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Asexual embryogenesis and plant regeneration in *Quercus*

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The Ohio State University, 1988

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ASEXUAL EMBRYOGENESIS AND PLANT REGENERATION
IN QUERCUS

DISSERTATION

Presented in Partial Fulfillment of the Requirements for
the Degree Doctor of Philosophy in the Graduate
School of The Ohio State University

By

Vicki Marie Gingas, B.S., M.S.

* * * * *

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1988

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CHAPTER I

INTRODUCTION

Economic Significance

Quercus species provide crops of major importance to forest and horticultural industries. In a 1983 Ohio survey, oaks led all other hardwoods in sawlog production yielding 165 million board feet or 52% of the total harvest (63). Quercus rubra L. (red oak) and alba L. (white oak) were top contributors. Veneer log production was dominated by white oak with 48% of the harvest followed by red oak with 14%. It is estimated that 40,000 red oaks are produced annually for use in commercial, municipal, and residential landscape situations (61).

Currently, oaks are chiefly produced by seedling propagation which poses several disadvantages. First, trees do not bear acorns until approximately 25 years of age and good harvests are possible only every 2 to 5 years (2). Second, seed viability can be low as mature acorns are prone to attacks of several insects and diseases prior to collection and during storage (1). Third, extensive genotypic and phenotypic variability results. In order to overcome poor uniformity, attempts have been made to

asexually propagate this species by conventional rooted cutting or grafting methods. Such efforts have been largely unsuccessful. Micropropagation of oaks has been attempted, but with limited plantlet regeneration (33,59).

As several woody species (walnut, tulip poplar, spruce, peach, apple, and filbert) have recently been regenerated via asexual embryogenesis, it seems possible that a protocol could be formulated for oaks (15,18,21,36,44,58). This would allow the establishment of large populations of genetically and phenotypically identical trees. It is the purpose of this research to determine the feasibility of utilizing such a system for the production of oaks.

The Origin of Asexual Embryos (Embryoids)

Asexual embryogenesis is defined as the process by which bipolar adventive embryos (embryoids) are produced from somatic cells of various tissues. Embryoids subsequently can be separated and grown into viable plantlets. This process was first described and studied in Daucus carota (52), but many herbaceous and woody genera have also been studied (57).

The exact origin of embryoids is the subject of much debate. Early work with carrot indicates that the process begins with a single totipotent cell (51). Recent studies on spruce concur (39). Others claim that embryoids arise from a multicellular complex (19). Neighboring cells

within a specific tissue function in concert with one another to form an aggregate which develops into an embryoid (32). Both single and multicellular origins have been identified in some plant species (32,42).

Regardless of the number of cells involved at initiation, it is well documented that embryogenic cells possess certain similar morphological and physiological characteristics. Cells are usually small, rich in cytoplasm, and contain large numbers of ribosomes and starch grains (64).

Cells that possess the ability to express totipotency are said to be "competent" (16). How cells become competent is uncertain. One theory suggests that asexual embryogenesis can follow one of two developmental pathways (49). First, if embryogenic cells are produced directly from explant tissue, then it is believed that these cells were predetermined for an embryogenic fate in a previous mitotic cell cycle. Such cells are called PEDC (pre-embryogenic determined cells). If exposed to proper stimulatory substances or if existing inhibitors are removed, the cells will continue embryogenic development (49). For example, when filbert cotyledons were cultured in an environment conducive to embryogenic development, cells in the external parenchyma gave rise to embryogenic cell groups which further developed into plantlets (43). Had the cotyledons not been exposed to an embryo-inducing

environment, these cells would have continued functioning as epidermal tissue. Studies with Trifolium repens have shown that after exposure to embryo-inducing substances, the pattern of division of hypocotyl epidermal cells is altered from regular anticlinal to irregular periclinal divisions. These cells subsequently gave rise to embryoids (32).

The second embryogenic path is taken if embryoids are produced from callus tissue produced from the explant. In this process, mature or differentiated cells are induced to form unorganized callus masses which contain embryogenic cells or cell complexes. Such cells are termed IEDC (induced embryogenic determined cells) (49). In this case, an inductive environment is required for re-entry of differentiated cells into the mitotic cycle. Inducers are also believed to be indirectly involved in the control of regulatory genes which determine the composition of newly synthesized RNAs (53). Thus, the fate of new callus cells is determined, some of which may be destined to undergo embryogenesis. Embryogenesis via IEDC has been demonstrated in many plant species (49). Regardless of whether embryoids results from PEDC or IEDC, their pattern of development into plantlets closely parallels that found in zygotic embryogenesis. One exception may be at the very early few-celled stage where the existence of a true suspensor has been questioned (64). Beyond this step,

however, continuity becomes apparent. Small clusters of tiny, cytoplasmically rich cells develop into definitive globular structures. Bipolarity becomes obvious during the heart stage as a distinct root axis and enlarging cotyledons are found. Many species then show elongation of the heart stage embryoid into the torpedo stage (17). However, this step is not evident in all plants (58). Finally, plantlet formation is achieved with elongation of the radicle and shoot development.

It is quite common for secondary and tertiary embryoid production to occur on the surface of primary embryoids (21,35,58). Such structures are thought to arise from single or multiple epidermal cells (64). Often, primary embryoids that are incapable of further development are most likely to produce secondary structures. This seems to be in agreement with what is known about the regulation of zygotic embryo development. If the young embryo does not receive signals for the termination of storage protein accumulation, the tissue remains embryonic and subsequent germinative stages will be greatly delayed (6). This same phenomenon may be occurring in asexual embryogenesis. It should be noted, however, that secondary and tertiary embryoids are capable of normal plantlet development (58).

