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ORIGINAL PAPER

Somatic embryogenesis and plant regeneration of northern red oak (*Quercus rubra* L.)

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Abstract A somatic embryogenesis protocol for plant regeneration of northern red oak (*Quercus rubra*) was established from immature cotyledon explants. Embryogenic callus cultures were induced on Murashige and Skoog medium (MS) containing 3% sucrose, 0.24% Phytagel^{TM}, and various concentrations of 2,4-dichlorophenoxyacetic acid (2,4-D) after 4 weeks of culture in darkness. A higher response (66%) of embryogenic callus was induced on 0.45 μM 2,4-D. Higher numbers of globular- (31), heart- (17), torpedo- (12), and cotyledon-stage (8) embryos per explant were obtained by culturing embryogenic callus on MS with 3% sucrose, 0.24% Phytagel^{TM}, and devoid of growth regulators after 8 weeks culture in darkness. Continuous sub-culturing of embryogenic callus on medium containing 2,4-D yielded only compact callus. Desiccation of embryos for 3 days in darkness at 25 ± 2°C followed by cold storage at 4°C in darkness for 8 weeks favored embryo germination and development of plantlets. Cotyledon-stage embryos subjected to desiccation and chilling treatment cultured on MS with 3% sucrose, 0.24 Phytagel^{TM}, 0.44 μM 6-benzylaminopurine (BA), and 0.29 μM gibberellic acid germinated at a higher frequency (61%) than with 0.44 μM BA alone and control cultures. Germinated plantlets developed a shoot and root, were acclimatized successfully, and maintained in a growth room for plantlet development.

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Keywords Embryogenic callus · Germination · Maturation · Oak · Somatic embryos

Abbreviations

BA	6-Benzylaminopurine
2,4-D	2,4-Dichlorophenoxyacetic acid
GA_3	Gibberellic acid
NRO	Northern red oak
MS	Murashige and Skoog medium

Introduction

Northern red oak (NRO) [*Quercus rubra* L.; Fagaceae] is an important hardwood tree species, native to the eastern United States and southeastern Canada, grown for its highly valued wood. Oaks are a major source of timber in the United States (Merkle and Nairn 2005; Pijut et al. 2007) and NRO is valued for flooring, cabinetry, interior finishing, caskets, furniture, and veneer. The acorns are consumed by insects, deer, and small mammals. The genetic improvement of NRO is limited by both conventional vegetative propagation and biotechnological methods. Propagation of oak species has several constraints, such as poor rooting of vegetative cuttings, delay in sexual maturity of trees for seed production, long rotation period between seed production, and difficulties in the establishment of seedling orchards (Wilhelm 2000). NRO acorns also have poor viability and are attacked by several genera of insect pests (Galford et al. 1991; Galford and Weiss-Cottrill 1991) prior to collection and during storage. Further, mature trees are also recalcitrant to in vitro regeneration. One of the ways to possibly overcome these problems is through somatic

embryogenesis, a prospective tool for genetic improvement of oak species. The main advantages of this system of regeneration includes mass propagation of elite oak genotypes, high multiplication rates, scale-up for large-scale production, genetic transformation, cryopreservation of embryos, and direct transfer to the field or greenhouse through artificial seeds. The combination of this technology could be very useful in an oak improvement program (Valladares et al. 2006).

Merkle and Nairn (2005) reported that somatic embryogenesis in North American oaks is infrequent and the reported studies resulted in few regenerated plants. Both juvenile and mature explant sources have been used for the initiation of somatic embryos of *Q. rubra*. Direct and indirect embryogenesis were induced from immature zygotic embryo explants using 2,4-dichlorophenoxyacetic acid (2,4-D) and 6-benzylaminopurine (BA), but plantlet regeneration was low (Gingas and Lineberger 1989). Leaf discs from 1.5 to 3-month-old seedlings were used as explants to induce embryos using α-naphthaleneacetic acid (NAA) and BA, but the majority of embryos did not have a viable apical bud and plantlet survival was low (Rancillac et al. 1991, 1996). Callus cultures obtained from male catkins using BA or 2,4-D did not regenerate embryos in NRO (Gingas 1991). Seckinger et al. (1979) reported the development of normal roots on organoids initiated from callus cultures on medium containing NAA and BA, although no shoots were observed.

