FOREST GENETICS
PROJECT ADVISORY COMMITTEE
MEETING

HANDOUTS

The Institute of Paper Chemistry
Continuing Education Center
Appleton, Wisconsin 54915

March 18-19, 1986
AGENDA
FOREST GENETICS
PROJECT ADVISORY COMMITTEE
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Tuesday, March 18, 1986

1:00 PM Opening Remarks
A. Introduction and Overview
   1. Review of PAC recommendations
   2. Review of recent accomplishments and new plan
B. Norway Spruce Somatic Embryogenesis-Tissue Culture Model
   1. The process of Norway spruce somatic embryo development
   2. The formation of plants from Norway spruce somatic embryos
   3. Quantification of Norway spruce somatic embryogenesis
   4. Window research

3:00 PM Coffee break

C. Norway Spruce Somatic Embryogenesis-Biochemical Model
   1. Chloroplasts in embryogenic and non-embryogenic callus
   2. Messenger RNA in embryogenic and non-embryogenic callus
   3. Additional biochemical parameters distinguishing embryogenic from nonembryogenic callus
      a. ethylene
      b. protein synthesis
      c. glutathione
      d. total reductants
D. Screening Pine Sources for Morphogenic Potential
E. The New Conceptual Plan

5:30 PM Cocktails and Dinner

7:00 PM F. Open Discussion

ADJOURN
<table>
<thead>
<tr>
<th>Time</th>
<th>Event</th>
</tr>
</thead>
<tbody>
<tr>
<td>7:00 AM</td>
<td>Breakfast (CEC Dining Room)</td>
</tr>
<tr>
<td>8:00 AM</td>
<td>Agenda</td>
</tr>
<tr>
<td>8:10 AM</td>
<td>PAC Deliberations</td>
</tr>
<tr>
<td>11:30 AM</td>
<td>ADJOURN</td>
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</tbody>
</table>

Lunch (CEC Dining Room)
FOREST GENETICS

Project Advisory Committee

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*date of retirement
/bsb
2/10/86
ATTENDANCE LIST

FOREST GENETICS
PROJECT ADVISORY COMMITTEE

The Institute of Paper Chemistry
Appleton, Wisconsin 54915

March 18-19, 1986

* * * * * *

PAC Committee

Mr. Robert Lazar
UNION CAMP CORPORATION

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John Carlson
Jud Conkey
Dean Einspahr
Russ Feirer
Debra Hanson
Morris Johnson
Lynn Kroll
Tom Noland
John Phythyon
Nagmani Ragnaswamy
Shirley Verhagen
Gary Wyckoff
Judy Wyckoff

Industrial Research Fellows

Steve Wann, Union Camp
Mike Becwar, Westvaco

IPC Administration

Harry Cullinan
Richard Matula
Wendall Smith
Douglas Wahren

IPC Students

Brent Earnshaw
Rene Kapik
Luke Nealey
"....all of us are aware that the embryos will not produce plants by the end of next year (though this will clearly be the ultimate measure of success)....biochemical approaches should not be abandoned..."

* * *

STUDENT RESEARCH

Brent Earnshaw, Ph.D.: biochem., WC

Luke Nealey, Ph.D.: biochem., LP

Rene Kapik, M.S.: phenols

Russ Feirer, Ph.D.: polyamines

* * *

COOPERATIVE PROGRAMS

N. C. State: R. Mott, H. Amerson
- organogenesis/polyamines

St. Norbert: John Phythyon
- embryogenesis/LP susp/biochem.

Yale: R. Slocum
- ADC/ODC in cultured cells

U. New Hampshire - discontinued

* * *

MODEL SPECIES TARGET SPECIES

pitch pine, PP loblolly pine, LP
Ponderosa pine, PO Douglas-fir, DF
Douglas-fir, DF
Norway spruce, NS
white spruce, WS(?)
larch, LA
wild carrot, WC

* * *
CODES

20(NS 384-1)21

sub specie plan time line explant
no. no. or type treatment

* * *

STATISTICS

Mean, std. deviation

Student’s t-test
- OK if used on pre-designated groups

Duncan’s New Multiple Range Test
- OK to use after the fact IF overlapping groups not re-grouped

N replicates, a = a at 95% confidence

* * *
STUDENT RESEARCH

1) Brent Earnshaw - A biochemically oriented Ph.D. program entitled "An investigation into the functions of glutathione and ascorbic acid in growth and development of wild carrot suspension cultures and plants."

2) Luke Nealey - An organic chemistry oriented Ph.D. program entitled "Isolation and characterization of xyloglucan from suspension cultured loblolly pine cell medium."

