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MICROPROPAGATION

In vitro propagation of northern red oak (*Quercus rubra* L.)

G. Vengadesan · Paula M. Pijut

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Abstract *In vitro* propagation of northern red oak (*Quercus rubra*) shoots was successful from cotyledonary node explants excised from 8-wk-old *in vitro* grown seedlings. Initially, four shoots per explant were obtained on Murashige and Skoog (MS) medium supplemented with 4.4 μM 6-benzylaminopurine (BA), 0.45 μM thidiazuron (TDZ), and 500 mg l^{-1} casein hydrolysate (CH) with a regeneration frequency of 64.7% after 3 wk. Subculturing explants (after harvesting shoots) to fresh treatment medium significantly increased shoot bud regeneration (16.6 buds per explant), but the buds failed to develop into shoots. A higher percentage (73.3%) of the explants regenerated four shoots per explant on woody plant medium (WPM) supplemented with 4.4 μM BA, 0.29 μM gibberellic acid (GA_3), and 500 mg l^{-1} CH after 3 wk. Explants subcultured to fresh treatment medium after harvesting shoots significantly increased shoot regeneration (16 shoots per explant). Shoot elongation was achieved (4 cm) when shoots were excised and cultured on WPM supplemented with 0.44 μM BA and 0.29 μM GA_3. *In vitro* regenerated shoots were rooted on WPM supplemented with 4.9 μM indole-3-butyric acid. A higher percentage regeneration response and shoot numbers per explant were recorded on WPM supplemented with BA and GA_3, than on MS medium containing BA and TDZ. Lower concentrations of BA and GA_3 were required for shoot elongation and prevention of shoot tip necrosis. Each cotyledonary node yielded approximately 20 shoots within 12 wk. Rooted plantlets were successfully acclimatized.

Keywords Cotyledonary node culture · Micropropagation · Plant regeneration · Rooting

G. Vengadesan
Hardwood Tree Improvement and Regeneration Center (HTIRC),
Department of Forestry and Natural Resources, Purdue University,
715 West State Street,
West Lafayette, IN 47907, USA

P. M. Pijut (✉)
HTIRC, Northern Research Station, USDA Forest Service,
715 West State Street,
West Lafayette, IN 47907, USA
e-mail: ppijut@purdue.edu
e-mail: ppijut@fs.fed.us

Introduction

The genus *Quercus* comprises nearly 400 species that are distributed throughout the temperate regions of the world (Johnson et al. 2002). Economically, oaks are a major source of timber for flooring, furniture, and cabinetry in the USA (Merkle and Nairn 2005; Pijut et al. 2007). Ecologically, oaks are important mast producers for many organisms, including insects, birds, and mammals (Johnson et al. 2002). The regeneration of oak species is reported to be gradually deteriorating as a result of extensive harvesting, irregular fructification, unavailability of seed every year, and predation. Northern red oak (NRO; *Quercus rubra* L.; Fagaceae) is native to North America in the eastern USA and southeastern Canada. NRO is one of the most important oaks for timber production, and the wood is of high value for veneer, furniture, flooring, general millwork, crates, caskets, handles, and wooden ware. The acorns are consumed by insects, deer, and small mammals. Natural regeneration of NRO throughout its range in the eastern USA is difficult because of several factors, such as interspecific competition, predation, disturbance, and reproductive mode (Crow 1988; Steiner 1995; Buckley et al. 1998). Acorn production is highly variable (3- to 5-y intervals) and populations of NRO do not produce acorns

abundantly until they are 25 y of age (Olson 1974). NRO acorns also have poor viability, are recalcitrant to long-term storage (Connor 2004), and several difficulties exist in establishment of seedlings in seed orchards. NRO acorns are attacked by several genera of insect pests (Galford et al. 1991; Galford and Weiss-Cottrill 1991) prior to collection and during storage. The red oak borer, a native wood-boring cerambycid beetle, is also a contributing factor to oak mortality in natural stands (Fierke et al. 2005).

Clonal reproduction of commercially important hardwood tree species, such as northern red oak, is vital in a tree improvement program in order to provide improved planting stock for production (plantation) forestry. *In vitro* and conventional vegetative propagation methods will be required to produce clones of elite genotypes or genetically improved (borer resistant) genotypes. NRO conventional propagation has several constraints, such as poor rooting of stem cuttings, graft incompatibility problems, and a delay in sexual maturity of trees for seed production. *In vitro* propagation could be useful in overcoming some of these difficulties in order to multiply elite or genetically improved germplasm.

