (*b*) 8.9 μM benzyladenine plus 0.0 μM meta-topolin, (*c*) 0.0 μM benzyladenine plus 4.1 μM meta-topolin, and (*d*) 8.9 μM benzyladenine plus 4.1 μM meta-topolin.

exudation and necrosis Shoots from juvenile material cultured on zeatin also had an increased prevalence of long leaf set and cessation of elongation (Fig. 3*a*). Long leaf set did not occur with shoots cultured on BA and MT, but occurrence ranged from 19.5–25.3% for those on medium supplemented with zeatin (Table 3).

Table 1. Dose effects of benzyladenine, meta-topolin, and zeatin alone and in combination on *in vitro* shoot elongation of two juvenile elite *Juglans nigra* L. genotypes after 8 wk of culture

Cytokinin (μM)			Genotype no. 55		Genotype no. 189	
BA	MT	Z	Length (cm)[a]	No. nodes[a]	Length (cm)[a]	No. nodes[a]
0	0	0	0.4 ± 0.04a	2.2 ± 0.2a	0.2 ± 0.03a	1.7 ± 0.2a
0	4.1	0	0.6 ± 0.05ab	3.5 ± 0.2bc	0.6 ± 0.05ad	4.1 ± 0.2bd
0	6.2	0	0.7 ± 0.06ad	3.8 ± 0.2bd	0.9 ± 0.08cde	4.0 ± 0.2bd
0	8.3	0	1.0 ± 0.09 cd	4.0 ± 0.3 cd	0.8 ± 0.08bde	4.1 ± 0.1bd
0	10.4	0	1.0 ± 0.07d	4.0 ± 0.2 cd	1.0 ± 0.1ef	3.7 ± 0.2bc
8.9	0	0	0.8 ± 0.07bd	4.7 ± 0.3 cde	0.5 ± 0.04ab	3.3 ± 0.2b
8.9	2.1	0	0.6 ± 0.05abc	4.3 ± 0.2 cde	0.8 ± 0.06bde	4.1 ± 0.3bd
8.9	4.1	0	1.7 ± 0.18e	6.4 ± 0.3f	1.3 ± 0.1f	5.1 ± 0.2d
8.9	6.2	0	0.9 ± 0.07bd	5.0 ± 0.3de	0.8 ± 0.1bde	4.4 ± 0.2bd
8.9	8.3	0	0.7 ± 0.05ad	5.0 ± 0.2de	0.8 ± 0.07bde	4.5 ± 0.3bd
8.9	10.4	0	0.8 ± 0.05bd	5.5 ± 0.2ef	0.8 ± 0.06bde	4.5 ± 0.2bd
0	4.1	2.5	0.7 ± 0.1ad	4.0 ± 0.4 cd	0.9 ± 0.09de	5.0 ± 0.3 cd
0	4.1	5	1.0 ± 0.1bd	4.2 ± 0.4 cd	0.8 ± 0.1bde	4.1 ± 0.4bd
0	4.1	12.5	0.8 ± 0.1ab	4.1 ± 0.4ab	0.8 ± 0.09abc	4.8 ± 0.3b

Nodal explants were cultured on Driver and Kuniyuki (DKW) medium supplemented with 0.005 μM indole-3-butyric acid, 200 mg L^{-1} casein hydrolysate, 50 mg L^{-1} adenine hemisulfate, 2 mL L^{-1} Plant Preservative Mixture™, 3% (*w/v*) sucrose plus 6-benzyladenine (BA), meta-topolin (MT), or zeatin (Z). Data were collected after 8 wk

[a] Values represent means ± SE for 36 explants per treatment. Means in each *column* followed by the *same letter* were not significantly different according to Tukey's multiple comparison test ($P \leq 0.05$)

Table 2. Dose effects of benzyladenine, meta-topolin, and zeatin alone and in combination on *in vitro* shoot elongation of two mature elite *Juglans nigra* L. genotypes after 8 wk of culture

