Direct Somatic Embryogenesis in Northern Red Oak (*Quercus rubra* L.)

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Abstract

Somatic embryo cultures of *Quercus rubra* L. were initiated from cotyledon explants of immature zygotic embryos. Acorns collected mid-July through August responded well. Embryogenic cultures were initiated, and globular and heart-shaped embryos were obtained on Murashige and Skoog (1962) (MS) medium supplemented with 4.4 µM BA + 2.9 µM GA_3, or 2.3 µM 2,4-D or 4.5 µM 2,4-D + 4.4 µM BA with 200 mg/l L-glutamine after 8 weeks culture in darkness. Embryos developed directly from the explants without callus development. Heart-shaped embryos developed into normal mature embryos with greenish cotyledons, apical bud, and root on MS medium supplemented with 4.4 µM BA + 2.9 µM GA_3 with 200 mg/l L-glutamine and the percentage of embryo development was low (4%). Heart-shaped embryos developed into abnormal embryos with petal-like lobes or bell-shaped formation on MS medium with 2.3 µM 2,4-D or 4.5 µM 2,4-D + 4.4 µM BA. MS medium containing 4.5 µM 2,4-D + 4.4 µM BA with 200 mg/l L-glutamine induced the maximum number of abnormal embryos per explant. MS medium without growth regulators failed to produce embryogenic tissue and somatic embryos. Prolonged culture of abnormal embryos induced the development of secondary embryos. Experiments on the germination and conversion of embryos into plants are in progress.

Introduction

Species of *Quercus* have a wide distribution throughout the Northern Hemisphere with considerable ecological and economic values. The genus comprises nearly 400 species. Economically, oaks are the major sources of timber for flooring, furniture, and cabinetry in the US (Merkle and Nairn, 2005). Both conventional vegetative propagation and biotechnological methods have 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). Seeds also have very poor viability and are attacked by insects (*Curculio* spp.) and pests prior to collection and during storage (Gingas and Lineberger, 1989). Further, mature trees are also recalcitrant to in vitro regeneration. One of the ways to overcome these problems is through using an in vitro culture method involving somatic embryogenesis along with cryopreservation. The main advantages of this system of regeneration includes mass propagation of elite forest oak genotypes, high multiplication rates, scale-up for large-scale production, and direct transfer to the field or greenhouse through artificial seeds.

Though the above method holds promising outcomes for the propagation of oak genotypes several problems such as poor regeneration ability of mature explant material, physiological status of the explants, tissue genotypes, and low conversion efficiencies exist. Both juvenile and mature explant sources have been used for the initiation of somatic embryos in *Q. rubra*. The most commonly used explants were the immature and mature zygotic embryos. Frequency of somatic embryo production is 87% when using these explants in the presence of 2,4-D (4.5 µM) and BA (4.4 µM). However, the highest percentage of somatic embryos was obtained on plant growth regulator (PGR) free medium. Tissues included leaf discs (from 6 to 8 week-old-seedlings) (Rancillac et al., 1991; 1996) and male catkins (Gingas, 1991). In both studies, the frequency of embryo production was 0 to 56% and 20%, respectively.

Objectives

The present study was undertaken to induce proliferating embryogenic cultures and somatic embryos from cotyledons of stock somatic embryos of *Q. rubra* of several genotypes using several growth regulators and additives. Explants were tested under varies light conditions, PGRs, and media formulations for the production of somatic embryos.

Materials and Methods

The cotyledons from somatic embryo cultures (Bosela and Michler, 2000) were separated and cut into small pieces (5 mm in size) and cultured in 100 x 20 mm petri dishes containing 25 ml of MS medium supplemented with 2,4-D (0.04, 0.45, 2.3, 4.5, 6.8, 9.1 µM), NAA (0.54, 2.7, 5.4, 8.1, 10.7 µM), BA (0.04, 0.44, 2.2, 4.4 µM), GA_3 (0.7, 1.4, 2.1, 2.9, 4.4, 5.8 µM) and 200 mg/l L-glutamine, 3% sucrose and 0.24% Phytagel (Gellan gum, Sigma, St. Louis, MO, USA). The medium was autoclaved at 121ºC at 15 lbs pressure after adjusting the pH to 5.7 with 0.1N NaOH. Explants were sub-cultured to fresh treatment medium at 3-week-intervals. Twenty-five explants were cultured per treatment and five explants were cultured per petri plate. The cultures were maintained in darkness. Control cultures were also initiated without PGRs and L-glutamine. Cultures were observed weekly and data recorded.