Somatic embryogenesis for various oak species has been induced from immature zygotic embryos (see review Wilhelm 2000). In *Q. suber* (Hernández et al. 2003a, b) and *Q. robur* (Toribio et al. 2004; Valladares et al. 2006) leaf explants from mature tree origin were used to induce somatic embryogenesis and percent conversion of the somatic embryos ranged from 0 to 70%. In *Q. suber*, somatic embryo maturation was improved by the addition of exogenous abscisic acid (ABA). However, germination was performed using desiccation and cold treatments (García-Martín et al. 2005).

Though the above methods hold promising outcomes for the propagation of oak genotypes, several problems such as the poor regeneration ability of mature explant material, physiological status of the explants, various genotypes, and low conversion into plantlets exists. Park et al. (1998) stated that high frequency somatic embryo induction, maturation, and germination are important for exploiting high-value clonal forestry and generating superior clonal varieties. The purpose of this study was to determine suitable growth regulator and culture conditions to induce proliferating embryogenic cultures, somatic embryos, and conversion of embryos into plantlets of *Q. rubra*.

Materials and methods

Plant material

Immature acorns (genotype # 45; year 2 maturation) were gathered mid-July 2006 from three NRO trees located at the Vallonia Nursery, Indiana Department of Natural Resources, Vallonia, IN, USA. Because of limited quantities and viability of sound acorns, the acorns from all trees were pooled in order to have enough zygotic embryos for experimentation.

Explant preparation and culture conditions

Immature acorns were washed vigorously in 1% (w/v) Alconox detergent (Alconox, Inc., White Plains, NY) for 20 min followed by three rinses with sterile, deionized water. Acorns were then immersed in 70% (v/v) ethanol for 1 min followed again by three rinses with sterile, deionized water. The acorns were then surface disinfected in 25% (v/v) bleach solution (5.25% sodium hypochlorite; Champion Packaging, Inc., Woodridge, IL) containing three drops of Tween® 20 (P-7949, Sigma-Aldrich, St. Louis, MO) for 20 min, and then finally rinsed three times with sterile, deionized water. After removing the pericarp aseptically, the completely filled immature cotyledons were excised, cut into small pieces (5 mm in size), and were used as the initial explant source to induce somatic embryos.

Induction of somatic embryogenesis

Explants were placed in Petri dishes (100 × 20 mm) containing 25 ml of modified Murashige and Skoog (Murashige and Skoog 1962) medium (MS; M499, *Phyto*Technology Laboratories, Shawnee Mission, KS) supplemented with 3% (w/v) sucrose, 0.24% (w/v) Phytagel™ (P-8169, Sigma-Aldrich, St. Louis, MO), 500 mg l^{-1} casein hydrolysate, 2,4-D (0, 0.04, 0.45, 2.3, or 4.5 μM) alone or in combination with BA (0, 0.44, or 4.4 μM), and the dishes sealed with Parafilm® M (Pechiney Plastic Packaging, Menasha, WI). These cultures were maintained in darkness at 25 ± 2°C for 4 weeks, and additional control cultures were maintained under a 16 h light photoperiod (50 μmol m^{-2} s^{-1}; cool-white fluorescent tubes) at 25 ± 2°C. All the cultures were transferred to fresh MS supplemented with 3% sucrose, 0.24% Phytagel™, devoid of growth regulators, and maintained under the same two conditions. All media were autoclaved at 121°C for 20 min after adjusting the pH to 5.7 with 0.1 N NaOH. Twenty-five explants were cultured per treatment with five explants per Petri dish. Control cultures were also initiated on MS devoid of any growth regulators. Cultures were observed weekly and data recorded. The experiment was replicated three times.