Cytokinin (μM)			Genotype no. 55		Genotype no. 189	
BA	MT	Z	Length (cm)[a]	No. nodes[a]	Length (cm)[a]	No. nodes[a]
0	0	0	0.02 ± 0.01a	0.2 ± 0.2a	0.02 ± 0.02a	0.3 ± 0.2a
8.9	4.1	0	0.2 ± 0.05ab	1.7 ± 0.4ab	0.06 ± 0.03a	0.7 ± 0.3a
8.9	6.2	0	0.4 ± 0.1b	2.8 ± 0.8b	0.09 ± 0.03a	0.6 ± 0.3a
8.9	8.3	0	0.1 ± 0.04a	1.1 ± 0.4ab	0.2 ± 0.04a	0.9 ± 0.3a
8.9	10.4	0	0.1 ± 0.05a	0.7 ± 0.4a	0.05 ± 0.02a	0.6 ± 0.3a
0	4.1	2.5	0.2 ± 0.04ab	1.2 ± 0.4ab	0.2 ± 0.05a	1.5 ± 0.4a
0	4.1	5	0.2 ± 0.04ab	1.7 ± 0.4ab	0.2 ± 0.05a	1.1 ± 0.3a
0	4.1	12.5	0.6 ± 0.04ab	1.3 ± 0.4ab	0.1 ± 0.04a	1.4 ± 0.4a

Nodal explants were cultured on Driver and Kuniyuki (DKW) medium supplemented with 0.005 μM indole-3-butyric acid, 200 mg L^{-1} casein hydrolysate, 50 mg L^{-1} adenine hemisulfate, 2 mL L^{-1} Plant Preservative Mixture™, 3% (*w/v*) sucrose plus 6-benzyladenine (BA), meta-topolin (MT), or zeatin (Z). Data were collected after 8 wk

[a] Values represent means ± SE for 36 explants per treatment. Means in each *column* followed by the *same letter* were not significantly different according to Tukey's multiple comparison test ($P \leq 0.05$)

Shoot multiplication and rooting Liquid culture resulted in improved microshoot multiplication. *In vitro* semi-solid elongated shoots that were defoliated and placed in a liquid DKW medium supplemented with 8.9 μM BA, 0.005 μM IBA, 200 mg L^{-1} casein hydrolysate, 50 mg L^{-1} adenine hemisulfate, 2 mL L^{-1} Plant Preservative Mixture™, and 4.1 μM MT on a rotary shaker proliferated. Lateral buds rapidly broke and elongated forming large masses of morphologically normal microshoots within 3–4 wk (Fig. 3*b*). Shoots elongated on semi-solid medium produced only a singular new shoot in 4–5 wk. Shoots of elite black walnut genotypes multiplied at a higher rate in liquid medium than those grown on the traditional semi-solid medium. Liquid culture-derived microshoots were successfully rooted *in vitro* (Fig. 3*c*).

Table 3. The effects of cytokinin treatment on *Juglans nigra* L. (black walnut) nodal explant health. Data combined for material from juvenile and mature *J. nigra* L. genotypes, respectively, after 8 wk of culture

Cytokinin (μM)			Juvenile				Mature			
BA	MT	Z	SE (%)	Nec (%)	MC (%)	LLS (%)	SE (%)	Nec (%)	MC (%)	LLS (%)
0	0	0	88.9	0	9.7	0	5.5	100	0	0
0	4.1	0	98.6	0	6.9	0	–	–	–	–
0	6.2	0	100	0	2.8	0	–	–	–	–
0	8.3	0	98.6	0	5.5	0	–	–	–	–
0	10.4	0	98.6	0	11.5	0	–	–	–	–
8.9	0	0	98.6	0	5.5	0	–	–	–	–
8.9	2.1	0	98.6	0	9.7	0	–	–	–	–
8.9	4.1	0	100	0	6.9	0	27.7	66.6	66.6	17.85
8.9	6.2	0	98.6	0	12.5	0	24.9	41.6	16.7	12.5
8.9	8.3	0	100	0	11.1	0	26.3	100	0	5
8.9	10.4	0	100	0	11.1	0	12.4	55	30	0
0	4.1	2.5	84.7	10.3	18.6	19.5	36.1	96.4	50	7
0	4.1	5	84.7	8.1	65.5	24.5	34.6	80	0	33.3
0	4.1	12.5	83.2	9.7	59.7	25.3	30.5	68.1	9	18.1