Induction

In order to express totipotency, competent cells must be exposed to environmental factors that will stimulate development or overcome repressors to the process. This event is termed "induction." Some of the factors known to influence the induction and early development of embryoids are growth medium constituents, growth regulators and light.

Commonly utilized plant tissue culture media contain various sources and concentrations of mineral salts and organic compounds to sustain plant growth in vitro. Manipulating such constituents can have a profound impact on embryogenesis. Many plant species have been induced on high salt media such as the formulation of Murashige and Skoog (MS) or Schenk and Hildebrandt (SH) (3), as well as the moderate salt medium, WPM (35). Callus induction from immature embryos of Picea abies was poor on a modified White's solution, a low salt medium (14). Once induced, some species require a reduction in salt concentration for further development of embryoids (31,58).

In wild carrot, it was determined that nitrogen must be supplied, at least partially, in the reduced form in order for the embryogenic process to commence (62). Amino acids are somewhat effective in furnishing reduced nitrogen, but ammonium compounds supported higher rates of embryogenesis. Others claim that the reduced nitrogen requirement is

dependent on the presence of 2,4-D in the culture medium (22). High phosphate concentrations (55) and low potassium levels (49) were found to be inhibitory to wild carrot embryogenesis.

In most tissue culture systems, sucrose is supplied (usually at 3% w/v) as the required carbon source. However, various forms and concentrations of carbohydrates have been found to impact on embryogenesis. In Citrus, galactose and lactose were more stimulatory to embryogenesis than sucrose (26). Embryogenic callus formation from immature corn embryos was enhanced when sucrose levels were increased up to 12% (3).

Other organics such as malt extract, coconut milk, and casein hydrolysate have been identified as potential enhancers of embryogenesis. Neither coconut milk nor casein hydrolysate stimulated embryo induction in Coffea arabica (50). Malt extract inhibited embryogenesis in Malus (21), but exhibited positive effects as Citrus (11).

For most plant species, auxin is essential to the induction of embryogenesis. The auxin 2,4-D has been the most widely utilized, with concentrations typically ranging from 0.01 mg l⁻¹ (11) to 2.0 mg l⁻¹ (35,39). Other auxins such as indole-3-butyric acid (IBA) (44,58), naphthaleneacetic acid (NAA) (18,21), and picloram (30) have been instrumental in embryoid formation. Once induction has occurred, it is recommended that explant

tissue be removed from auxin completely (45) or at least transferred to reduced concentrations (18), as continued exposure can result in developmental abnormalities.

Some species apparently do not require auxin for induction. Embryoids successfully developed on Ilex aquifolium immature zygotic embryo tissue (19) as well as on nucellar and endosperm tissue of 50-day-old Malus species in the absence of any growth regulators (21).

Cytokinins have proved to be essential to induction in some species. Embryogenic callus formed from nucellar tissue of 30-day-old Malus seeds only in the presence of an auxin and BA (21). In Norway spruce, auxins supplemented with various cytokinins were more effective in inducing embryogenic callus than auxins alone (15). The effective range of cytokinins has been 0.1 to 1.0 mg l⁻¹ (18,40). On the other hand, cytokinins were not necessary for the formation of embryoids in chestnut (12) or Citrus (11).

Whether or not light is stimulatory or inhibitory to embryogenesis appears to be species-dependent. Malus (21) and walnut (58) were successfully regenerated in darkness. Spruce (15,40), Ilex (20), pecan (35), and chestnut (12) produced embryoids in the presence of light with intensities ranging from 10 to 70 uE m⁻² s⁻¹. Induction occurred when grape (28) and Coffea (50) were cultured in darkness on hormone-containing media and then transferred to basal media in light.

Germination

Once embryoids have reached the late heart or torpedo stages, they must be exposed to proper environmental conditions in order for germination (root and shoot development) to occur. As embryoid development closely parallels that of zygotic embryos, it is believed that factors affecting seed development and germination may regulate in vitro embryoid germination. In the seed, storage proteins are synthesized and accumulated in cotyledons of the developing embryo. At some point, gene expression is altered, storage protein levels decrease, and the embryo is triggered into a germinative rather than embryogenic mode (6). Desiccation followed by rehydration has been implicated as the environmental signal that results in the switch to germinative development in Brassica napus, Phaseolus vulgaris, and Ricinus communis (9,37). Absciscic acid was thought to be the regulating factor in the transition from embryogeny to maturity (7). In rapeseed, ABA levels may suppress germination early in seed development, but once the seed has reached the desiccation stage, only high ABA levels can block germination upon rehydration (10). In grape (Vitis longii 'Microsperma'), after 14 and 21 days of dehydrated storage, 28% and 20% of the embryoids, respectively, produced plantlets after rehydration on basal medium as compared with 5% of the controls (13).

In several woody species, seeds require a chilling treatment in order for germination to occur (1). For this reason, researchers have tried cold treating recalcitrant embryoids. For walnut embryoids, it was found that 8 to 10 weeks of 2 to 4° C encouraged epicotyl elongation (58). Grape embryoids treated at 4° C for 8 weeks exhibited a 94% germination rate compared to 2% of the control treatment (47).

Chilling has been thought to either stimulate the production of promoter substances such as gibberellins or reduce inhibitors such as ABA so that germination may proceed (27). Abscissic acid levels were higher in grape embryoids that were never cold-treated compared to those that were chilled (46). When ABA was applied to chilled grape embryoids, germination rates decreased (54). Working with the same species, it was found that chilling stimulated synthesis of gibberellic acid (GA) precursors and decreased the formation of GA conjugates (41). This would allow increased synthesis of free GAs which may positively influence germination.