Germination and conversion of somatic embryos

Somatic embryos, developed to the cotyledon-stage, were gently separated from the original explant and cultured on MS supplemented with 3% sucrose, 0.24% Phytagel™, devoid of growth regulators in darkness for 2 weeks at 25 ± 2°C. After 2 weeks embryos were placed horizontally in small empty Petri dishes (60 × 15 mm) for desiccation. Five embryos were placed per dish and kept in a desiccation chamber. Desiccation conditions [50.5% relative humidity (RH)] were attained by placing 50 ml super-saturated $Ca(NO_3)_2 \cdot 4H_2O$ solution in small plastic containers at the bottom of the desiccation chamber. The RH was monitored with a meter (Traceable Hygrometer/Thermometer, Fisher Scientific, Pittsburgh, PA), the desiccators were sealed with Parafilm®, and maintained in darkness for 3 or 7 days at 25 ± 2°C. After 3 or 7 days desiccation, embryos were transferred to MS supplemented with 3% sucrose, 0.24 Phytagel™, devoid of growth regulators, and maintained in cold storage at 4°C in darkness for 8 weeks. Embryos not subjected to desiccation and cold storage served as controls. Twenty-five cotyledon-stage embryos from a single embryogenic line were used for desiccation and cold storage treatments, and the experiment was replicated three times. After desiccation and cold storage, somatic embryos were transferred to Petri dishes (100 × 20 mm) containing 25 ml of MS supplemented with 3% sucrose, 0.24 Phytagel™ without growth regulators. The somatic embryo cultures were maintained under a 16 h photoperiod as mentioned previously for 2 weeks. Cotyledonary-stage embryos subjected for desiccation and cold treatment from each replicate were then transferred to GA-7 Magenta boxes (Magenta Corp., Chicago, IL) containing 50 ml MS supplemented with 3% sucrose, 0.24 Phytagel™, and 0.44 μM BA alone and in combination with 0.29 μM gibberellic acid (GA_3), and maintained under a 16 h photoperiod as mentioned above. MS devoid of growth regulators served as control treatment. The experiment was replicated three times.

Transplantation of plantlets

Germinated embryos with a developed shoot (~10 mm height) and root (~30 mm length) were removed from the medium. The roots were gently washed under running tap water and the plantlets transferred to plastic pots (10 × 9 cm) containing autoclaved, moistened ProMix HP (Premier Horticulture, Inc., Quakertown, PA) for acclimatization. The potted plants were placed in 3.8 l zip-lock plastic bags to provide a high relative humidity. The plantlets were maintained in a growth room under a 16 h photoperiod at 25 ± 2°C, and the plants were watered every other day. Plantlets were gradually acclimatized (over a period of 4–6 weeks) 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, embryo formation, and germination were subjected to analysis of variance and significant ($P < 0.05$) means were determined with Tukey test to distinguish differences between treatment means at the $\alpha = 0.05$ level using SAS software (SAS Institute Inc 2001).

Results and discussion

Induction of somatic embryogenesis

Immature cotyledon explants cultured on MS supplemented with 3% sucrose, 0.24% Phytagel™, and various concentrations of 2,4-D became swollen and produced compact white to yellowish embryogenic callus when cultured in darkness. Callus initiation occurred from the cut ends of the explants within 3 weeks of culture in darkness. Embryogenic callus yielded globular-, heart-, torpedo-, and cotyledon-stage somatic embryos when transferred to MS containing 3% sucrose, 0.24% Phytagel™, and devoid of growth regulators after 8 weeks of culture in darkness. Explants cultured on MS containing 0.45 μM 2,4-D yielded a higher percentage (66 ± 3.8) of embryogenic callus (Table 1) after 4 weeks of culture in darkness (Fig. 1a). Embryogenic callus obtained on 0.45 μM 2,4-D when transferred to MS devoid of growth regulators yielded a higher number of globular embryos (31 ± 4.1; Fig. 1b). Globular embryos developed into heart-stage embryos (17 ± 4.0; Fig. 1c), and these embryos further developed into late heart-stage (Fig. 1d), into torpedo-stage embryos (12 ± 1.7; Fig. 1e), and finally into normal mature white to yellowish opaque cotyledon-stage embryos (8 ± 2.1; Fig. 1f). The percentage of explant response and the number of somatic embryos per explant was highest at 0.45 μM 2,4-D (Table 1) for the various concentrations of 2,4-D tested. Increasing or decreasing 2,4-D concentration resulted in a decline for both explant response and number of embryos per explant (Table 1).