Nodal explants were cultured on Driver and Kuniyuki (DKW) medium supplemented with 0.005 μM indole-3-butyric acid, 200 mg L^{-1} casein hydrolysate, 50 mg L^{-1} adenine hemisulfate, 2 mL L^{-1} Plant Preservative Mixture™, 3% (*w/v*) sucrose plus 6-benzyladenine (BA), meta-topolin (MT), or zeatin (Z). Data were collected after 8 wk

SE shoot elongation, *Nec* necrosis, *MC* microbial contamination, *LLS* long leaf set

Values represent means for 36 explants per treatment.

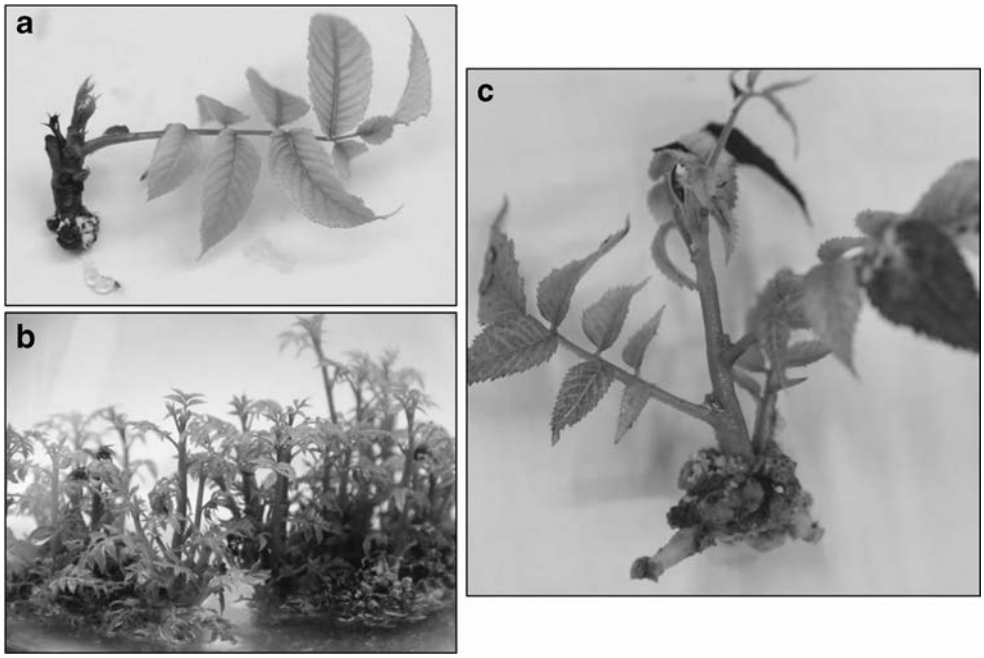

Figure 3. Growth abnormalities effecting shoot elongation and culture establishment, and successful shoot multiplication of *Juglans nigra* L. (*a*) Nodal explant displaying long leaf set and cessation of active growth; (*b*) a mass of rapidly proliferating microshoots in culture on a rotary shaker in liquid DKW medium supplemented with 0.005 μM indole-3-butyric acid, 8.9 μM benzyladenine, 4.1 μM meta-topolin, 200 mg L^{-1} casein hydrolysate, 50 mg L^{-1} adenine hemisulfate, 2 mL L^{-1} Plant Preservative Mixture™, and 3% (*w/v*) sucrose, and (*c*) a healthy, successfully rooted microshoot.