3) Rene Kapik - An independent study (M.S.) topic entitled "Phenolic components of the primary cell wall and their possible role in the regulation of growth."

4) Russell Feirer - A biochemically oriented Ph.D. program investigating the role of polyamines and associated enzymes in plant development (in cooperation with the University of Wisconsin, Madison).
COOPERATIVE INVESTIGATIONS

1) North Carolina State - A cooperative study with Dr. Ralph Mott and Dr. Henry Amerson on variation of polyamine levels during organogenesis of loblolly pine.

2) University of New Hampshire - A cooperative study with Dr. Subhash Minocha on the use of nuclear proteins (histones) as biochemical markers for monitoring somatic embryogenesis (discontinued).

3) St. Norbert College - A cooperative study with Dr. John Phythyon on the role of methionine and S-adenosylmethionine on potential embryogenesis of loblolly pine cell suspensions.

4) Yale University; Williams - A cooperative study with Dr. Robert Slocum who is assisting in efforts to characterize and localize ADC/ODC in cultured plant cells.
CODES

Tissue response and the results of many studies may be altered or complicated by the genetic differences between cell lines and/or the length of time in culture. To aid the reader (reviewer) in understanding, and the investigator in reporting/analyzing, it is important to be aware of the tissue source used for each study. An example and explanation of our standard tissue identification coding system is presented below; however, at times only part of the code may appear in a text.

All cell lines in excess of one year old:

Example: 20(NS 384-1)21

20 = subcultured 20 times
NS = Norway spruce
384 = research plan (RP384)\(^a\)
-1 = time of initiation or treatment identification
2 = line or genetic source, e.g., seedling No. 2
I = Immature embryo; explant type (only used if cell line derived from more than one explant within a research plan).

All cell lines less than one year old:

Example: 5(NS1)2 -- the RP No. is deleted.

<table>
<thead>
<tr>
<th>Species Codes</th>
<th>Explant Codes</th>
</tr>
</thead>
<tbody>
<tr>
<td>LP = loblolly pine</td>
<td>C - cotyledon</td>
</tr>
<tr>
<td>DF = Douglas-fir</td>
<td>H - hypocotyl</td>
</tr>
<tr>
<td>PP = pitch pine</td>
<td>B - bud</td>
</tr>
<tr>
<td>PO = pond pine</td>
<td>I - immature embryo</td>
</tr>
<tr>
<td>NS - Norway spruce</td>
<td>E - mature embryo</td>
</tr>
<tr>
<td>SP - slash pine</td>
<td>N - nucellus</td>
</tr>
<tr>
<td>WC - wild carrot(^b)</td>
<td>G - gametophyte</td>
</tr>
</tbody>
</table>

\(^a\)Each experiment initiated by any team member has an approved research plan with an identifying number. The tissue source origin (clone, seed lot, etc.) and initiation date is recorded under that number in the investigator's IPC research notebook and is available in the Tissue Culture Research Plan files.

\(^b\)All current WC lines, with the exception of eight explained below, originated from one genetic source obtained from D. F. Wetherell in March, 1982. This single genetic line has been regenerated numerous times by initiating new callus from induced somatic embryos. The coding system for this line remains essentially the same as for the conifers with the code (WC8-3)R\(#\), where in this case the R\(#\) is the number of regeneration times. The eight "exceptions" were initiated from IPC greenhouse plants grown from wild seed sources, and are coded WC1, WC2, WC3, etc.
STATISTICS

Where statistics beyond means and standard deviations (S.D.) were used in the evaluation of results to be presented, the data were subjected to analysis of variance (ANOVA) followed by Duncan's New Multiple Range Test for multiple comparison of means. Values with a common superscript letter are not significantly different from each other ($P \leq 0.05$). The number of replications is indicated by $N$. 
PROJECT TITLE: The Mass Production of Conifers

PROJECT STAFF: *Becwar, Feirer, Johnson, Verhagen
* Wann

PRIMARY AREA OF INDUSTRY NEED: Raw Materials

PROGRAM AREA: Increased wood production by embryogenesis and bioengineering

PROJECT RATIONALE:

Major increases can be obtained in tree growth and forest production through the clonal propagation of "elite" trees and through the creation of new genetic combinations. Planned genetic combinations are ones that are difficult to produce using conventional techniques but are expected to result in individuals of exceptional disease resistance and special site and/or climatic adaptability. Production of plantlets from cell cultures will open the way to the badly needed genetic gains described above through genetic engineering. Cell cultures form the basis for a second-generation technology which, some day, probably will replace existing practices.