Gingas and Lineberger (1989) reported induction of normal polar embryoids from immature zygotic embryos of NRO when explants were cultured for 2 weeks on a modified MS medium devoid of growth regulators or with 0.05 μM 2,4-D in combination with 0, 0.04, or 0.44 μM BA and cultured under 16 h light conditions. Embryoids also developed on medium containing 4.52 μM 2,4-D and 4.44 μM BA, but the highest embryoid numbers developed

Table 1 Effect of 2,4-dichlorophenoxyacetic acid (2,4-D) on somatic embryo induction from immature cotyledon explants of northern red oak

2,4-D (μM)	Embryogenic callus response (%)	Number of embryos per explant per developmental stage			
		Globular	Heart	Torpedo	Cotyledon
0.00	11 ± 1.7 d	5 ± 0.9 c	3 ± 0.6 c	2 ± 0.6 bc	1 ± 0.3 b
0.04	45 ± 3.2 b	20 ± 3.5 b	11 ± 3.2 b	7 ± 2.3 bc	4 ± 1.2 b
0.45	66 ± 3.8 a	31 ± 4.1 a	17 ± 4.0 a	12 ± 1.7 a	8 ± 2.1 a
2.30	29 ± 2.9 c	15 ± 2.0 bc	8 ± 1.2 b	5 ± 1.2 bc	3 ± 0.9 b
4.50	19 ± 2.1 cd	7 ± 1.4 c	4 ± 1.2 c	3 ± 0.9 bc	2 ± 0.9 b

Column means followed by different letters differ significantly according to Tukey's test. $\alpha = 0.05$. Cultures were initially maintained in darkness for 4 weeks and then transferred to growth regulator-free medium and maintained in darkness. Twenty-five explants were used for each treatment and the experiment was repeated three times. Mean values ± SE

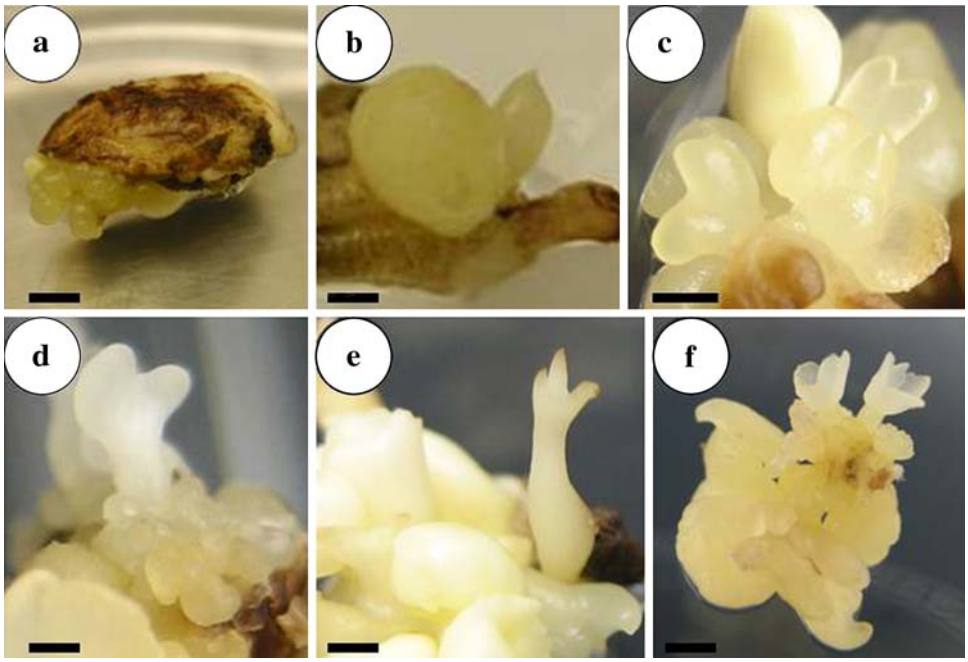

Fig. 1 Various developmental stages of northern red oak somatic embryos induced from immature cotyledons. **a** Embryogenic callus obtained from immature cotyledon explant on MS medium containing 0.45 μM 2,4-D after 4 weeks culture in darkness (bar = 1.5 mm). **b** Globular-stage embryo (bar = 1 mm). **c** Heart-stage embryo (bar = 2.5 mm). **d** Late heart-stage embryo (bar = 2.5 mm). **e** Torpedo-stage embryo (bar = 2.5 mm). **f** Cotyledon-stage embryo (bar = 5 mm)