In vitro propagation of *Q. rubra* has been reported using stem segments and embryonic axes, but the *in vitro* raised microshoots did not produce roots (Rancillac et al. 1991; Schwarz and Schlarbaum 1993). Vieitez et al. (1993) achieved plant regeneration in *Q. rubra* using shoot tip and nodal segments from both juvenile and adult trees, but shoot cultures exhibited arrest of growth and apical necrosis. High shoot proliferation rates were reported using apical and nodal segments of *Q. rubra* from 2- and 18-mo-old seedlings (Ostrolucka and Bezo 1994). Sanchez et al. (1996) investigated *in vitro* rooting of *Q. rubra* shoot cultures established from epicormic shoots of a 40-y-old tree. Micropropagation using cotyledonary nodes of *in vitro* raised seedlings has not been reported for NRO. The objective of this study was to develop an efficient *in vitro* propagation methodology through multiple shoot formation and subsequent rooting from cotyledonary nodes of *Q. rubra*.

Materials and Methods

Plant material. Mature acorns from 14 individual trees of northern red oak were collected mid-September 2005 from the Jasper Pulaski Nursery in Medaryville, IN. Because of limited quantities and viability of sound acorns, the acorns from all trees were pooled and used for experimentation.

Explant preparation. Mature acorns were washed vigorously with 1% (*w*/*v*) Alconox (Alconox Inc., White Plains, NY) detergent for 20 min followed by three rinses (3 min each) with sterile distilled water. Acorns were then disinfected with 70% (*v*/*v*) ethanol for 3 min followed again by three rinses (3 min each) with sterile distilled water. The acorns were then surface disinfected with 30% (*v*/*v*) bleach solution (5.25% sodium hypochlorite; Champion Packaging, Inc., Woodridge, IL) containing three drops of Tween 20 (P7949, Sigma, St. Louis, MO) for 20 min and then rinsed three times (3 min each) with sterile distilled water. The pericarp was then removed aseptically. After removing the pericarps, the acorns were again disinfected with 70% ethanol for 1 min, rinsed three times (3 min each) with sterile distilled water, surface disinfected with 20% bleach solution (plus three drops of Tween 20) for 15 min, and then rinsed five times (3 min each) with sterile distilled water. The embryos were carefully excised aseptically and the cotyledons were separated without damage. Embryos were placed in 25×150 mm culture tubes (single embryo per tube) containing 15 ml of woody plant medium (WPM; L154, PhytoTechnology, Shawnee Mission, KS; Lloyd and McCown 1981) supplemented with 3% (*w*/*v*) sucrose (S5390, Sigma), 0.24% Phytagel (P8169, Sigma), and devoid of growth regulators for *in vitro* germination. Cotyledonary node explants (0.5 cm) were excised from 8-wk-old *in vitro* grown seedlings and used as the explant source for shoot proliferation.

In vitro shoot multiplication. Cotyledonary node explants were cultured on Murashige and Skoog (MS) medium (M499, PhytoTechnology; Murashige and Skoog 1962) supplemented with 6-benzylaminopurine (BA; B9395, Sigma) at 0, 0.44, 4.4, 8.8, or 17.6 μM alone and in combination with thidiazuron (TDZ; T888, PhytoTechnology) at 0, 0.045, 0.45, or 4.5 μM (Table 1) or WPM supplemented with BA at 0, 0.44, 4.4, 8.8, or 17.6 μM alone and in combination with gibberellic acid (GA_3; G7645, Sigma) at 0, 0.029, 0.29, or 2.9 μM (Table 2). All media contained 500 mg l^{-1} casein hydrolysate (C7290, Sigma), 3% sucrose, and 0.24% Phytagel. Fifteen explants were cultured per treatment with a single explant per culture tube (25×150 mm; 15 ml of medium). Explants were transferred to fresh treatment medium at 3-wk intervals. Individual shoots (1 cm long) harvested from each explant were cultured in Magenta GA-7 vessels (Magenta Corp., Chicago, IL) containing 50 ml WPM supplemented with 0.44 μM BA and 0.29 μM GA_3 for shoot elongation. After harvesting the shoots, the original explants were transferred to fresh treatment medium for further shoot proliferation. The experiments were repeated three times.