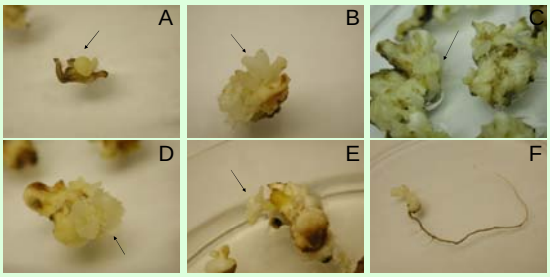

Figure 1. Direct somatic embryogenesis in *Quercus rubra* L. **A** Globular & **B** heart-shaped somatic embryos (arrow) produced on MS medium supplemented with 2,4-D 2.3 µM or 2,4-D 4.5 µM + BA 4.4 µM, or BA 4.4 µM + GA_3 2.9 µM along with 200 mg/l L-glutamine after 8 weeks of culture in darkness. **C** Normal somatic embryos (arrow) with light greenish cotyledons, apical bud, and radicle obtained on MS medium with 1.0 mg/l BA and 1.0 mg/l GA_3 along with 200 mg/l L-glutamine after 12 weeks culture in darkness. **D** Abnormal embryos with petal-like lobes or bell-shaped formation (arrow) obtained on MS medium containing 2,4-D 2.3 µM or 2,4-D 4.5 µM + BA 4.4 µM along with 200 mg/l L-glutamine after 12 weeks culture in darkness. **E**. Embryo loosely attached to the surface of cotyledon explant. **F** Matured somatic embryo with well developed root.

Table 1. Effect of 2,4-D and BA on northern red oak somatic embryo production.

PGRs (µM)		Explant response[y,z]	Average number of embryos per explant
2,4-D	BA		
0	0	0	0
0.45	0	0	0
2.3	0	1	3
4.5	0	0	0
6.8	0	0	0
0.04	0.04	10	2
0.45	0.44	20	3
2.3	0.44	0	0
4.5	0.44	0	0
6.8	0.44	0	0
0.45	2.2	0	0
2.3	2.2	2	1
4.5	2.2	0	0
6.8	2.2	0	0
0.45	4.4	0	0
2.3	4.4	0	0
4.5	4.4	55	7
6.8	4.4	0	0
0	2.2	0	0
0	4.4	20	1

[y] Cotyledon explants of somatic embryos were cultured in MS medium supplemented with various concentrations and combination of 2,4-D, BA, and 200 mg/l L-glutamine to produce somatic embryos in darkness.
[z] Five explants were cultured per plate and twenty-five explants were used per treatment.

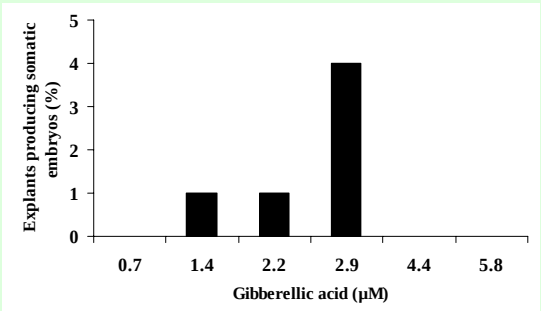

Figure 2. Effect of BA, and GA_3 on embryo production: Cotyledon explants of somatic embryos were cultured on MS medium supplemented with various concentrations of GA_3 plus 4.4 µM BA along with 200 mg/l L-glutamine to produce proliferating somatic embryos. Five explants were cultured per plate and twenty-five explants were used per treatment.