RESULTS TO DATE:

Appropriate new media have been developed for initiating callus production and for growing cells in suspension. Procedures for establishing cell lines have been developed. Biochemical and morphological characterization of embryogenesis is under way.

Use of wild carrot, coffee, and, now, Norway spruce somatic embryogenesis as well as natural Douglas-fir, loblolly pine, and aspen zygotic embryo development as model systems has assisted in establishing tissue culture requirements and in developing needed biochemical markers. Loblolly pine organogenesis has also been used in the development of markers. Excised conifer embryos have been used to determine the nutrient requirements of developing embryos.

Inhibitor studies have demonstrated the importance of polyamines in embryogenesis and determined that polyamine biosynthesis in wild carrot and aspen is controlled mainly through the arginine/agmatine pathway. Furthermore, there are indications that spermidine may be the polyamine most involved in development. Studies of natural pine embryogenesis indicated that polyamines play an essential role in the development of pine embryos in maturing cones. This was also demonstrated in the case of aspen seed development. Tests of the LM medium for growing conifer cells in suspension showed that the medium could also be used to support somatic embryogenesis of wild carrot.

* Industrial Research Fellow
Immature embryo cell lines have been produced and are being monitored to determine whether they have an improved potential for embryogenesis. The immature pine seed extracts that enhanced wild carrot somatic embryogenesis showed no desirable effects on loblolly pine suspensions but did appear to increase loblolly pine organogenesis.

Research into energy charge levels of the wild carrot and loblolly pine suspension cells revealed that the pine cells in culture have a high energy charge and should have adequate ATP levels to drive biosyntheses. Ascorbic acid and glutathione investigations indicated that wild carrot cultured cells may have more control of their internal redox status than do pine cells in culture, particularly old cell lines. Most indications are that the cell lines obtained to date are deficient in too many respects to have much capacity for organized development.

Somatic embryogenesis has been observed in Norway spruce, first in Sweden and now in our own laboratory, along with Douglas-fir (low frequency). Also, somatic embryogenesis in larch has been observed in Canada. The ability to obtain embryogenic and nonembryogenic Norway spruce callus from the same genotype is facilitating biochemical and histological comparisons of the two types. Aside from the visual and tactile differences, embryogenic callus has been found to evolve less ethylene, contain more total reductants and be devoid of chloroplasts when compared to nonembryogenic callus. Characterizations such as these are expected to lead directly to the development of biochemical markers which can be applied to the species relevant to the goal of the project.

A new conceptual research plan has been aimed at taking advantage of current progress in conifer tissue culture occurring throughout the world.

**PLANNED ACTIVITY FOR THE PERIOD:**

Plans for the coming year are detailed in a new conceptual plan appearing in Progress Report 13. This plan builds upon recent advances in the regeneration of plants from cultured conifer tissue to accelerate the attainment of project goals. A major change in the use of model systems is the adoption of the newly developed Norway spruce somatic embryogenesis system in lieu of the wild carrot model used in the past. Associated with the new model is the concept of a "juvenility window", i.e., that not only the right explant must be used but that it must also be of the right physiological age to be successful. The unique white mucilaginous callus observed to be embryogenic in Norway spruce will be sought also in loblolly pine and Douglas-fir; some has already been seen in the latter.

In addition to the visual/tactile characteristics of known embryogenic conifer callus, biochemical markers will be used as well. Some, like ethylene evolution rate, will be used as a primary marker whereas others, like polyamine and glutathione status, will be used as secondary markers if they prove to be useful indicators of competence in the spruce model system. Several other potential markers (biochemical, cytological, and physical) remain under investigation for possible use in mainstream screening for competence in new pine and fir cell
lines. Exploratory tissue culture research aimed at improvement and future exploitation of current protocols will be also conducted along with the main effort to initiate new embryogenic cell lines from immature zygotic embryos and from nucellar and gametophytic tissue. Embryogenic callus obtained will be converted to embryogenic cell suspensions, if possible, to further enhance the mass production potential. Even low frequency somatic embryogenesis, if obtained, may be used as a starting point to initiate mass production. Additionally, some effort will be expended to demonstrate the conversion of conifer somatic embryos into complete plants.

SHORT TERM GOALS:

Goals for the 1986-87 conifer cell and tissue culture program:

1. Finish biochemical characterization of wild carrot somatic embryogenesis and of incompetent loblolly pine cells; no new investigations will be started on these systems.

2. Characterize the Norway spruce somatic embryogenesis model in terms of physical, biochemical, and cytological/histological parameters; include European larch and Douglas-fir callus with latent potential where appropriate.