from explants cultured in 16 h light on modified MS without plant growth regulators. Rancillac et al. (1996) obtained somatic embryos of NRO with 5.4 μM NAA and 0.09 μM BA using leaf disc explants cultured in the light. In contrast to these results, in the present study addition of BA along with 2,4-D produced neither embryogenic callus nor somatic embryos (data not shown). In the present study, continuous culture of embryogenic callus on MS medium containing 2,4-D produced only compact, yellowish callus, and control cultures maintained under a 16 h light photoperiod yielded brownish, yellow, compact callus, and both types of callus were non-embryogenic. Gingas (1991) reported that MS medium containing 4.52 μM 2,4-D induced yellow callus, but failed to yield somatic embryos in NRO when male catkins were used as explants. In NRO the requirement of BA to induce somatic embryos may depend on the type of explant, genotype, plant growth regulators, and induction culture conditions (16 h light vs. dark).

A significantly higher percentage of globular-, heart-, and torpedo-stage embryos were obtained on medium [basal medium comprised of macronutrients of Sommer et al. (1975) and micronutrients of MS] supplemented with 2.26 and 4.5 μM 2,4-D under a 16 h photoperiod in *Q. suber* (Bueno et al. 1992). Manzanera et al. (1993) reported that 2,4-D was the best growth regulator to induce embryogenic callus from hypocotyls of immature zygotic embryos of *Q. suber* compared with BA and NAA combination, and somatic embryo development occurred on growth regulator-free medium. The concentration of 2,4-D specifically at 2.3 and 4.5 μM proved effective to induce embryogenesis using zygotic embryos as explants in *Q. suber* and *Q. robur* (Manzanera 1992; Manzanera et al. 1993). Sánchez et al. (2005) stated that 2.3 μM 2,4-D induced somatic embryos

from immature zygotic embryos of *Q. suber*, and further embryo development occurred on growth regulator-free medium at 16 h light photoperiod. Our study revealed that a lower concentration of 2,4-D (0.45 μM) induced a higher percentage of embryogenic callus, and somatic embryos then occurred on growth regulator-free medium in the dark. Medium containing either low concentration of plant growth regulators or devoid of plant growth regulators favor unicellular origin of somatic embryos and decreases the possibility of somaclonal variation (Wilhelm 2000). The role of 2,4-D in cell division and embryogenic potential was reported by Lelu and Bornman (1990). In contrast to our observations, immature zygotic embryos collected during July and August and cultured in darkness on MS or woody plant medium (WPM) (Lloyd and McCown 1980) containing 1.36–9.05 μM 2,4-D was ineffective to induce embryogenic callus in *Q. robur* (Chalupa 1990), but embryos showing a bipolar structure were developed in the presence of 4.44 μM BA or in combination with 2.89 μM GA_3. Immature embryos of *Q. acutissima* harvested 6 weeks post-fertilization yielded embryogenic callus on modified MS medium supplemented with 2.0 μM indole-3-butyric acid (IBA) or 4.4 μM BA and 2.0 μM IBA, and somatic embryos were induced on growth regulator-free MS medium under a 16 h photoperiod (Kim et al. 1994). Kim et al. (1997) reported that immature embryos cultured on MS medium supplemented with 4.4 μM BA and 4.1 μM IBA yielded a higher frequency (91.2 and 90.9%) of somatic embryos under light cultivation (16 h photoperiod) in *Q. acutissima*.

In the present study, explants cultured on MS supplemented with 2,4-D plus BA in darkness produced only friable, whitish-brown callus and were non-embryogenic. Somatic embryos were obtained only on MS medium supplemented with 0–4.5 μM 2,4-D, whereas Gingas and Lineberger (1989) obtained somatic embryos of *Q. rubra* using immature zygotic embryos cultured on modified MS medium containing various combinations of 2,4-D and BA, with the highest number of embryos induced on plant growth regulator-free MS medium under a 16 h light photoperiod. Zygotic embryos cultured on modified WPM supplemented with 0.5–5.0 μM BA and 2.5–10.0 μM 2,4-D developed somatic embryos in *Q. petraea* (Jörgensen 1993). Similarly, 2,4-D and BA were used to initiate somatic embryos from immature and mature zygotic embryos in other *Quercus* species (Hübner et al. 1995; Manzanera et al. 1996; Endemann and Wilhelm 1999). Kim et al. (1994) reported that the addition of 4.4 μM BA was beneficial to induce embryogenic callus using immature embryo explants of *Q. acutissima*, however our study revealed that BA was not essential to induce embryogenic callus in *Q. rubra*.