Various forms of GA have been applied to embryoids to enhance germination. Apple embryoids were not stimulated by GA₃ applications, even though zygotic embryos were (21). Citrus embryoid germination was enhanced by GA₃ (11), although its effectiveness could be questioned as it was added to media prior to autoclaving. Gibberellic acid

applications substituted for chilling treatments of grape embryoids, although germination percentages were half that of the cold-treated embryoids (47).

Norway spruce embryoids were stimulated to form plantlets by the application of cytokinins (60). Application of this hormone to some woody plant seeds is known to overcome dormancy (27). The mechanism of this reaction is uncertain.

Removal of embryoids from explant tissue stimulated germination of Ilex (20) and grape (34) embryoids. It has been suggested that the explant produces an inhibitor to germination (56).

White spruce embryoids germinated when transferred to basal medium at 1/3 strength (30). Similar results were obtained with walnut embryoids when cultured on 1/2 strength basal medium (58).

CHAPTER II
INDUCTION OF ASEXUAL EMBRYOGENESIS AND EARLY
EMBRYOID DEVELOPMENT IN QUERCUS

Introduction

Experiments were conducted to assess the asexual embryogenic potential of plants within the genus Quercus. Explant source and stage of development, culture media, growth regulators, and the presence or absence of light were evaluated as to their influence on the induction and early development of embryoids. Results of first-year studies on red oak provided a preliminary understanding of the inductive process and factors that impact upon it. Such data enabled experimental refinement in second-year studies and prompted the examination of an additional species, white oak.

Materials and Methods

Experiments were conducted over 21 months (May 1986 to February 1988) which allowed collection of explant material over 2 growing seasons.

Year 1. Expanding internodes, immature leaves, unfertilized ovules, and developing zygotic embryo tissues

were used as explant material. Internodes and leaves were collected from a red oak tree south of Vivian Hall on The Ohio State University campus on May 9, 1986. Tissues were washed in a 0.5% Alconox solution for 20 minutes, transferred to a 0.5% sodium hypochlorite solution for 20 min, and rinsed twice with sterile distilled water. Internodes were cut into 1.0 cm segments and leaves into 1.0 cm² pieces. One-year-old immature acorns were collected on May 16 and 23, 1986. Involucres were removed and acorns were surface-sterilized in 0.5% sodium hypochlorite for 15 min and rinsed twice with sterile distilled water. Unfertilized ovules were extracted. From June 5 to August 20, 1986, developing acorns were collected from the same tree at weekly intervals. Involucres were removed and acorns were surface-sterilized in 0.5% sodium hypochlorite for 15 min and rinsed twice with sterile distilled water. From these acorns, embryo tissues of various developmental stages were extracted. Zygotic embryos collected 3-5 weeks post-fertilization were removed from the seed coat. Time of fertilization was defined as the point at which one ovule continued development and the other five began to degenerate. Those collected after this time were removed from the seed coat and halved so that each piece contained one cotyledon.

All explants were cultured in test tubes containing 12.5 ml of medium solidified with 0.66% Difco Bacto-agar.

Six medium treatments included Woody Plant Medium (WPM) with 3% sucrose and modified Murashige and Skoog (MS) medium containing 200 mg l⁻¹ casein hydrolysate and 3% sucrose. Each medium was supplemented with 1 mg l⁻¹ 2,4-D + 1 mg l⁻¹ BA, 1 mg l⁻¹ 2,4-D + 0.1 mg l⁻¹ BA, or 10 mg l⁻¹ 2,4-D + 0.1 mg l⁻¹ BA. After 4-5 weeks, all zygotic explants were transferred to WPM containing 0.1 mg l⁻¹ BA.

Ten explants per treatment per harvest date were cultured (a total of 60 internodes, 60 leaf pieces, 120 ovules, and 660 embryos or embryo pieces). Half of the explants were grown in darkness and half were illuminated for 16 hr day⁻¹ with cool white fluorescent bulbs providing 80 to 100 uE m⁻² sec⁻¹. All cultures were maintained at 24° C. After approximately 4 weeks, explants were transferred to fresh media. Later-stage zygotic explants grown in light were transferred 3 to 5 days after initial culturing due to medium discoloration.

Callus clumps that developed from embryoids were removed from solid media and placed in test tubes containing 1.5 ml of liquid modified MS medium (previously described) without growth regulators. Tubes were housed on a rotating wheel (2 rpm) under 50 uE m⁻² sec⁻¹ of continuous cool white fluorescent lighting. Callus cultures were transferred to fresh media every 4 weeks.

Data from the factorial experiment were analyzed using the SAS GLM procedure (48).

Year 2. Immature red oak embryos 2 to 5 weeks post-fertilization and white oak embryos approximately 7 weeks post-fertilization were collected and extracted as described for Year 1. All explants were cultured in test tubes containing 12.5 ml of the previously described modified MS medium. Red oak explants were subjected to the following growth regulator treatment combinations for 2 weeks under light conditions previously described: 1.0 mg l⁻¹ 2,4-D + 1.0 mg l⁻¹ BA, 0.1 mg l⁻¹ 2,4-D + 0.1 mg l⁻¹ BA, 0.01 mg l⁻¹ 2,4-D + 0.1 mg l⁻¹ BA, 0.01 mg l⁻¹ 2,4-D + 0.01 mg l⁻¹ BA, and 0.1, 0.01, and 0 mg l⁻¹ 2,4-D without BA. White oak explants were treated for 2 weeks in darkness (due to a phenolic buildup) on 1.0 mg l⁻¹ 2,4-D + 1.0 mg l⁻¹ BA and 0.1 mg l⁻¹ 2,4-D + 0.1 mg l⁻¹ BA. After initial treatments, all explants were transferred to the modified MS medium lacking growth regulators and grown in light. Embryoids and callus cultures derived from explants were transferred to 1/2 modified MS medium solidified with 0.3% Gelrite.