Rooting success was dependent on auxin concentration and type. Microshoots cultured with 50 μM IBA without IAA resulted in the greatest frequency of adventitious root formation (40%) for juvenile material from both genotypes (Table 4). There was, however, only a marginally significant difference ($P \leq 0.05$) among treatments for the average number and length of adventitious roots. Successfully rooted microshoots were acclimatized to ambient growth room conditions but did not survive the transition to the greenhouse.

Discussion

Culture establishment was successful for material from both elite black walnut genotypes of juvenile origin. Significantly longer shoots (1.3–1.7 cm) and number of nodes (5.1–6.4) were recorded when nodal explants were cultured on DKW medium supplement with 8.9 μM BA plus 4.1 μM MT. Cytokinin type and concentration greatly influenced growth and survival of black walnut *in vitro* cultures. Other black walnut researchers have reported similar effects. Bosela and Michler (2008) found that zeatin was superior to BA at concentrations ranging from 2.5 to 25 μM for rapid shoot elongation of seedling black walnut nodal sections, although zeatin and BA at elevated concentrations resulted in shoot necrosis, which was an issue unfavorable to *in vitro* rooting protocols. The incorporation of thidiazuron (TDZ) at 0.05–0.1 μM in the medium led to an increased rate of morphological abnormalities such as fasciation (Bosela and Michler 2008). Van Sambeek *et al.* (1997) reported, however, successfully establishing black walnut cultures

Table 4. The effects of auxin-concentration and -type on root formation of *Juglans nigra* L. *in vitro* microshoots after 5 wk

Auxin (μM)		Genotype no. 55			Genotype no. 189		
IBA	IAA	No. roots[a]	Root length (cm)[a]	Root formation (%)	No. roots[a]	Root length (cm)[a]	Root formation (%)
0	0	0a	0a	0	0a	0a	0
30	0	0.6 ± 0.2ab	0.14 ± 0.04bc	27.5	0.5 ± 0.2ab	0.1 ± 0.03 ac	22.5
30	50	0.3 ± 0.1ab	0.04 ± 0.02ab	15	0.3 ± 0.1ab	0.03 ± 0.2ab	12.5
30	100	0.1 ± 0.03a	0.03 ± 0.02ab	7.5	0.3 ± 0.1ab	0.02 ± 0.2ab	10
50	0	0.8 ± 0.2b	0.2 ± 0.05c	40	0.9 ± 0.2b	0.2 ± 0.04c	40
50	50	0.7 ± 0.2b	0.1 ± 0.04bc	27.5	0.7 ± 0.2b	0.2 ± 0.04bc	30
50	100	0.8 ± 0.2b	0.2 ± 0.04c	35	0.8 ± 0.2b	0.2 ± 0.04c	32.5

IBA indole-3-butyric acid, *IAA* indole-3-acetic acid

[a] Values represent means ± SE for 40 explants per treatment. Means in each *column* followed by the *same letter* were not significantly different according to Tukey's multiple comparison test ($P \leq 0.05$)

supplemented with 0.3 μM TDZ demonstrating the high response variation among *Juglans* genotypes to various cytokinins. Also, in contrast to the findings presented here, where an absence of exogenous cytokinin application resulted in reduced shoot elongation, Heile-Sudholt *et al.* (1986) saw no significant difference between the control (zero BA) and BA-treated (8.8–44.4 μM) black walnut shoots.