Quercus rubra stem, leaf petiole, and leaf disc explants were used to induce somatic embryos, and embryos with cotyledons and a root pole were obtained only from leaf discs cultured on MS medium supplemented with 5.4 μM NAA and 0.09 μM BA after 6–8 weeks under a 16 h photoperiod without subculture, but the embryos failed to undergo apical bud elongation (Rancillac et al. 1991). Rancillac et al. (1996) also achieved somatic embryogenesis from leaf discs collected from juvenile NRO plants on MS medium supplemented with 5.4 μM NAA and 0.09 μM BA enriched with casein hydrolysate, and reported that light was necessary for somatic embryogenesis. Similarly, NAA and BA were used to induce somatic embryos using leaf explants of mature trees and seedlings in other *Quercus* species (Fernández-Guijarro et al. 1995; Cuenca et al. 1999; Hernández et al. 2003a; Valladares et al. 2006). In the present study, embryos developed in darkness and proved that light was not essential for somatic embryogenesis in this species. Gingas (1991) cultured catkins in continuous light after 48 h dark incubation to induce somatic embryos, but obtained only callus. In other *Quercus* species the combination of an auxin and BA was used to induce somatic embryos from immature and mature zygotic embryos (see review Wilhelm 2000) and a combination of BA and GA_3 induced somatic embryos in *Q. robur* (Chalupa 1987). From the present study, BA and the requirement of light was not essential to induce somatic embryos of NRO, and the variation of growth regulator type and concentration, basal medium formulation, and culture conditions could depend on the tissue of origin used as explant source, genotype, and the nature or type of explant for *Quercus* species. Genotype and harvesting time significantly affected somatic embryo induction in *Q. suber* (Hernández et al. 2003a). Similarly, Cuenca et al. (1999) reported variation among genotypes on induction frequency of somatic embryogenesis in *Q. robur*. The collection date of explants and seed family influenced somatic embryo frequency using immature embryos as explant source for *Q. acutissima* (Kim et al. 1997). The frequency of initiating somatic embryos was associated with zygotic embryo developmental stage (Wilhelm 2000). The present investigation demonstrated that immature cotyledon explants, gathered mid-July and cultured in the dark on MS medium supplemented with only 2,4-D, were capable of inducing embryogenic callus and somatic embryos developed after transfer to plant growth regulator-free medium. Similarly, seasonal effect on production of somatic embryos in *Q. suber* was reported by Bueno et al. (1992) where immature embryo explants gathered in August produced a higher frequency of somatic embryos.

Germination and conversion of somatic embryos

Cotyledon-stage embryos subjected to desiccation treatment for 7 days became brown and did not survive in cold storage. Embryos desiccated for 3 days and subjected to cold storage for 8 weeks (cultured on MS devoid of growth regulators) turned opaque white. Cotyledon-stage embryos not subjected to desiccation and cold storage treatment (control cultures) failed to germinate and re-callused.

After cold storage to break dormancy, embryos cultured on MS devoid of growth regulators under a 16 h photoperiod, increased in size and the cotyledons developed anthocyanins and the root started to elongate and was white (Fig. 2a). Two weeks after culture (after cold storage), embryos transferred to MS medium supplemented with 3% sucrose, 0.44 μM BA and 0.29 μM GA_3 germinated, and a normal shoot and root developed resulting in a higher germination frequency (61% ± 2) which was significantly higher ($P > 0.05$) than 0.44 μM BA alone (24% ± 2.1) or medium devoid of growth regulators (control) (8.6% ± 2.3) (Fig. 3). Germination occurred with the emergence of a primary green shoot and a white root, and simultaneous growth of leaves and root occurred with the gradual elongation of the hypocotyl (Fig. 2b). Generally a long primary root without root hairs developed. The embryos developed true shoots with new leaves and roots (Fig 2c). Embryos