3. Attempt to establish embryogenic cell suspensions of Norway spruce.

4. Attempt to obtain complete plants of somatic embryos from Norway Spruce.

5. Establish new cell lines from immature embryos plus nucellar and gametophytic explants of loblolly pine, Douglas-fir, and other conifers based on the "juvenility window" concept, and use markers to determine embryogenic competence potential. Emphasis will be placed on loblolly pine and Douglas-fir. However, hard pines that are easily vegetatively propagated will also be explanted. At certain stages of explant development deemed to be critical, extra collections and clones will be used. Temporary manpower will be employed to assist in this work.

6. Determine which characteristics of mature conifer explants must be changed to enable their use as sources of embryogenic callus.

7. Conduct exploratory tissue culture research on topics such as regeneration from single cells and protoplasts, methods of transformation, molecular biology techniques at the nucleic acid level, and oligosaccharides to assist the production and exploitation of embryogenic cell lines.

8. Conduct research on potential markers of embryogenic competence at the callus stage. Topics such as ultrastructure, total reductants, SDS-PAGE, media parameters, isozymes, peroxide, and organogenesis will be evaluated in the conifer model systems.
STUDENT RELATED RESEARCH:

Brent Earnshaw - A biochemically oriented Ph.D program entitled "An Investigation into the Functions of Glutathione and Ascorbic Acid in Growth and Development of Wild Carrot Suspensions in Cultures and Plants".

Luke Nealey - An organic chemistry oriented Ph.D program entitled "Isolation and Characterization of Xyloglucan from Suspension Cultured Loblolly Pine Cell Medium".

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Rene Kapik - An independent study (MS) topic entitled "Phenolic Components of the Primary Cell Wall and Their Possible Role in the Regulation of Growth".
CONCEPTUAL PLAN FLOW CHART

Explants
Norway Spruce  Douglas Fir  Loblolly Pine  Others

- spring (gametophyte/cotyledon) - x x x x
- summer (incomplete embryos)  x x x x
- fall (stored embryos)  x - x x

Tissue Culture System

Exploratory Tissue Culture
regeneration from single cells
protoplast isolation and culture
regeneration from protoplasts
method of transformation
- e.g.: electroporation
- Agrobacterium
- microinjection
molecular biology marker techniques
- e.g.: restriction fragment polymorphism
oligosaccharides

Primary Markers
visual
- tactile
- ethylene

Embryogenic callus
+ x/-
Embryogenic suspensions
++ x/-
Embryos
Plants

Potential Markers
phenolics/total reductants
ultrastructure
chloroplasts
pH cells/medium
osmolarity
oxygen uptake
medium redox potential
isoenymes
SDS-PAGE
organogenesis
peroxide

Secondary Markers
GSH/ascorbic acid
polymamines/enzymes
NORWAY SPRUCE SOMATIC EMBRYO DEVELOPMENT

1) Protocol used to induce somatic embryo development.
2) Method used to quantify the level of somatic embryogenesis.
3) Embryogenic capacity of callus lines.
4) Proembryo to cotyledonary stage development of somatic embryos.

Summary of callus lines established in Norway spruce.

<table>
<thead>
<tr>
<th>Code</th>
<th>Collection site</th>
<th>Number explants</th>
<th>Established callus lines</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(1985 date)</td>
<td>E</td>
<td>(both)</td>
</tr>
<tr>
<td>NS1</td>
<td>Appleton (7/12)</td>
<td>18</td>
<td>12</td>
</tr>
<tr>
<td>NS2</td>
<td>Appleton (7/19)</td>
<td>10</td>
<td>5</td>
</tr>
<tr>
<td>NS3</td>
<td>Appleton (7/26)</td>
<td>20</td>
<td>0</td>
</tr>
<tr>
<td>NS4</td>
<td>Argonne (8/2)</td>
<td>18</td>
<td>5</td>
</tr>
<tr>
<td>NS5</td>
<td>Appleton (8/2)</td>
<td>18</td>
<td>0</td>
</tr>
<tr>
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<td>Appleton (8/9)</td>
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<td>0</td>
</tr>
<tr>
<td>NS7</td>
<td>Appleton (8/16)</td>
<td>45</td>
<td>0</td>
</tr>
<tr>
<td>NS8</td>
<td>Argonne (8/26)</td>
<td>88</td>
<td>4</td>
</tr>
</tbody>
</table>

a Lines maintained for at least 6 months. E = embryogenic white callus. NE = nonembryogenic green callus. Value in parentheses indicates number of lines in which both callus types are available from the same genotype.
NORWAY SPRUCE SOMATIC EMBRYO DEVELOPMENT PROTOCOL