Rooting of in vitro shoots. Elongated shoots (4 cm) were cultured in Magenta GA-7 vessels containing 50 ml WPM supplemented with indole-3-butyric acid (IBA; I5386, Sigma) at 0, 2.5, 4.9, or 14.7 μM for root induction. Cultures were initially placed in a dark incubator at 26°C for 5 d then transferred to 16-h photoperiod. Ten shoots were used per treatment with a single shoot per culture vessel. The experiments were repeated three times. MS

Table 1 Influence of BA and TDZ on multiple shoot proliferation and regeneration from cotyledonary node explants of northern red oak

Plant growth regulators (μM)		Explant response (%)	No. of shoots per explant after 3 wk	No. of shoots per explant after 6 wk	Shoot length (cm)
BA	TDZ				
0.0	0.0	0.0 h	0.0 de	0.0 f	0.0 d
0.44	0.0	31.3±2.0 d	0.7±0.3 de	0.7±0.3 ef	0.4±0.03 bc
4.4	0.0	44.7±3.1 b	1.3±0.3 cd	1.6±0.4 de	0.5±0.07 bc
8.8	0.0	23.3±3.5 e	1.0±0.1 cd	1.0±0.3 de	0.9±0.3 a
17.6	0.0	14.0±3.2 f	0.6±0.4 de	0.7±0.3 ef	0.4±0.04 bc
0.0	0.045	9.6±2.6 g	1.3±0.3 cd	1.6±0.4 de	0.4±0.02 bcd
0.0	0.45	16.0±2.1 f	2.0±0.5 bc	4.0±0.6 cd	0.3±0.08 bcd
0.0	4.5	7.6±1.4 g	1.0±0.2 cd	5.0±0.5 bc	0.2±0.06 cd
0.44	0.045	37.0±3.7 c	2.0±0.6 bc	4.3±0.9 bc	0.3±0.02 bcd
4.4	0.045	32.0±4.6 d	2.0±1.2 b	7.3±1.5 b	0.6±0.2 ab
8.8	0.045	29.0±3.0 d	2.0±0.6 bc	3.0±0.6 cd	0.4±0.08 bc
17.6	0.045	17.5±3.7 f	1.0±0.3 cd	2.3±0.3 cd	0.4±0.04 bc
0.44	0.45	42.6±5.0 b	2.3±0.4 bc	6.7±1.4 b	0.5±0.03 bc
4.4	0.45	64.7±5.2 a	4.0±0.6 a	16.6±0.8 a	0.7±0.14 ab
8.8	0.45	38.7±2.0 c	2.0±0.5 bc	6.0±2.1 b	0.3±0.05 bcd
17.6	0.45	23.6±3.4 e	1.0±0.3 cd	3.0±0.6 cd	0.4±0.01 bcd
0.44	4.5	33.0±4.3 d	2.0±0.6 bc	3.6±0.9 cd	0.4±0.01 bc
4.4	4.5	36.3±2.7 c	2.0±0.1 bc	3.7±0.3 cd	0.3±0.02 bcd
8.8	4.5	15.6±2.3 f	2.0±0.8 bc	3.0±0.3 cd	0.2±0.01 bcd
17.6	4.5	18.6±2.9 f	1.0±0.1 cd	1.0±0.4 de	0.3±0.02 bcd

Cotyledonary node explants were cultured on Murashige and Skoog medium supplemented with 3% sucrose, 0.24% Phytagel, BA and TDZ, and 500 mg l^{-1} casein hydrolysate at 16-h photoperiod at 25±2°C. MS medium devoid of plant growth regulators served as control. Fifteen explants were used per treatment, the experiments were repeated three times, and data scored after 3 and 6 wk in culture. Values represent means ± standard error. Means in each *column* followed by the same *letters* are not significantly different according to Tukey's multiple comparison test ($P \leq 0.05$) *BA* 6-benzylaminopurine, *TDZ* thidiazuron, *MS* Murashige and Skoog

basal and WPM basal devoid of growth regulators served as controls in all experiments.

All media were autoclaved at 121°C at 1.05 kg cm^{-2} pressure for 20 min after adjusting the pH to 5.7 with 1 N NaOH. All cultures (except during the dark root induction period) were maintained at 25±2°C with a 16-h photoperiod, 50 μmol m^{-2} s^{-1} provided by cool-white fluorescent lamps (Phillips F30T12/CW/RS, Kirby Risk Electrical Supply, Lafayette, IN).

Acclimatization. Shoots with well-developed roots were removed from the culture medium and the roots gently washed under running tap water to remove any traces of Phytagel. The plantlets were then transplanted into 10×9-cm plastic pots containing a moist, autoclaved medium (ProMix HP, Premier Horticulture, Inc., Quakertown, PA) and placed in 1-gal Zip-loc™ plastic bags to provide a high relative humidity. Potted plants in bags were placed under a 16-h photoperiod (50 μmol m^{-2} s^{-1}) at 25±2°C. Plantlets were gradually acclimatized (over a period of 4 to 6 wk) to room temperature and humidity conditions by progressively opening the bag until plants were ready to be completely removed from the bags.