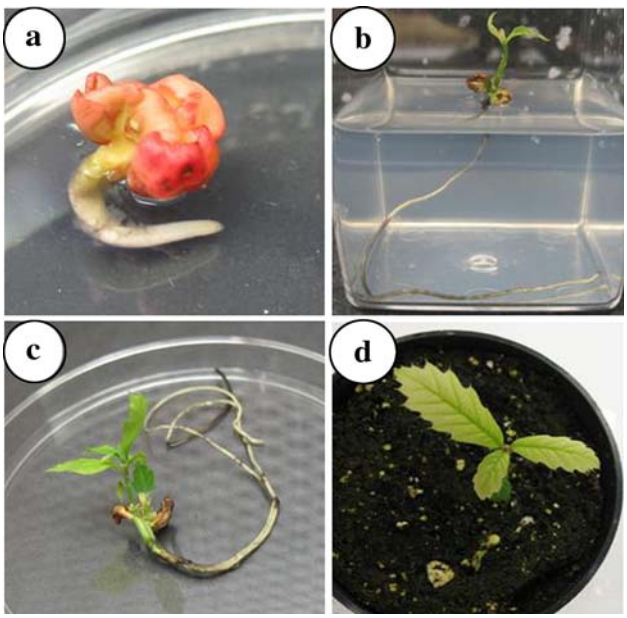

Fig. 2 Germination of northern red oak cotyledon-stage embryo into a plantlet. **a** Somatic embryo germination on MS containing 3% sucrose, 0.24% Phytagel™, and devoid of growth regulators after 2 weeks culture at 16 h photoperiod. **b** Embryo germination on MS medium containing 3% sucrose, 0.24% Phytagel™, 0.44 μM BA, and 0.29 μM GA_3 at 16 h photoperiod. **c** Germinated embryo with normal shoot and root. **d** Acclimatized plantlet

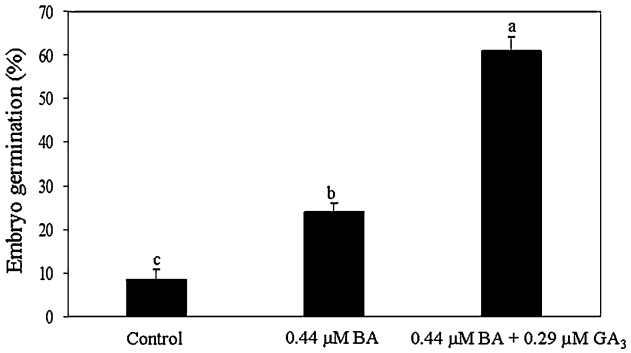

Fig. 3 Germination response of northern red oak cotyledon-stage embryos. White-yellowish opaque cotyledon-stage embryos were cultured on MS supplemented with 3% sucrose, 0.24% Phytagel™, and either BA (0.44 μM) alone or in combination with GA_3 (0.29 μM). Twenty-five embryos were used per treatment and the experiment was repeated three times. Vertical bars indicate the standard error of the mean for three experiments

germinated on medium supplemented with 0.44 μM BA and devoid of GA_3 did not continue to develop further.

Cork oak somatic embryos subjected to cold treatment at 4°C for 2 months in darkness on Schenk and Hildebrandt (1972) macronutrients and MS micronutrients yielded higher germination rates (80–100%) (García-Martín et al. 2001). Bueno et al. (1992) reported that 3.78 μM abscisic acid (ABA) stimulated somatic embryo maturation in *Q. suber*, and cold storage at 5°C in darkness for 10 weeks was the best treatment to break dormancy of somatic embryos. Manzanera et al. (1993) stated that cold storage treatment at 5°C in darkness for 10 weeks and 2 weeks at 2°C induced germination of somatic embryos in *Q. suber*, and desiccation by the addition of 0.3 or 0.7 M sorbitol completely hampered the germination of somatic embryos. Similarly, cold treatments prior to germination were used to germinate somatic embryos of walnut (Deng and Cornu 1992). Fernández-Guijarro et al. (1995) reported that the duration of cold storage was important for both improving germination percentage and synchronized germination of somatic embryos. Desiccation and chilling treatments have been reported to stimulate the accumulation of storage products for post-embryogenic development (Wilhelm 2000). Tang et al. (2000) and Pond et al. (2002) reported that the quality of germinated embryos improved after desiccating somatic embryos prior to germination. In *Q. robur*, epicotyl dormancy of somatic embryos was broken by chilling treatment (Manzanera et al. 1993).