HM (Hakman, et al., 1985) medium
+ 2 mg/l 2,4-D
+ 1 mg/l BA
2 wk subculture

HM + 1% activated charcoal
no growth regulators
1 wk

HM + 1 μM each IBA & ABA

Subculture for somatic embryo development on callus

Disperse for counting somatic embryos and development
SOMATIC EMBRYO DISPERSION AND COUNTING TECHNIQUE

Callus, 50-100 mg wet weight

Liquid medium, 1 ml

Agitate, 15 sec

Liquid medium with 1.2% LMP agarose, 1 ml at 38°C

Mix

Pour over solidified 0.6% agarose medium, 2 ml

Cool to room temp.

Count somatic embryos

Calculate somatic embryos/mg wet weight callus

Somatic embryo development
### Differences in somatic embryogenesis among Norway spruce callus lines

<table>
<thead>
<tr>
<th>callus line</th>
<th>callus type</th>
<th>Somatic embryos counted per mg wet weight callus</th>
</tr>
</thead>
<tbody>
<tr>
<td>(NS1)11</td>
<td>W</td>
<td>1.5 A</td>
</tr>
<tr>
<td>(NS1)12</td>
<td>W</td>
<td>0.8 B</td>
</tr>
<tr>
<td>(NS1)10</td>
<td>W</td>
<td>0.8 B</td>
</tr>
<tr>
<td>(NS1)8</td>
<td>W</td>
<td>0.7 BC</td>
</tr>
<tr>
<td>(NS1)5</td>
<td>W</td>
<td>0.7 BCD</td>
</tr>
<tr>
<td>(NS1)7</td>
<td>W</td>
<td>0.7 BCD</td>
</tr>
<tr>
<td>(NS1)13</td>
<td>W</td>
<td>0.6 BCD</td>
</tr>
<tr>
<td>(NS2)6</td>
<td>W</td>
<td>0.5 BCD</td>
</tr>
<tr>
<td>(NS2)5</td>
<td>W</td>
<td>0.5 CDE</td>
</tr>
<tr>
<td>(NS1)9</td>
<td>W</td>
<td>0.5 CDEF</td>
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<tr>
<td>(NS1)1</td>
<td>W</td>
<td>0.4 DEFG</td>
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<tr>
<td>(NS2)4</td>
<td>W</td>
<td>0.2 EFGH</td>
</tr>
<tr>
<td>(NS1)6</td>
<td>W</td>
<td>0.2 FGH</td>
</tr>
<tr>
<td>(NS2)3</td>
<td>W</td>
<td>0.2 FGH</td>
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<td>(NS8)</td>
<td>W</td>
<td>0.2 GH</td>
</tr>
<tr>
<td>(NS1)2</td>
<td>W</td>
<td>0.2 GH</td>
</tr>
<tr>
<td>(NS4)4</td>
<td>W</td>
<td>0.1 H</td>
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<td>W</td>
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<td>(NS5)17</td>
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<td>0.003 H</td>
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<td>(NS1)4</td>
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<tr>
<td>(NS5)5</td>
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<td>0 H</td>
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<td>(NS5)15</td>
<td>G</td>
<td>0 H</td>
</tr>
<tr>
<td>(NS5)20</td>
<td>G</td>
<td>0 H</td>
</tr>
<tr>
<td>(NS7)</td>
<td>G</td>
<td>0 H</td>
</tr>
</tbody>
</table>

*a = white to translucent and G = green

bMean value of four observers on four callus pieces per line.

### SYSTEMS FOR MAINTENANCE OF NORWAY SPRUCE EMBRYOGENESIS

1) Liquid suspensions cultures.

2) "Bead Culture": A possible alternative to liquid suspensions.
The Formation of Plants from Norway Spruce Somatic Embryos

Table 1

Differences in frequency of somatic embryos (S.E.'s) and developmental capacity among Norway spruce callus lines.