Statistical analyses. All experimental data on percent response, shoot formation, and rooting were statistically analyzed with analysis of variance based on the linear model for factorial designs (Anderson and McLean 1974) using the General Linear Model procedure of SAS software version 9.1.3 (SAS Institute Inc. 2005), and significant differences between means were determined by the Tukey's test at the 5% level of probability for all experiments.

Results and Discussion

Zygotic embryos isolated from mature acorns germinated within 2 wk of culture. Approximately 60% germination was achieved in 4 wk on WPM. Cotyledonary node explants excised from 8-wk-old in vitro seedlings on MS and WPM, supplemented with BA and TDZ, and BA and GA_3 induced multiple shoots. The number of shoots formed varied with the type and concentration of growth regulators and media formulation used.

Effect of BA and TDZ on shoot multiplication. Four axillary shoot buds developed from single cotyledonary nodal explants (Fig. 1*A*) and 64.7% of explants responded on MS supplemented with 4.4 μM BA and 0.45 μM TDZ after 3 wk of culture without basal callusing (Table 1). Subculturing explants, after harvesting shoots, to fresh

Table 2. Influence of BA and GA_3 on multiple shoot proliferation and regeneration from cotyledonary node explants of northern red oak

Plant growth regulators (μM)		Explant response (%)	No. of shoots per explant after 3 wk	No. of shoots per explant after 6 wk	Shoot length (cm)
BA	GA_3				
0.0	0.0	0.0 k	0.0 c	0.0 e	0.0 h
0.44	0.0	31.7±2.6 e	1.3±0.3 bc	1.6±0.3 de	0.9±0.1 e
4.4	0.0	44.6±4.0 c	1.7±0.4 bc	2.3±0.5 cd	0.7±0.1 f
8.8	0.0	22.3±3.5 f	1.3±0.3 bc	1.6±0.4 de	0.4±0.1 g
17.6	0.0	13.0±3.2 h	1.0±0.1 bc	1.5±0.3 de	0.4±0.08 g
0.0	0.029	7.6±1.5 j	0.6±0.3 bc	1.0±0.5 de	1.3±0.05 cd
0.0	0.29	11.6±1.4 i	1.3±0.4 bc	2.0±0.3 cd	1.2±0.2 de
0.0	2.9	19.0±2.1 f	2.0±0.6 b	2.0±0.5 cd	1.5±0.3 ab
0.44	0.029	34.0±2.6 d	2.0±0.2 b	3.3±0.3 c	1.3±0.01 cd
4.4	0.029	56.3±4.5 b	2.0±0.5 b	6.3±1.2 b	1.4±0.3 bc
8.8	0.029	26.0±3.8 e	2.0±0.3 b	3.6±0.3 c	0.7±0.2 f
17.6	0.029	15.7±3.4 f	1.3±0.4 bc	2.0±0.6 cd	1.2±0.1 de
0.44	0.29	36.0±3.2 d	1.7±0.3 bc	3.0±0.6 c	1.4±0.1 bc
4.4	0.29	73.3±7.5 a	4.0±0.6 a	16.0±0.5 a	1.7±0.08 a
8.8	0.29	29.6±2.9 e	2.0±0.5 b	4.0±0.5 c	1.5±0.1 ab
17.6	0.29	19.4±3.6 f	2.0±0.7 b	3.6±0.8 c	0.5±0.1 fg
0.44	2.9	32.0±3.1 e	1.6±0.3 bc	3.0±0.6 c	1.5±0.2 ab
4.4	2.9	41.7±4.4 c	2.0±0.1 b	3.3±0.3 c	0.5±0.1 fg
8.8	2.9	24.7±3.2 f	2.0±0.6 b	3.6±0.9 c	0.8±0.05 ef
17.6	2.9	15.0±2.9 g	1.5±0.3 bc	2.3±0.3 cd	0.8±0.2 ef

Cotyledonary node explants were cultured on woody plant medium supplemented with 3% sucrose, 0.24% Phytagel, BA and GA_3, and 500 mg l^{-1} casein hydrolysate at 16-h photoperiod at 25±2°C. WPM devoid of plant growth regulators served as control. Fifteen explants were used per treatment, the experiments were repeated three times, and data scored after 3 and 6 wk in culture. Values represent means ± standard error. Means in each *column* followed by the same *letters* are not significantly different according to Tukey's multiple comparison test ($P \leq 0.05$)
BA 6-benzylaminopurine, *GA_3* gibberellic acid, *WPM* woody plant medium

treatment medium enhanced shoot bud proliferation. Maximum proliferation of 16.6 shoot buds per explant (Fig. 1*B*) was obtained at the end of the first subculture (6 wk after initial culture; Table 1), but the shoot buds failed to develop further. Among the various concentrations of BA and TDZ examined alone, highest shoot regeneration response of 44.7% (4.4 μM BA) and 16% (0.45 μM TDZ) were recorded with an average of 1.6 shoots per explant for BA and 4.0 shoots per explant for TDZ after 6 wk (Table 1).