Conclusion

Cotyledons of *Q. rubra* somatic embryos have a capacity for somatic embryogenesis when cultured under various growth regulator conditions. Experiments performed for the induction of somatic embryogenesis in *Q. rubra* proved the requirement for both BA and GA_3 for the induction of embryogenic tissue and development of normal somatic embryos in darkness. Addition of GA_3 with BA was essential for normal embryo development. In the presence of 2,4-D or 2,4-D + BA, several abnormal developmental stages of somatic embryos could be observed simultaneously in culture.

Results and Discussion

Cotyledon explants from somatic embryos produced embryogenic tissue. After 4 weeks culture on MS medium supplemented with BA and GA_3, 2,4-D, or 2,4-D and BA, along with 200 mg/l L-glutamine cotyledons became swollen and turned white. Globular (Fig 1A) and heart-shaped (Fig 1B) somatic embryos developed directly on the cultured explants after 6 weeks and no callus production occurred. Heart-shaped embryos produced normal, light-green cotyledonary stage embryos with two cotyledons, apical bud, and radicle at a low frequency (4%) on MS medium supplemented with 4.4 µM BA, 2.9 µM GA_3 with 200 mg/l L-glutamine (Fig 1C; Fig 2) after 12 weeks in darkness. Addition of 2.9 µM GA_3 along with BA was reported to produce somatic embryos in *Q. robur* (Chalupa, 1990).

Explants cultured on MS medium containing NAA, NAA and BA, or without PGRs were non-embryogenic without somatic embryo production. Previously in *Q. rubra* leaf disc explants produced organogenic callus on MS medium containing 5.4 µM NAA and 0.09 µM BA which produced somatic embryos after 6 to 8 weeks at 16 h photoperiod without subcultures, however the embryos failed to undergo apical bud elongation (Rancillac et al., 1991). Gingas and Lineberger (1989) reported production of normal somatic embryos from immature zygotic embryos (collected 4 to 7 weeks post-fertilization) when cultured in growth-regulator-free medium. Rancillac et al. (1996) also achieved somatic embryogenesis from leaf discs collected from juvenile red oak plants on MS medium supplemented with NAA and BA enriched with casein hydrolysate, and reported that light is necessary for somatic embryogenesis. In contrast in the present study, embryos developed after incubation in darkness. This variation may be a result of the genotype and the nature or type of explant (Cotyledons of somatic embryos were used in our study).

On MS medium containing 2.3 µM 2,4-D or 4.5 µM 2,4-D + 4.4 µM BA along with 200 mg/l L-glutamine heart-shaped embryos developed into abnormal embryos with petal-like lobes or bell-shaped structures (Fig 1D). This medium resulted in the highest number of embryogenic cultures after 8 weeks in darkness. The number of somatic embryos per explant (7 embryos) and the percentage response (55%) was highest on MS medium with 4.5 µM 2,4-D + 4.4 µM BA and 200 mg/l L-glutamine (Table 1). Abnormal embryos were found distributed on the surface of the explants and the embryos were loosely attached to the original tissue at the root pole (Fig 1E). Prolonged culture of abnormal embryos led to the development of secondary embryos. Similar poly-cotyledonary, fused embryos, or other abnormalities were commonly observed during somatic embryo development in other *Quercus* species (Wilhelm, 2000). Gingas and Lineberger (1989) observed similar types of abnormal embryos in northern red oak and reported that the same concentrations of 2,4-D and BA generated the highest number of embryos when immature zygotic embryo explants were cultured under a 16 h photoperiod. It was observed in the current study that 2,4-D alone was ineffective for stimulation of high frequency somatic embryogenesis. Embryos were not observed in other concentrations of 2,4-D tested. Gingas (1991) reported that 2,4-D failed to produce embryogenic callus and somatic embryos in this species when catkins were used as explants. The total number of abnormal embryos per explant was higher compared to the total number of normal somatic embryos per explant.

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