<table>
<thead>
<tr>
<th>(NS-1) callus line</th>
<th>S.E.'s per mg wet weight callus</th>
<th>Development at 21 days on HM IBA/ABA visible S.E.'s</th>
<th>Development at 21 days on HM IBA/ABA cotyledonary S.E.'s</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.4 ± 0.3</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>0.2 ± 0.1</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>4 (green)</td>
<td>0</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>5</td>
<td>0.7 ± 0.3</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>6</td>
<td>0.2 ± 0.2</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>7</td>
<td>0.7 ± 0.3</td>
<td>+ (callusing)</td>
<td>-</td>
</tr>
<tr>
<td>8</td>
<td>0.7 ± 0.2</td>
<td>+ (callusing)</td>
<td>+</td>
</tr>
<tr>
<td>9</td>
<td>0.5 ± 0.2</td>
<td>+ (callusing)</td>
<td>+</td>
</tr>
<tr>
<td>10</td>
<td>0.8 ± 0.3</td>
<td>+ (proembryos)</td>
<td>-</td>
</tr>
<tr>
<td>11</td>
<td>1.5 ± 0.7</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>12</td>
<td>0.8 ± 0.4</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>13</td>
<td>0.6 ± 0.2</td>
<td>+ (callusing)</td>
<td>-</td>
</tr>
</tbody>
</table>
Table 2

<table>
<thead>
<tr>
<th>Embryos Transferred by 3/5</th>
<th>Cell Line</th>
<th>Normal</th>
<th>Aberrant</th>
</tr>
</thead>
<tbody>
<tr>
<td>11 (NS-1)5</td>
<td></td>
<td>3</td>
<td>8</td>
</tr>
<tr>
<td>3 (NS-1)1</td>
<td></td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>2 (NS-1)9</td>
<td></td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>Totals 16</td>
<td></td>
<td>8</td>
<td>8</td>
</tr>
</tbody>
</table>

Table 3

<table>
<thead>
<tr>
<th>Type of Development</th>
<th>Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Shoot Growth</td>
<td>3</td>
</tr>
<tr>
<td>Root Growth</td>
<td>4</td>
</tr>
<tr>
<td>Shoot and Root Growth</td>
<td>1</td>
</tr>
</tbody>
</table>
Objective: Quantify growth and development of embryogenic callus of Norway spruce for:

1. Evaluation of biochemical experiments designed to determine the importance of various metabolic pathways to somatic embryogenesis.

2. Evaluation of other treatments (physical, nutritional, or hormonal) aimed at enhancing somatic embryogenesis in marginally competent lines.

Embryogenic Norway spruce callus: composition

1. Extracellular, mucilaginous substance
2. Callus or suspensor single cells
3. Dedifferentiated "growth centers" (callus)
4. Somatic embryos

Definition of gravimetric measurements

Wet weight - extracellular material and tissues
Fresh weight - tissues washed free of extracellular material
Dry weight - oven-dried material from fresh weight determination
mass increase, %

composition, %

extracellular mass
intracellular mass
dry mass

(NSI)8
- wet weight
○ fresh weight
□ dry weight

Time, weeks
Current protocol for biochemical experiments on embryogenic spruce callus

1. Callus pieces (40-50 mg wet wt.) are placed for two subculture intervals (2 weeks each) on HM basal medium containing 2 mg/L 2,4-D, 1 mg/L BA, and the biochemical of interest.

2. Transfer to HM medium containing 1% charcoal for 1 week.

3. Transfer to HM medium containing 1 M IBA and ABA for 2 weeks.

4. Disperse (ala wild carrot) and count somatic embryos.

Total time per experiment = 7 weeks (compare with 3 weeks for carrot)

The effect of GSH and BSO on somatic embryogenesis in Norway spruce (NS1)

<table>
<thead>
<tr>
<th>Treatment</th>
<th>embryos/mg wet wt.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.82 a</td>
</tr>
<tr>
<td>10^-5 mM BSO</td>
<td>0.47 b</td>
</tr>
<tr>
<td>10^-4 mM BSO</td>
<td>0.32 bc</td>
</tr>
<tr>
<td>5 x 10^-3 mM GSH</td>
<td>0.20 c</td>
</tr>
</tbody>
</table>

Conclusions

1. Embryogenic spruce callus undergoes significant changes in gravimetric composition as a function of time.

2. At any given time, little difference exists between cell lines in their gravimetric composition.

3. The number of embryos per unit mass varies in some lines with respect to time.

4. The growth and development of embryogenic spruce callus is not a steady state process. Rather, callus composition and the number of somatic embryos are dependent upon the duration of the subculture interval.

5. The rapid (within one week) differentiation of embryos upon subculture suggests that shorter transfer intervals are beneficial.
Embryogenic Callus Initiation Frequency, %

embryogenesis fails for practical reasons

"window" of embryogenic potential

embryogenesis fails for theoretical reasons

June

July

August

Time
Culture method and conditions used in attempts to induce embryogenic callus from mature embryos of Norway spruce.