TDZ along with BA stimulated the development of large numbers of multiple shoot buds, but this combination failed to favor continued shoot growth and development. The results of the present study suggest that TDZ caused cell division, but not elongation as reported by Bowen-O'Connor et al. (2007) for *Acer grandidentatum*. Huetteman and Preece (1993) reported that inhibition of shoot elongation and shoot fasciation was a result of the high cytokinin activity of TDZ and the presence of phenyl groups in TDZ. Cotyledonary node explants cultured continuously on TDZ supplemented medium resulted in the formation of suppressed or fasciated shoots in *Pterocarpus marsupium* (Husain et al. 2007). Although TDZ has been shown to increase shoot production, many other reports have demonstrated that TDZ does not benefit or hinders shoot production and elongation (Yusnita et al. 1990; Lu 1993; Kartsonas and Papafotiou 2007). In contrast, TDZ either alone or in combination with other plant growth regulators was successfully used for *in vitro* propagation of *Acer* and *Fraxinus* species (Kim et al. 1997; Brassard et al. 2003).

Effect of BA and GA_3 on shoot multiplication. Cotyledonary node explants cultured on WPM with 4.4 μM BA and 0.29 μM GA_3 initially developed four shoots per explant (Table 2) after 3 wk of culture (Fig. 1*C*). Subculturing explants, after harvesting shoots, to fresh treatment medium increased shoot proliferation. A maximum of 16 axillary shoots (1 cm long) per explant (Fig. 1*D*) proliferated at the end of the first subculture (6 wk after initial culture; Table 2). Further subculture did not improve any shoot proliferation. Growth regulator concentrations significantly ($P=0.001$) effected the percent response of explants and shoot number per explant (Tables 1 and 2). Each cotyledonary node yielded approximately 20 shoots within 12 wk. The combination of BA (22.19 μM) and GA_3 (2.89 μM) increased shoot numbers per cotyledonary node explant in *Quercus leucotrichophora* and *Quercus glauca*, but the shoot numbers were not significantly different compared to BA (22.19 μM) alone (Purohit et al. 2002b).