Results and Discussion

Year 1. Internode, expanding leaf, and ovular explants of red oak failed to produce embryogenic structures. A hard, dense, non-regenerative callus formed on the ends of

internode segments. A similar type of callus occurred on foliar tissue. However, after 2 months in culture, all foliar tissue became necrotic. A white or tan friable callus developed on ovular surfaces after 4 to 5 weeks in culture. This callus turned brown after several months in culture.

Obtaining ovular and zygotic explant tissue elucidated the embryogeny of the red oak seed. Studies on the fruit development of Q. rubra are not detailed (29), but this process closely paralleled that found in Q. velutina (black oak), a closely related species (38). As a member of the subgenus Erythrobalanus, red oak seed maturation occurred over a two-season period. Pollination occurred approximately early to mid May the first year and fertilization took place early to mid June the following season, as was previously reported by others (38). Just prior to fertilization, the ovary consisted of three locules, each of which contained 2 ovules (Plate I). Approximately 14 to 21 days post-fertilization, the embryo was a transparent heart-shaped structure with distinct cotyledons and root axis (Plate II). At 4 to 5 weeks of age, the embryo had consumed all endosperm, cotyledons greatly expanded and starch accumulation was apparent (Plate III). Until maturity, the cotyledons continued to increase in size, eventually surrounding the embryo axis.

Plate I. One-year-old red oak ovary
containing 6 ovules.

Plate II. Heart-stage red oak embryo
2-3 weeks post-fertilization.

Plate III. Late heart-stage red oak
embryo 4-5 weeks post-
fertilization.

High starch content resulted in an opaque appearance (Plate IV).

In first-year work with red oak, numerous embryoids were generated from immature zygotic embryo explants. Most embryoids were produced directly from cotyledon surfaces of the explant and began to appear after 3 to 4 weeks in culture (Plate V). Embryoids were off-white and nodular in shape, approximately 1.0 mm in diameter, similar to those found in pecan (35). Repetitive asexual embryogenesis was common in all cultures. Secondary and tertiary embryoids formed on the cotyledons of other embryoids (Plate VI).

Analysis of variance revealed the significance of explant stage of development, light, and medium type on total numbers of embryoids produced. A significant interaction between stage of development and light was found (Figure 1). The highest embryoid numbers were generated from tissues explanted 4 to 7 weeks post-fertilization and grown in light. The percentage of embryogenic explants was highest for this explant age when grown in light (Table 1).

During this most embryogenic period, the immature zygotic embryo is heart-shaped (Plate III) and transforms from a tiny translucent structure to one with enlarged cotyledons accumulating starch.

Highest embryoid numbers were obtained from explants cultured on the modified MS medium supplemented with 1.0 mg

Plate IV. Red oak embryo 9 weeks post-fertilization composed primarily of cotyledon tissue.

Plate V. Red oak embryoids forming
from the cotyledons of the
zygotic embryo explant.

Plate VI. Secondary and tertiary
embryoids forming from the
cotyledons of primary red
oak embryoids.

Figure 1. The interaction between explant stage of development and the presence or absence of light and its effect on total number of embryoids generated (*significant at $p > 0.01$).

Table 1. Percentage of embryogenic explants as influenced by explant age and light.

Explant age ^z	Light %	Dark %
1	10	10
2	17	10
3	10	17
4	87	73
5	87	83
6	80	40
7	67	27
8	6	13
9	20	13
10	8	0
11	10	6

^zweeks post-fertilization.

1^{-1} 2,4-D + $1.0 \text{ mg } 1^{-1}$ BA. This medium treatment was significantly different than the other media-hormone combinations (Figure 2).

When embryoids of various developmental stages could be removed from one another with a teasing needle, they were transferred to WPM containing $0.1 \text{ mg } 1^{-1}$ BA. This medium was chosen since the highest numbers of embryoids were achieved on this medium initially. After several weeks, many embryoids became polar with distinct root axes and enlarged cotyledons. Polar embryoids ranged from 0.5 to 1.2 cm in length. Less than 3% rooted (Plate VII), but showed no further development and eventually died. Of all embryoids regenerated, only one resulted in epicotyl elongation. Details of germination studies are discussed in Chapter III.

It was apparent that many of the embryoids were abnormal and incapable of normal plantlet development. Some of the anomalies included fused embryoids (Plate VIII) and polycotyledony, as others have described (11).

From some of the embryoids that never developed beyond the rooting stage, a white translucent callus proliferated. This event was sporadic and could not be attributed to any treatments. Callus maintained on solid medium turned brown and was non-regenerative. If removed from solid media and placed in liquid MS, the callus continued to multiply and could be subcultured. After approximately 6 weeks in

Figure 2. Effect of culture media (formulation + mg l^{-1} 2,4-D and BA) on total number of embryoids generated (*significant at the 0.05 level according to LSD test).

Plate VII. Rooting red oak embryoid.

Plate VIII. Abnormal red oak embryoids
fused at the radicles.

liquid culture, globular (Plate IX) and some heart-shaped (Plate X) structures were identified. Development beyond the heart stage has not been obtained in the liquid cultures to date.

Year 2. In order to refine the protocol for induction and further development of embryoids in Quercus, a second season of experimentation was conducted. Red oak zygotic embryos were collected at times corresponding to the most embryogenic stages identified during first year studies. Explant material had developed at a faster rate than the previous year's tissues, indicating that the 4 to 7 week post-fertilization range is an approximation. A more accurate approach would be to begin screening embryo development 2 weeks post-anthesis until structures resembling Plate III are found. Several environmental factors could account for varied rates of development. However, it is believed that genetic variability may be responsible in this case. As seed was extremely difficult to obtain from red oaks during the second year, explants were taken from a different tree than the one used in Year 1. It is well known that acorn development and size varies with race within this species (2).