In accordance with our results, Kim et al. (1997) and Sasaki et al. (1988) reported that BA and GA_3 were effective for epicotyl formation and shoot elongation, respectively, for *Q. acutissima*. Kim et al. (1994) achieved embryo germination on WPM containing 0.44 μM BA and the addition of 0.4 μM ABA and 0.29 μM GA_3 to MS

medium favored a higher germination frequency in *Q. acutissima* than an osmoticum treatment. MS liquid medium containing 0.04 μM BA promoted shoot elongation and development of the radicle for *Q. suber* somatic embryos (González-Benito et al. 2002). Valladares et al. (2006) used 0.44 μM BA for germination of somatic embryos of *Q. robur*. The influence of BA on germination and conversion of somatic embryos has been reported for several other *Quercus* species (see review Wilhelm 2000). It was obvious from our study that the combination of BA and GA_3 favored simultaneous development of both the shoot and root and yielded a higher germination frequency for *Q. rubra* somatic embryos. In our study, approximately 61% ± 2.8 of the embryos germinated and developed normal shoots and roots on MS containing BA and GA_3 under a 16 h photoperiod, and embryos grown on MS supplemented with only 0.44 μM BA did not favor shoot elongation. Both BA and GA_3 promoted shoot elongation in somatic embryos of *Q. acutissima* (Kim et al. 1992). One micromolar BA induced both shoot and root development for *Q. suber* somatic embryos, and the conversion rate was 15% (Fernández-Guijarro et al. 1995). Plant conversion percentage was also improved by increasing sucrose concentration and addition of ABA in *Q. robur* (Cuenca et al. 1999) and *Q. suber* (García-Martín et al. 2001).

Somatic embryos cultured on MS medium with a high concentration of sorbitol (0.6 M) and BA (0.88–2.7 μM) increased germination frequency in *Q. robur* (Chalupa 1990). Germination of somatic embryos in *Q. rubra* was achieved by desiccation with either sorbitol or by air drying (Gingas and Lineberger 1989). Rancillac et al. (1996) reported very low frequency of embryo maturation and germination derived from leaf discs on growth regulator-free media after four to five subcultures for *Q. rubra*. Somatic embryos cultured in sorbitol supplemented maturation medium enhanced the conversion frequency in *Q. robur* (Sánchez et al. 2003). Various somatic embryo cell lines for *Q. robur* exhibited different germination response as a result of the varied genetic background of diverse families (Wilhelm et al. 1999). The present study showed that desiccation followed by cold storage enhanced germination of *Q. rubra* somatic embryos.

Transplantation of plantlets

Germinated plantlets with a normal shoot and root grown on MS medium containing 0.44 μM BA and 0.29 μM GA_3 were transplanted after 3–4 weeks in culture into a potting mix (Fig. 2d) and were maintained under lights in the growth room in the lab. The transplanted plants appeared normal and continued to grow showing the development of new leaves.

Conclusion

In the present study, a protocol was developed for somatic embryogenesis and plant regeneration for NRO. Higher percentage of embryogenic callus was initiated on MS medium containing a low concentration of 2,4-D (0.45 μM), and globular-, heart-, torpedo- and cotyledon-stage somatic embryos occurred on growth regulator-free medium. Addition of GA_3 with BA was essential for a higher rate of embryo germination. Desiccation and cold treatment conditions in this investigation favored higher germination frequency (61%) than previous reports in this species (Wilhelm 2000). This embryogenesis system offers an avenue for genetic manipulation studies of NRO, and somatic embryos (Fig. 2a) could possibly be encapsulated to use as artificial seeds as reported for *Q. serrata* (Ishii et al. 1999) and *Q. robur* (Wilhelm et al. 1999) for mass propagation forestry. A NRO genetic transformation system will provide a platform to accelerate an oak tree improvement program (Shekhawat et al. 2008).

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