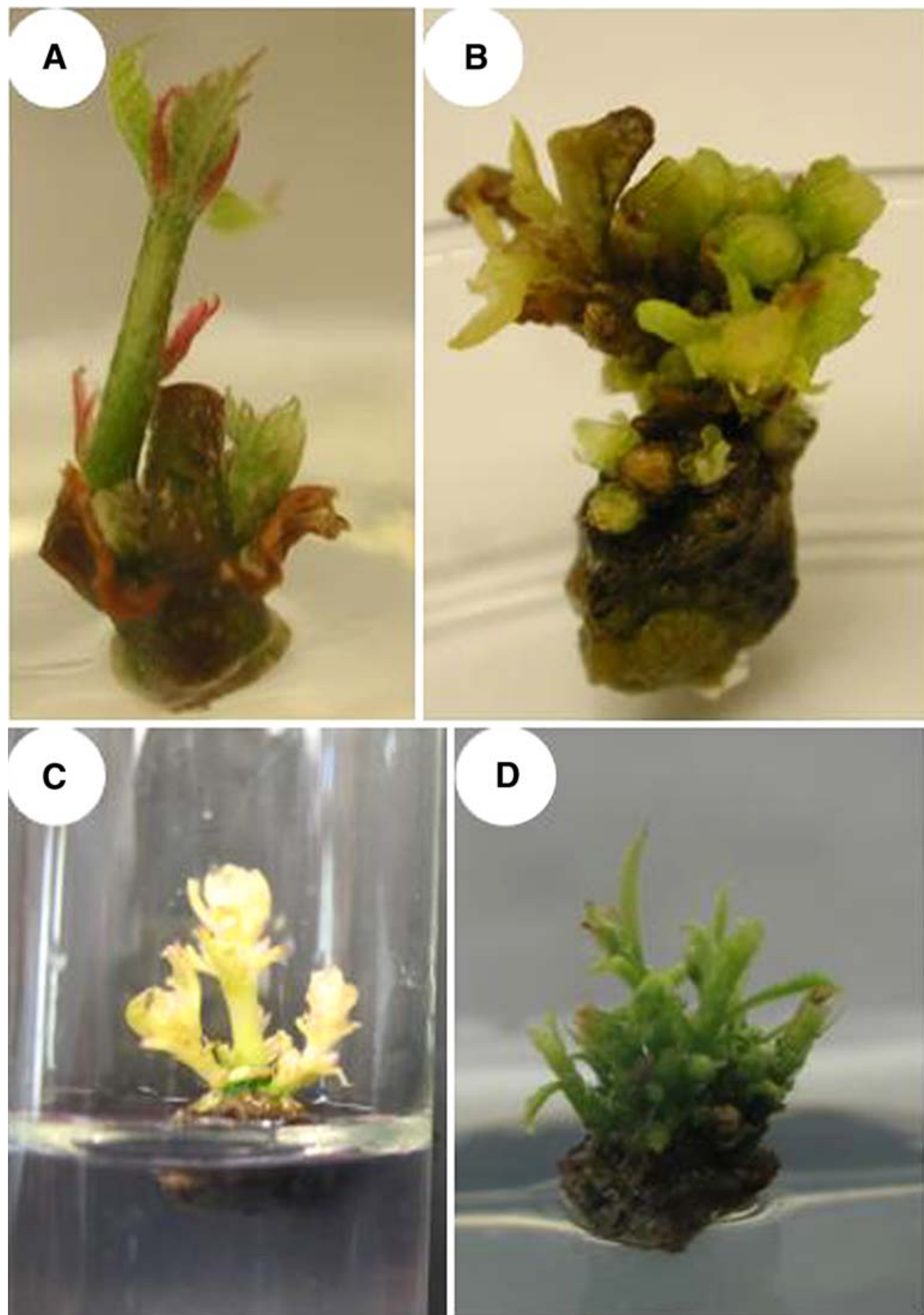

Figure 1. *In vitro* propagation of *Q. rubra*. (*A*) Multiple shoot induction from cotyledonary node explant on MS with 4.4 μM BA and 0.45 μM TDZ after 3 wk. (*B*) Shoot bud induction after subculturing (6 wk after initial culture). (*C*) Multiple shoot induction from cotyledonary node explant cultured on WPM with 4.4 μM BA and 0.29 μM GA_3 after 3 wk. (*D*) Shoot proliferation after subculturing (6 wk after initial culture).

Ostrolucka and Bezo (1994) reported that a combination of BA (4.4 μM) and GA_3 (1.4 μM) induced higher numbers of shoots in *Quercus robur* and *Quercus virgiliana*. Shoot bud cultures were established for *Q. robur* and *Quercus petraea* using a combination of BA (2.2 μM), GA_3 (0.3 μM), and IBA (2.5 μM; Pevalek-Kozlina and Jeleska 1986). Our observation revealed that a combination of BA and GA_3 was essential for efficient shoot bud induction and growth for *Q. rubra*.

Effect of BA on shoot multiplication. In the present study, 4.4 μM BA promoted production of two shoots per explant. Percentage response (73.3%) of explants and shoot numbers (16.0±0.5) were significantly higher (P=0.001) with the combination of 4.4 μM BA and 0.29 μM GA_3 than with 4.4 μM BA alone (Table 2). BA (0.88 to 3.5 μM) induced a higher number of shoots in *Q. robur* than kinetin, and higher concentrations (8.8 to 22.0 μM) resulted in the formation of short shoots (Chalupa 1984, 1988). Vieitez et

al. (1985) and Favre and Juncker (1987) reported that 4.4 μM BA significantly influenced shoot number and proliferation in *Q. robur*, and BA was reported to be the best plant growth regulator for shoot multiplication in *Quercus shumardii* (Bennett and Davies 1986).

Low concentrations of BA (0.88 to 2.64 μM) favored higher shoot multiplication in other *Quercus* species (San-Jose et al. 1990; Chalupa 1993; Meier-Dinkel et al. 1993). Juncker and Favre (1994) reported that 0.44 μM BA was best for axillary bud induction and shoot elongation than 0.49 μM 2iP and 0.45 μM zeatin in *Q. robur*, and higher concentrations of BA exhibited vitrification and basal callogenesis which inhibited further shoot growth. Similarly, shoot number per explant was significantly increased by BA, and a higher concentration (4.4 μM) led to hyperhydricity, malformation, and twisted leaves in *Q. robur* (Puddephat et al. 1997). Higher shoot multiplication was achieved in *Q. rubra* and *Quercus cerris* on media supplemented with BA (0.44 to 2.2 μM) or BA combined with naphthaleneacetic acid (NAA; 0.54 to 2.7 μM; Ostrolucka and Bezo 1994). A maximum of six shoots per embryonic axis explant was achieved in *Q. rubra* on medium supplemented with 4.4 μM BA, and the addition of 1.0 and 10.0 μM NAA to 4.4 μM BA reduced both shoot number and explant response (Schwarz and Schlarbaum 1993). In contrast, BA in combination with low concentration with NAA improved shoot multiplication rate in *Quercus suber* (Manzanera and Pardos 1990). BA (0.89 μM) induced an average of three shoots in *Q. rubra* from shoot-tip explants excised from 3-mo-old seedlings (Vieitez et al. 1993). Efficient *in vitro* multiplication of shoots was reported using 0.89 μM BA in *Q. robur* by culturing explants (obtained from mature origin) horizontally (Vieitez et al. 1994). In the present study, BA alone did not favor higher shoot proliferation, and MS or WPM devoid of growth regulators did not induce multiple shoot induction. Multiple shoot proliferation from cotyledonary node explants was successful in other tree species (Buendia-Gonzalez et al. 2007; Nayak et al. 2007).