As numerous abnormal embryoids were regenerated during Year 1 and no normal plantlets resulted, it was presumed that 2,4-D levels may have been excessive and responsible, at least in part, for such anomalies. For this reason,

Plate IX. Globular red oak embryoids
from liquid culture.

Plate X. Heart-stage embryoid from
liquid culture.

2,4-D rates were greatly reduced and a control treatment included.

Fifty-seven percent of the red oak explants were lost due to a high incidence of endophytic bacterial contamination which appeared 4 to 6 weeks after culture initiation. Of the surviving explants, 46% were embryogenic (Table 2). Highest rates of survival and embryogenesis were found among explants cultured 3 weeks post-fertilization. Embryogenic explants generated hundreds of embryoids over an 8-month period. High frequency repetitive embryogenesis was common in most cultures making total embryoid counts difficult.

Initially, red oak embryoids were produced directly from the cotyledons or radicles of the explants or other embryoids (Plate XI). However, after 10 to 12 weeks in culture, callus proliferation became abundant in most cultures and embryoids began to arise indirectly. Callus was creamy white and finely granular (Plate XII) from which pale yellow globular embryoids were generated (Plate XIII). Regeneration via callus increased over time. Maintenance of callus cultures was successfully achieved on half-strength modified MS medium solidified with Gelrite.

White oak embryoids arose directly from the cotyledons of explant tissue (Plate XIV). Repetitive embryogenesis was both direct, primarily from radicles, and indirect (Plate XV). Embryoids of this species were whiter in

Table 2. Number of surviving² and embryogenic explants categorized by explant age and treatment.

Initial ¹ Treatment		Explant age ²							
2,4-D	BA	2		3		4		5	
		Survived	Embryogenic	Survived	Embryogenic	Survived	Embryogenic	Survived	Embryogenic
0	0	3	2	3	3	3	0	2	0
0.01	0	1	0	2	2	2	0	4	1
0.1	0	1	0	1	0	2	0	1	0
0.01	0.01	2	1	4	3	1	1	4	0
0.1	0.1	3	2	3	3	1	1	2	0
1.0	1.0	0	0	3	3	2	1	2	1
Total		10	5	16	14	11	3	15	2

¹Based on 5 explants per treatment combination.

²Weeks post-fertilization.

³mg l⁻¹.

Plate XI. Secondary red oak embryoids
developing from the radicle of
a primary embryoid.

Plate XII. Callus derived from red
oak embryoids.

Plate XIII. Globular embryoids arising
from red oak callus.

Plate XIV. Embryoids forming from the cotyledons of the white oak zygotic embryo explant.

Plate XV. Secondary and tertiary white
oak embryoids developing from
primaries.

color, and callus much coarser and nodular (Plate XVI). Only 20% of the white oak explants were embryogenic. This could be due to collection of the explants beyond the most embryogenic period. Pollination and fertilization of the white oak occurs in one season (subgenus Lepidobalanus) (1) and early embryo development is supposed to lag behind that of black oak (38), which justifies late collection. However, the 7-week post-fertilization embryos once excised, were further advanced in development than was expected.

Heart-shaped embryoids of both species were separated from embryogenic clumps and cultured in Petri plates. As some embryoids became necrotic due to hypoxia, a different solidification protocol had to be identified. The callus maintenance medium was found to be suitable for developing embryoids. This reduced salt medium was thought also to be favorable as plantlet development under such conditions has been enhanced in some species (30).

As others have found (20), isolation of individual heart-shaped embryoids from embryogenic clusters was necessary for further development into polar structures. Polar embryoids exhibited distinct root axes and enlarged cotyledons resembling the zygotic embryo. Only a small number of white oak embryoids reached this stage of development. In contrast, hundreds of normal, polar red oak structures were generated (Plate XVII). Forty percent

Plate XVI. Callus derived from white
oak embryoids.

Plate XVII. Isolated polar red oak
embryoids.

of those structures resulted from explants that were never exposed to growth regulators (Table 3). This includes Quercus in the limited list of woody species that do not require auxin for the induction of asexual embryogenesis (20).

If maintained on 1/2 modified MS medium at 24° C, approximately 23% of the polar embryoids rooted. None resulted in shoot formation. Embryogenic callus proliferated from many rooted and unrooted polar embryoids (Plate XVIII).

Several plantlets have been regenerated from embryoids produced during the Year 2 study. Germination data are presented in Chapter III.

Table 3. Total number and percentage of polar red oak embryoids derived from explants cultured on various medium treatments.

Treatment	No. Polar Embryoids	%
MS	192	40.0
MS + 0.01 mg l ⁻¹ 2,4-D	12	2.5
MS + 0.01 mg l ⁻¹ 2,4-D + 0.01 mg l ⁻¹ BA	35	7.0
MS + 0.01 mg l ⁻¹ 2,4-D + 0.1 mg l ⁻¹ BA	168	34.0
MS + 1.0 mg l ⁻¹ 2,4-D + 1.0 mg l ⁻¹ BA	82	16.5

Plate XVIII. Callus proliferating from
rooted red oak embryoids.

CHAPTER III
EMBRYOID GERMINATION AND PLANT REGENERATION
IN QUERCUS

Introduction

Results from induction studies on Quercus clearly indicated that polar embryoids derived from immature zygotic embryo explants were incapable of epicotyl elongation if maintained at room temperature on basal medium or basal medium + 0.1 mg l⁻¹ BA. This meant that either no meristem existed or that it was arrested by epicotyl dormancy. As morphology of the polar embryoids closely resembled that of the zygotic embryo, the latter seemed to be most probable. Therefore, experiments were conducted to evaluate the use of dormancy-breaking treatments that are traditionally employed on seeds, in an attempt to stimulate shoot development in oak embryoids. As in induction studies, first-year experiments were considered preliminary, and two of the three experiments were repeated on embryoids generated the second year.