BA has been the most reported cytokinin used to promote *Juglans in vitro* shoot elongation at a wide range of concentrations. *Juglans regia* juvenile nodal explants were successfully multiplied *in vitro* using 0.44–8.9 μM BA (Chalupa 1981; Gruselle *et al.* 1987; McGranahan *et al.* 1988; Revilla *et al.* 1989; Jay-Allemand *et al.* 1992; Heloir *et al.* 1996; Saadat and Hennerty 1996; Vahdati *et al.* 2004). Using 8.9 μM BA, Pijut (1997) successfully elongated nodal explants of *J. cinerea* (butternut) from seedling origin. BA (0–12.5 μM) has been used to promote shoot elongation of *J. nigra* (Somers *et al.* 1982; Caruso 1983; Heile-Sudholt *et al.* 1986; Long *et al.* 1995; Bosela and Michler 2008). The present report, however, is the first to document the benefits of MT on culture establishment and shoot elongation for any *Juglans* species, specifically for black walnut.

The use of BA and MT individually during culture initiation resulted in black walnut shoot elongation; however, the positive interaction between the two had the greatest influence on mean length of shoots. One potential explanation for this observation was the way these cytokinins were metabolized and perceived by actively growing black walnut shoots. As noted previously, BA at optimal- and supra-optimal concentrations was slow to induce shoot elongation and had an increase of necrosis in black walnut cultures, respectively (Bosela and Michler 2008) necessitating fine-tuning of BA levels. Meta-topolin, even at higher equimolar concentrations relative to BA, remained as effective without inducing morphological abnormalities. These observations have been reported in other species and were believed to be a result of the rapid turnover of MT and its translocation within plant tissues (Kaminek *et al.* 1987; Bairu *et al.* 2009; Amoo *et al.* 2011). When both cytokinins were available, black walnut shoots presumably could have responded rapidly to MT, as a result of its increased bioactivity, while the uptake of the more metabolically stable BA, at levels below toxicity, was delayed but integral to continued elongation after the turnover of MT. Although, beneficial for black walnut, the positive effects of MT were not ubiquitous across species. It was reported that micropropagation of *Crataegus aronia* L. (spiny hawthorn) and other woody species was less effective using MT than BA for shoot elongation (Aremu *et al.* 2012; Nuri Nas *et al.* 2012).

While 8.9 μM BA plus 4.1 μM MT in semi-solid DKW medium was suitable for repeated subcultures, more rapid shoot multiplication was achieved using a liquid culture system. Black walnut microshoots, typically apically dominant when cultured on semi-solid medium, lost this dominance when partially submerged in liquid culture medium on a rotary shaker. This resulted in the release of lateral buds that quickly elongated forming large masses of morphologically normal shoots. Somers *et al.* (1982) reported similar findings with black walnut shoots cultured in a liquid medium containing 22.2 μM BA, but the condition of the shoots was not described. Other reports of black walnut grown on a paper bridge in stationary liquid medium found that shoots remained short and thickened, inhibiting rooting trials (Heile-Sudholt *et al.* 1986). In the present study, when explants were cultured on a rotary shaker in liquid medium, no hyperhydricity, an inherent problem in liquid culture systems, was observed. Hyperhydricity and vitrification was overcome through mechanical agitation and aeration on the rotary shaker. Hyperhydricity has been an issue in previous *Juglans* studies associated with inadequate mineral salt concentration (Bosela and Michler 2008), supra-optimal BA levels (Revilla *et al.* 1989), or carbohydrate dilution (Gruselle *et al.* 1987). The improved multiplication rate found in liquid shake culture suggested this to be the result of better nutrient and water availability, stability and availability of cytokinins, a reduction in the severity of pH and plant growth regulator gradients, loss of apical dominance, and a dilution of inhibitory substances (e.g., phenolics) (see review by Ascough and Fennell 2004; Hart *et al.* 2016). The dilution of phenolics is of particular importance for black walnut cultures, as it has long been an issue in developing routine micropropagation systems.