Shoot elongation. WPM with 4.4 μM BA and 0.29 μM GA_3 did not favor further shoot growth. Shoots (1 cm long) cultured on WPM containing 0.44 μM BA and 0.29 μM GA_3 favored shoot elongation with an average height of 4 cm, data not shown (Fig. 2*A*). Similarly, the addition of 2.89 μM GA_3 enhanced shoot height in *Q. leucotrichophora* and *Q. glauca* (Purohit et al. 2002b) and slightly longer shoots in *Quercus floribunda* (Purohit et al. 2002a). The addition of IBA or indole-3-acetic acid at 0.06 or 0.57 to 0.89 μM BA slightly increased shoot number and length, and culturing decapitated shoots in a horizontal orientation promoted shoot elongation in *Q. rubra* (Vieitez et al. 1993). Shoot-tip necrosis is a common physiological disorder with some *in vitro* cultures of tree species (Vieitez et al. 1989). Explants cultured in a vertical orientation exhibited shoot growth and apical necrosis in *Q. rubra* (Vieitez et al. 1993), and similar observations were reported by Rancillac et al. (1991). In the present study, a low concentration of BA (0.44 μM) and GA_3 (0.29 μM) promoted shoot elongation and prevented shoot-tip dormancy and necrosis.

Effect of subculture. The number of shoots developed during the first subculture was distinctly higher than the initial culture (Tables 1 and 2), and the shoot number was higher compared to other *Quercus* species using nodal explants. San-Jose et al. (1988) reported that recycling the explants is helpful for species that are recalcitrant to *in vitro* propagation, such as *Q. robur*, and repeated culturing increased multiplication efficiency considerably in *Q. petraea* (San-Jose et al. 1990). Subculturing explants in a

Figure 2. Elongation and rooting of *in vitro* produced shoots. (*A*) *In vitro* shoot elongation on WPM containing 0.44 μM BA and 0.29 μM GA_3 after 4 wk. (*B*) Rooting of microshoot on WPM with 4.9 μM IBA after 4 wk. (*C*) Acclimatized plantlet.

horizontal orientation seems to reinvigorate oak cultures compared to a vertical orientation (Vieitez et al. 1994). Subsequent recycling of mother shoots enhanced multiplication rates in *Q. rubra* cultures (Vieitez et al. 1993). In contrast, subculturing cotyledonary node explants after harvesting shoots decreased the shoot number in *Q. floribunda* (Purohit et al. 2002a). Puddephat et al. (1997) reported that transferring nodal explants to fresh medium did not increase the number of explants producing shoots on medium devoid of BA in *Q. robur*. Repeated transfer of radiata pine explants was suggested as an efficient method for micropropagation (Aitken-Christie and Jones 1987), and such a procedure caused activation and conditioning of meristems (Boulay 1985). Shekhawat et al. (1993) reported transferring explants to fresh medium is essential for sustained shoot growth as well as to prevent defoliation in *Prosopis cineraria*.

Effect of salt formulation. Cotyledonary node explants cultured on WPM supplemented with BA and GA_3 had the highest shoot regeneration frequency in this study. WPM and MS medium with half-strength macronutrients enhanced shoot induction and growth in *Q. rubra* and other *Quercus* species (Ostrolucka and Bezo 1994). Chalupa (1984) reported WPM was better for shoot growth and development than MS or Gresshoff and Doy (GD) medium (Gresshoff and Doy 1972) for *Q. rubra*. High frequency shoot multiplication was achieved in *Q. leucotrichophora*, *Q. glauca*, and *Q. floribunda* using WPM in comparison to MS medium (Purohit et al. 2002a, b). Eight macronutrient formulae were tested for multiple shoot induction for *Q. robur*, and GD medium responded better, forming healthy shoots, whereas other macronutrients produced necrosis or succulent shoots (Vieitez et al. 1985). Chalupa (1993) recorded higher shoot multiplication efficiency with broad-leaved tree medium and WPM, than MS and Schenk and Hildebrandt medium (Schenk and Hildebrandt 1972) which developed short shoots in *Q. robur* and *Q. petraea*. San-Jose et al. (1990) and Vieitez et al. (1994) used GD medium successfully for clonal propagation of *Q. petraea* and *Q. robur*, respectively.