Materials and Methods

Over a 2-year period, germination studies were conducted on polar red oak embryoids derived from the induction experiments described in Chapter II.

Year 1. All media were solidified with 0.66% Difco Bacto-agar. All experiments were carried out in presterilized plastic Petri plates (15 x 100 cm). Light was defined as a 16 hr photoperiod of 80 to 90 $\mu\text{E m}^{-2} \text{s}^{-1}$ of cool white fluorescent light.

In a chilling study, 134 embryoids were cultured on WPM or WPM + 0.1 mg l^{-1} BA and stored in darkness at 4° C for 10 weeks. As a control, 115 embryoids were cultured in light on the same media at 24° C. To evaluate the effect of elevated osmoticum on germination, 28 embryoids were cultured in light on each of 3 media: WPM, WPM + 6% (.33M) sorbitol, and WPM + 11% (.60M) sorbitol. After 2 weeks, all embryoids were transferred to WPM. Fifteen embryoids per treatment were cultured in light on WPM supplemented with GA₄₊₇ or GA₃ (both filter sterilized and added after autoclaving) at 0.01 and 0.1 mg l^{-1} . Embryoids were transferred to fresh media every 2 weeks.

Year 2. All media were solidified with 0.3% Gelrite. All experiments were carried out in presterilized plastic Petri plates. Light was defined as a 16 hr photoperiod at 80 to 90 $\mu\text{E m}^{-2} \text{s}^{-1}$ of cool white fluorescent light, unless otherwise specified.

Seventy embryoids were cultured on 1/2-strength modified MS medium (described in Chapter II) and stored in darkness at 4° C for 10 weeks. Embryoids were then transferred to fresh medium and cultured at 24° C under lights.

In a high osmoticum study, 26, 30, and 30 embryoids were cultured, respectively, on 1/2-strength modified MS, 1/2-strength modified MS + 11% (.60M) sorbitol, and 1/2-strength modified MS + 16% (.87M) sorbitol in darkness at 22° C. After 2 weeks, all embryoids were transferred to 1/2-strength modified MS and grown in light.

To evaluate the effect of desiccation and rehydration on germination, 80 embryoids were placed in empty plastic Petri plates, wrapped in parafilm, and kept in darkness at 22° C for 2 weeks. Forty embryoids were cultured in the same manner for 3 weeks. Embryoids were plated as doublets (larger sizes), triplets, or quadruplets (smaller sizes). Relative humidity within Petri plates lacking medium but containing embryoids was measured by a digital hygrometer (Hanna Instruments-HI8064). All desiccated embryoids were rehydrated on 1/2-strength modified MS medium. When rooting became apparent, cultures were moved to low light ($2-3 \text{ uE m}^{-2} \text{ s}^{-1}$). When shoots became visible, cultures were moved to the higher light intensity. Percent moisture content was calculated as described by the International Seed Testing Association (4).

As epicotyls elongated and leaves began to expand, plantlets were removed from sterile conditions and transplanted into Super Cell Cone-tainers (Ray Leach Cone-tainer Nursery) filled with a mix of peat, perlite, and shredded bark (1:1:1 v/v). Plants were covered with a plastic film tent and grown under a 16 hr photoperiod of $30-40 \mu\text{E m}^{-2} \text{ s}^{-1}$. Plants were watered once every 3 days and fertilized weekly with 50 ppm of a 20 N - 20 P_2O_5 - 20 K_2O formulation.

Results and Discussion

Year 1. As red oak seed dormancy must be broken by a cold stratification period of $0-4^\circ \text{C}$ for 30-70 days in order for germination to occur (1), it was believed that embryoids may require a similar treatment. Walnut embryoids were found to possess an epicotyl dormancy, just as their zygotic counterparts, which could be overcome by a chilling period (58). Low temperatures are thought to release seeds from dormancy by stimulating the production of promotor substances such as gibberellins or causing a decrease in inhibitors such as abscisic acid (4). Ten weeks of storage at 4°C did not appear to stimulate germination of red oak embryoids. No epicotyl development was apparent on treated nor control embryoids. Highest rooting percentages were found among cold-treated embryoids cultured on WPM (Table 4). Roots of such embryoids

Table 4. Rooting percentage of embryoids subjected to chilling, osmotic and gibberellin treatments.

Chilling Treatment			Osmotic Treatment		Gibberellin Treatment		
Temperature	Medium	% Rooting	Sorbitol (M)	% Rooting	Form	Concentration	% Rooting
4° C	WPM	14.4	0	0	GA ₃	0.01 mg l ⁻¹	0
	WPM + 0.1 mg l ⁻¹ BA	2.5	0.33	7		0.1 mg l ⁻¹	7
24° C	WPM	0.9	0.60	7	GA ₄₊₇	0.01 mg l ⁻¹	7
	WPM + 0.1 mg l ⁻¹ BA	2.7				0.1 mg l ⁻¹	7

continued elongation for approximately one month, with death of the embryoid occurring shortly thereafter.

It is well established that desiccation plays a role in switching zygotic embryos from the embryogenic phase to the germinative phase (9,24). Drying suppresses developmental protein synthesis and accumulation of storage compounds (24). Upon rehydration, mRNAs for germinative proteins increase and storage protein degradation commences (9,25, 37). Such events are typical biochemical processes associated with germination (4). As desiccation in vitro has been induced by use of an osmoticum (9), embryoids were cultured on WPM plus various sorbitol concentrations. No epicotyl development resulted. Seven percent of the embryoids within each sorbitol treatment rooted compared with 0% of the control (Table 4). Survival of rooted embryoids was similar to that described in the chilling study.