Physiological age influenced mean shoot length and overall survival of black walnut nodal explants. Mature material from elite genotypes often failed to elongate after bud break, and long-term culture could not be established. Other studies with black walnut, however, have reported it was possible to establish cultures from mature origin. Using dormant branches from mature trees, Van Sambeek *et al.* (1997) developed a solution that forced elongation of lateral buds that were then successfully cultured *in vitro*. Explants from mature origin remained recalcitrant to micropropagation techniques, a result of many endogenous and exogenous factors known to inhibit culture initiation (Hackett 1987; McCown 2000). Increased phenolic and endogenous microbiota exudation from the wound site in mature nodal explants led to the necrosis of shoot cultures before establishment could occur. The microbial biodiversity associated with walnuts was significant in *J. regia* mature stem, leaf, and fruit samples; 26.1% (970 individuals) of all isolates were classified as endophytes (Pardatscher and Schewiegkofler 2009). Differences in phenolic content between mature and juvenile shoots have been reported in *J. regia* × *J. nigra* hybrids (Claudot *et al.* 1992), and microbial contamination has hindered previous black walnut micropropagation attempts (Cummins and Ashby 1969; Somers *et al.* 1982; Heile-Sudholt *et al.* 1986; Van Sambeek *et al.* 1997). In contrast, entophytic bacteria were found to promote growth in *Prunus avium* (sweet cherry) cultures,

and even enhanced rooting in difficult-to-root genotypes (Quambusch *et al.* 2016). These conflicting reports require the elucidation of the interactions between black walnut and its endophytes to further improve micropropagation. Future studies must mitigate these deleterious effects before routine *in vitro* propagation is achieved.

In vitro cultured juvenile black walnut shoots remained recalcitrant to adventitious root formation. Repeatable and routine rooting has long been an impediment to black walnut micropropagation. However, independent of genotype, the present study demonstrated rooting as high as 40% when microshoots were exposed to 50 μM IBA and cultured in a slurry of half-strength DKW medium with vermiculite. A two-step rooting protocol whereby microshoots were cultured on separate induction medium in the dark, and expression medium in the light, resulted in healthier roots produced at higher frequencies (71–100%) for *J. regia* cultivars and hybrids at similar IBA concentrations (14.76–50 μM) (Jay-Allemand *et al.* 1992; Ripetti *et al.* 1994; Scaltsoyiannes *et al.* 1997; Leslie *et al.* 2010). Unfortunately, these techniques have not been transferrable to *J. nigra*. Black walnut microshoots placed in the dark for as few as 3 d turned necrotic. Failure to form adventitious roots could be explained by the relatively high concentrations of cytokinins in the shoot proliferation stage. BA metabolites that tend to accumulate in rooting zones can inhibit rooting, while MT metabolism does not lead to any deleterious side effects (Werbrouck *et al.* 1995; Valero-Aracama *et al.* 2010). Shoot proliferation exclusively with MT resulted in a lower multiplication rate but could have resulted in increased rooting in acclimatization. Further study is needed to improve *in vitro* rooting of black walnut microshoots. In the present study, successfully rooted microshoots were acclimatized to ambient growth room conditions where roots continued to grow, but shoot growth was not observed. Acclimatized plantlets did not survive the transition to the greenhouse for further growth and development. Failure to acclimatize to greenhouse conditions could have been a result of excess callus formation at the base of the microshoot limiting full vascular continuity with the shoot. While rooted plants survived in the growth room, the root system may not have been extensive enough to support the microplants under high stress greenhouse conditions. Improving root induction to limit callus formation could increase survival during acclimatization. Survival could also be improved through a more prolonged acclimatization period allowing microplants to tolerate increasingly stressful conditions.

Conclusion

The addition of MT to culture initiation medium, and the use of liquid culture on a rotary shaker, greatly increased shoot proliferation of black walnut explants; however, future research is needed to improve rooting frequency of microshoots and optimize acclimatization before commercial micropropagation of elite genotypes is practical. Experiments aimed at examining the lingering effects of elevated BA concentrations found in the shoot elongation medium or the influence of the type of cut made to the base of the microshoot prior to root induction could yield positive results.

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