Rooting of microshoots and acclimatization. *In vitro* shoots (4 cm long) produced roots when grown on WPM containing IBA. IBA at 4.9 μM produced a prominent root (2.1 cm long) with lateral hairy roots (Fig. 2*B*) after 3 wk of culture with a rooting response of 30% (Table 3). IBA at 2.5 and 14.7 μM induced more callusing at the base of the shoot with the production of healthy roots, but the plantlets did not survive acclimatization, and this may be a result of a lack of vascular connection. The rooted plantlets were acclimatized (Fig. 2*C*) with 30% survival, grew well, and appeared healthy. We showed that stabilized *in vitro* shoot cultures initiated from cotyledonary nodes of *Q. rubra* can be rooted using 4.9 μM IBA. Microshoots maintained in darkness initially for 5 d improved rooting response in this species as reported by Sanchez et al. (1996). Shoots cultured on half-strength WPM supplemented with 122.5 μM IBA for 24 h, and subsequent transfer to auxin-free medium containing 1% activated charcoal gave best rooting efficiency for *Q. rubra* (Sanchez et al. 1996). WPM with half-strength macronutrients supplemented with various concentrations of IBA did not induce rooting, but dipping the basal region of shoots in 4.92 mM IBA for 1 min favored rooting at a frequency of 40% for *Q. rubra* (Vieitez et al. 1993). A low concentration of IBA (1.47 μM) and NAA (0.54 μM) induced the highest rooting percentage (90%) for *Q. rubra* (Ostrolucka and Bezo 1994). Generally, darkness induces shoot senescence and causes a low survival percentage of plantlets (Rugini et al. 1988) as observed in this study. Rooted plants were acclimatized by transferring them into plastic pots containing a mixture of sterile peat and perlite (Fig. 2*C*), and 30% of the plantlets survived. Further experiments will be carried out to improve the survival percentage of rooted plants.

The present study is the first successful report of *in vitro* propagation of northern red oak via cotyledonary node explants, and the number of shoots produced was higher using this protocol than in previous studies. This will be important in a breeding and selection program where limited acorns are produced of improved selections. The maximum number of shoots per explant and explant

Table 3 Influence of indole-3-butyric acid on rooting response of *in vitro* regenerated shoots from cotyledonary node explants of northern red oak

IBA (μM)	Response (%)	No. of roots/shoot	Root length (cm)
0.0	0.0 d	0.0 d	0.0 b
2.5	50.0±5.7 a	6.5±1.4 a	2.0±0.7 a
4.9	30.0±3.2 c	1.5±0.2 c	2.1±0.3 a
14.7	43.0±8.8 b	3.7±0.6 b	2.0±0.5 a

In vitro raised shoots (4 cm long) were cultured on woody plant medium containing 3% sucrose, 0.24% Phytagel, and IBA. Initially, the cultures were maintained in darkness for 5 d at 26°C and then transferred to 16-h photoperiod. Ten shoots were used per treatment, the experiments were repeated three times, and data scored after 3 wk in culture. Values represent means ± standard error. Means in each *column* followed by the same *letters* are not significantly different according to Tukey's multiple comparison test ($P \leq 0.05$)
IBA indole-3-butyric acid

response was obtained on WPM containing 4.4 μM BA and 0.29 μM GA_3, and subculturing the original explants markedly increased the shoot number and growth. It is interesting to note that a combination of plant growth regulators can result in better shoot proliferation than when used alone. The variation in shoot proliferation response of *Quercus* species is dependent on the type of plant growth regulator as reported by Bowen-O'Connor et al. (2007) and depends on the genotype as reported by Vieitez et al. (1993). Genotypes showed considerable differences in their physiological requirements for maximum *in vitro* shoot proliferation and elongation. The individual shoots were rooted on WPM containing 4.9 μM IBA, and 30% of the rooted plants survived acclimatization. Thus, cotyledonary nodal explants obtained from *in vitro* germinated mature zygotic embryos proved to be an excellent explant for *in vitro* propagation for northern red oak.

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