<table>
<thead>
<tr>
<th>Culture Method/Condition</th>
<th>Medium supplements, mg/L&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Whole embryos</strong></td>
<td></td>
</tr>
<tr>
<td>1. Standard&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2 2,4-D</td>
</tr>
<tr>
<td>2. dark</td>
<td>&quot;</td>
</tr>
<tr>
<td>3. on embryogenic callus</td>
<td>&quot;</td>
</tr>
<tr>
<td>4. on filter paper rafts placed on embryogenic callus</td>
<td>&quot;</td>
</tr>
<tr>
<td>5. standard</td>
<td>&quot; 2,4-D; 100-1900 NO&lt;sub&gt;3&lt;/sub&gt;&lt;sup&gt;-&lt;/sup&gt;; 0-1000 NH&lt;sub&gt;4&lt;/sub&gt;&lt;sup&gt;+&lt;/sup&gt;; + 1500 Glu</td>
</tr>
<tr>
<td>6. standard</td>
<td>2 2,4-D - amino acids; + 400 Glu</td>
</tr>
<tr>
<td>7. standard</td>
<td>1 NAA</td>
</tr>
<tr>
<td><strong>Sectioned embryos (cots, hypocots, radical)</strong></td>
<td></td>
</tr>
<tr>
<td>1. standard</td>
<td>2 2,4-D</td>
</tr>
<tr>
<td>2. standard</td>
<td>&quot; 2,4-D; 100-1900 NO&lt;sub&gt;3&lt;/sub&gt;&lt;sup&gt;-&lt;/sup&gt;; 0-100 NH&lt;sub&gt;4&lt;/sub&gt;&lt;sup&gt;+&lt;/sup&gt;; + 1500 Glu</td>
</tr>
</tbody>
</table>

<sup>a</sup>Bal medium = HM + 1 mg/L BA  
<sup>b</sup>Standard culture method refers to physical environment after Hakman et al.

Possible scenario for obtaining embryogenic callus in Douglas-fir

1. Culture immature embryos from late July/early August by Bormran protocol containing 2/0.2 and/or 1/0 (mg/L) 2,4-D/BA. (callus induction)
2. Transfer callus to HM medium containing 1% charcoal. (LFSE formation)
3. Transfer LFSEs to auxin containing medium. (embryogenic callus induction)

**Results to Date**

1. Embryogenic callus can be induced on mature embryos, but only infrequently.
2. Somatic embryos of Norway spruce appear to be better adapted to in vitro conditions than zygotic embryos.
3. When cultured on initiation medium, low frequency somatic embryos produce embryogenic callus.
Ultrastructural examination of embryogenic vs non-embryogenic Norway Spruce cells

1. To better characterize embryogenic conifer cells
2. Aid in our understanding and interpretation of visual marker (white vs green calli)

DEVELOPMENT OF NEW MARKERS AND TECHNIQUES

DNA \rightarrow mRNA \rightarrow protein (enzymes) \rightarrow ethylene
\rightarrow chloroplasts
\rightarrow GSH
\rightarrow polyamines

Isolation of mRNA from tissues (callus)

1. For use in in vitro translations (possible markers)
2. To generate cDNA probes
   a. Serve as possible markers (northern blotting)
   b. For use in RFLP (restriction fragment length polymorphism) analysis
   c. Used as genetic probes (analysis of genome-southern blotting)
Steps in genetic engineering

a. Identification and characterization of trait
b. Isolation of mRNA and DNA (trait specific)
c. Clone DNA
d. Transfer DNA to host plant
e. Identification and selection of transformed host plants
f. Plant regeneration
g. Stable expression, replication and transmission of introduced trait
Isolation of trait-specific gene (mRNA or cDNA): Techniques

1. Use related cDNA probe (another species)
2. Complementation of deficient mutant
3. Screen gene library with artificial gene fragment or antibody
4. "Plus-minus" screening
   a. differential hybridization
   b. cascade hybridization
mRNA preparation from embryo

\[ \downarrow \] cDNA in plasmid vector

cDNA clone library streaked onto 2 identical nitrocellulose filters, plus master plate

\[ \downarrow \] retain master plate

\[ \downarrow \] individual colonies streaked in grid pattern

\[ \downarrow \] pick desired colonies

\[ \downarrow \] example of clone containing a sequence abundant in both embryo and callus mRNA

\[ \downarrow \] prepare DNA-print

\[ \downarrow \] nitrocellulose DNA-print

\[ \downarrow \] prepare DNA-print

\[ \downarrow \] nitrocellulose DNA-print

\[ \downarrow \] hybridize with \( ^{32}P \)-cDNA from embryo mRNA

\[ \downarrow \] wash, etc.