In many species of seed, gibberellin treatments successfully overcome dormancy. The mode of action is believed to be a stimulation of RNA synthesis which is responsible for the production of germinative proteins (4). Since others have found GA treatments to substitute for chilling in the breaking of embryoid dormancy (23,47), embryoids were cultured at various concentrations of GA₃ and GA₄₊₇. The GA₄₊₇ formulation was included as it was found to be effective in breaking dormancy of Japanese

pear leaf buds (65). None of the treatments resulted in epicotyl development. Seven percent of the embryoids rooted within all treatments with the exception of the 0.01 mg l⁻¹ GA₃ treatment which resulted in no rooting.

Of all embryoids generated, only one resulted in epicotyl elongation (Plate XIX). By observing this structure, it was uncertain whether or not the leafy shoot was connected to the root system by a normal, continuous vascular system. The vast majority of this "plantlet" was composed of cotyledonary tissue from which roots and shoots emerged. It must be noted that shoot development occurred only after the rooted embryoid was subjected to a second cold treatment of 6 weeks duration. This additional treatment cannot be deemed responsible for shoot formation, as other rooted embryoids failed to produce shoots upon exposure to a second stratification period.

The shoot of this "plantlet" developed a total of 4 leaves while being maintained in vitro. After 2 months in culture, foliar abscission occurred and cotyledonous structures began to emerge from the shoot apex. It appeared as though the embryoid was returning to an embryogenic growth phase. Efforts to reinstate the germinative phase with a third cold treatment were unsuccessful and resulted in death of the "plantlet."

Year 2. Chilling and sorbitol experiments were repeated on polar red oak embryoids derived from second-

Plate XIX. Epicotyl bearing leaves
emerging from red oak
embryoid.

year explants. As found previously, chilling did not result in epicotyl development (Table 5). Sixty-seven percent of the embryoids survived the chilling treatment and of them, 43% rooted. Comparatively, the second-year study produced a 3-fold rooting increase over that of the first year.

Since the first-year sorbitol experiment resulted in no shoot development, the study was repeated with a control, the highest sorbitol concentration from the first year (11%), and an elevated sorbitol level (16%). The control resulted in no epicotyl development, in contrast with the sorbitol treatments, which resulted in the development of viable plantlets (Table 6). Radicle elongation commenced immediately following removal from the sorbitol-containing media. The primary root elongated rapidly, often accompanied by the development of lateral roots. Epicotyl elongation became apparent 2 to 3 weeks after "rehydration" on 1/2-strength basal medium. It should be noted that epicotyl elongation occurred on an additional 69% and 18% of the surviving embryoids cultured on 11% and 16% sorbitol, respectively. However, these shoots failed to produce true leaves. Instead, new cotyledons or cotyledonous structures developed at the apex of the epicotyl (Plate XX). Similar results have been found by Finkelstein et al. (8) in immature zygotic embryos of rapeseed excised and cultured in vitro at various times

Table 5. Survival, rooting, and plantlet development of second-year polar red oak embryoids subjected to 4° C for 10 weeks.

	Number of Embryoids	Percent
Surviving ^z	47	67
Producing roots only	20	43 ^y
Producing plantlets	0	0 ^y

^zOf 70 initially cultured.

^yOf surviving embryoids.

Table 6. Effect of sorbitol treatments on survival, rooting, and plantlet development of second-year polar red oak embryoids.

	% Sorbitol					
	0%		11%		16%	
	No. of Embryoids	%	No. of Embryoids	%	No. of Embryoids	%
Total cultured	24	-	30	-	30	-
Surviving	22	92	13	43	11	37
Producing roots only	5	23*	11	85*	6	55*
Producing plantlets	0	0*	1	8*	1	9*
Producing cotyledonous shoots ^y	0	0*	9	69*	2	18*

*Of surviving embryoids.

^yAlso producing roots.

Plate XX. Cotyledonous structures which developed from elongating epicotyl of red oak embryoid treated with sorbitol.

post-anthesis. Apparently, such structures are transitional between the embryonic and germinative phases. If drying occurs prematurely at an early embryo stage, tissues may not be desiccation tolerant and synthesis of both embryonic and germinative proteins can result (24). In rapeseed, many of these structures eventually formed true leaves and developed into normal plants. To date, this has not been the case for such red oak structures.

It was clear that exposure to sorbitol decreased embryoid survival (Table 6). The largest and most opaque embryoids survived treatment and were capable of further development.

As an alternative to desiccation via use of an osmoticum, a study was conducted in which embryoids were removed from culture medium and permitted to air dry. This was accomplished by allowing embryoids to equilibrate with the environment inside sterile, empty, sealed Petri plates for 2 and 3 weeks. Temperature within the plates remained constant at $22 \pm .6^{\circ}$ C. Relative humidity rose to $> 95\%$ during the first 24 hours and remained at this level for 8 days (Figure 3). By day 14, relative humidity dropped to 38% and continued to decline to approximately 20% by day 21.

Plantlets with well-developed epicotyls and roots (Plate XXI) were produced from embryoids subjected to both desiccation durations (Table 7). Within 2 to 3 days after

Figure 3. Relative humidity levels within sealed Petri dish containing only red oak embryoids over 3-week duration.

Plate XXI. Normal epicotyl emerging
from between cotyledons of
red oak embryoid.

Table 7. Effects of desiccation duration² on survival, rooting, and plantlet development of polar red oak embryooids.

	Desiccation Duration			
	2 Weeks		3 Weeks	
	No. of Embryooids	%	No. of Embryooids	%
Total cultured	80	-	44	-
Surviving	32	40 ^y	11	25 ^y
Producing roots only	29	91 ^y	6	55 ^y
Producing plantlets	3	9 ^y	2	18 ^y
Producing cotyledonous shoots ^x	15	47 ^y	0	0 ^y

²Weeks kept in empty, sealed Petri plates.

^yOf surviving embryooids.

^xAlso producing roots.

rehydration on 1/2-strength basal medium, radicle elongation began. Shoot meristem emergence became apparent within 2 to 4 weeks post-rehydration and closely resembled that found in the mature sexual embryo (Figure 4).

Cotyledonous shoots developed from 47% of the surviving embryoids subjected to the 2-week desiccation treatment, while the 3-week treatment resulted in none (Table 7). This appeared to indicate that the switch to complete germinative development occurred within this 2-3 week desiccation time frame. Even though 2 weeks may have been insufficient to bring about normal germination in some plantlets, the 3-week treatment clearly decreased embryoid survival. Again, increased size and opaqueness of embryoids entering desiccation seemed to positively influence survival. Hardy embryoids exited desiccation with a rubbery texture, while non-survivors were brittle.

The formula used to calculate percent moisture content in seeds was applied to embryoids to determine the percent weight loss during desiccation. Attempts to use this as a predictor of critical dehydration necessary to trigger the switch from the embryonic to germinative phase proved unsuccessful. Percent weight loss ranged from 76 to 98% after the two-week desiccation treatment. However, only 1 of 56 embryoids survived upon rehydration and it failed to produce roots or shoots. This is believed to be due to individual plating which resulted in humidity levels too

Figure 4. Comparison of epicotyl emergence from between cotyledons of red oak embryoid (top) and germinating red oak acorn (bottom).

low to maintain a sufficient moisture content, therefore decreasing embryoid viability. Acorns of Quercus species have been categorized as being "recalcitrant" or "desiccation sensitive" as they cannot tolerate drying below 30% moisture content and remain viable (4,5). Membrane degradation and enzyme alterations are believed to be responsible for decreased viability resulting from critical water loss (5).

Plantlets derived from the osmoticum and desiccation-rehydration studies were successfully acclimated to non-sterile conditions (Plate XXII). It was necessary to maintain a high relative humidity around above-soil plant parts for several weeks. To date, healthy, viable plantlets have been growing in soil mix for approximately 1-1/2 months.

It should be noted that all viable plantlets originated from explants cultured at the same stage of development (3 weeks post-fertilization) and on the same induction medium (MS + 0.1 mg l⁻¹ 2,4-D + 0.1 mg l⁻¹ BA).

Plate XXII. Plantlet derived from red oak embryoid growing in non-sterile conditions.

SUMMARY AND CONCLUSIONS

Results of this study clearly indicate that asexual embryogenesis offers an alternative to sexual propagation methods for Quercus. Much refinement of the germination protocol would be necessary in order for the system to be commercially adaptable. However, 2 years of pioneering research have answered many crucial questions regarding induction and germination procedures as well as elucidated developmental processes within this genus.

The induction of embryoid formation was repeatable over two seasons as well as with different genotypes within the Q. rubra species and with representative members of each of the two subspecies, Erythrobalanus and Lepidobalanus. Immature zygotic embryos were clearly the most regenerative explant source, especially when extracted and cultured at the mid-late heart or early cotyledon stages. Embryoids were produced from explants cultured on both modified MS medium and WPM, but highest numbers resulted from the modified MS treatments. Growth regulators were not essential to the induction process in Quercus. Elevated 2,4-D levels used in first-year studies were actually detrimental and could have accounted, at least in part, for

the failure to regenerate viable plants. Embryoids arose under both light and dark conditions. However, induction efficiency was greater in the presence of light.

Regardless of whether origin was direct or indirect, embryoid morphological development from the heart stage to elongation of the radicle and epicotyl bore a striking resemblance to that of the zygotic embryo. A few differences noted in the 2 processes included smaller cotyledon size of the polar embryoids versus acorns and smaller size of plantlets versus seedlings.

Physiologically, embryoids differed from seeds in their environmental requirements for terminating epicotyl dormancy. Cold stratification, a common acorn treatment, proved ineffective in breaking embryoid dormancy. Instead, desiccation-rehydration treatments, chemical or air drying, were the only effective means for allowing germination to proceed. Further studies need to be conducted to pinpoint how much drying is necessary to switch on germinative development without adversely affecting viability.

White oak embryoids were not included in germination studies primarily because few developed into polar structures exhibiting starch-laden cotyledons and distinct root axes. It is possible that once white oak polar embryoids are regenerated, germination problems could be minimal as seed from members of the Lepidobalanus subgenus do not possess epicotyl dormancy.

Discrepancies between first- and second-year data from both induction and germination studies are numerous. Differences could be attributed to formulations of culture media utilized, growth regulator concentrations, genotypic variability, or diligence in adhering to transfer schedules. First-year studies were designed to provide preliminary information about explant sources and chemical and environmental treatment effects. Such data were utilized in refining second-year studies which emphasized more precise techniques and observations. In spite of variability witnessed, the information derived from these 2 years of experimentation will hopefully further understanding of the asexual embryogenesis process in woody plants and how it may be manipulated for commercial production purposes.

Follow-up studies to this work might include adaptation of the induction protocol to a liquid batch culture system which would allow for the production of greater embryoid numbers and synchronization of developmental stages. Further desiccation experimentation might focus on rapid versus slow drying processes as well as options for maintenance of constant relative humidity levels with the use of desiccants, saturated salt solutions or concentrated acid solutions. Regenerated plants must be acclimated to greenhouse and field conditions where qualitative and quantitative evaluations can be made.

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