\[ \downarrow \] autoradiograph

\[ \downarrow \] hybridize with \( ^{32}P \)-cDNA from embryo mRNA

\[ \downarrow \] wash, etc.

\[ \downarrow \] autoradiograph

Autoradiograph on X-ray film

clones containing a sequence that is abundant in embryo but not callus

Plus and minus screening.
REVERSE TRANSCRIPTASE AND P-LABELLED NUCLEOTIDES

mRNA from embryo

(\alpha_p, mRNA)

\downarrow

\alpha_p, cDNA

\downarrow

HYBRIDIZE

\uparrow

\alpha_p, cDNA REMAINS UNHYBRIDIZED

HIDROXYAPATITE
CHROMATOGRAPHY

cDNA LIBRARY

CLONED IN pBR322
(1% \alpha_p)

HYBRIDIZE

\uparrow

\alpha_p, cDNA

AUTORADIOPHGRAPHY
## ETHYLENE TEST

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Ethylene Evolution,* nL/mg fr. wt./day</th>
<th>N</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Embryogenic Callus</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Nonembryogenic Callus</td>
<td></td>
</tr>
<tr>
<td>5(NS1)8</td>
<td>0.15c</td>
<td>5</td>
</tr>
<tr>
<td>4(NS1)8</td>
<td>n.a.</td>
<td>3</td>
</tr>
<tr>
<td>4(NS1)2</td>
<td>0.32c</td>
<td>3</td>
</tr>
<tr>
<td>4(NS1)3</td>
<td>n.a.</td>
<td>3</td>
</tr>
<tr>
<td>4(NS1)9</td>
<td>0.18c</td>
<td>3</td>
</tr>
<tr>
<td>7(NS7)X</td>
<td>n.a.</td>
<td>5</td>
</tr>
<tr>
<td>5(NS1)12</td>
<td>0.17c**</td>
<td>5</td>
</tr>
<tr>
<td>4(NS1)12</td>
<td>0.36c</td>
<td>3</td>
</tr>
</tbody>
</table>

*n.a. = not available  
** = some green nonembryogenic callus present also.

## RATE OF PROTEIN SYNTHESIS IN NORWAY SPRUCE CALLUS

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Embryogenic</th>
<th>Non-embryogenic</th>
</tr>
</thead>
<tbody>
<tr>
<td>(NS1) 1</td>
<td>688 ± 518</td>
<td>32 ± 6</td>
</tr>
<tr>
<td>(NS1) 5</td>
<td>764 ± 257</td>
<td>60 ± 12</td>
</tr>
</tbody>
</table>
GLUTATHIONE TEST

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Emb. Callus</th>
<th>Nonemb. Callus</th>
<th>GSH,* n moles/g protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>10(NS1)6</td>
<td>134&lt;sup&gt;c&lt;/sup&gt;</td>
<td>434&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.35&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>10(NS1)7</td>
<td>141&lt;sup&gt;c&lt;/sup&gt;</td>
<td>473&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.31&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>10(NS1)8</td>
<td>120&lt;sup&gt;c&lt;/sup&gt;</td>
<td>325&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.27&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

*<sup>N = 3; H<sub>2</sub>O extracts; ANOVA and Duncan's New Multiple Range Test run separately for the fresh weight basis and the protein basis.</sup>

TOTAL REDUCTANTS TEST

SPECIFICITY

<table>
<thead>
<tr>
<th>Compound</th>
<th>A&lt;sub&gt;760&lt;/sub&gt;/m mole</th>
</tr>
</thead>
<tbody>
<tr>
<td>catechin</td>
<td>17.6</td>
</tr>
<tr>
<td>ferulic acid</td>
<td>13.5</td>
</tr>
<tr>
<td>caffeic acid</td>
<td>11.9</td>
</tr>
<tr>
<td>ascorbic acid</td>
<td>5.5</td>
</tr>
<tr>
<td>glutathione</td>
<td>3.2</td>
</tr>
<tr>
<td>p-coumaric acid</td>
<td>1.5</td>
</tr>
<tr>
<td>glucose</td>
<td>0.3</td>
</tr>
</tbody>
</table>
### TOTAL REDUCTANTS TEST

**Reductants, A760/g fr. wt.**

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Embryogenic Callus</th>
<th>Nonembryogenic Callus</th>
<th>N</th>
</tr>
</thead>
<tbody>
<tr>
<td>10(NS1)6</td>
<td>30d</td>
<td>518b</td>
<td>3</td>
</tr>
<tr>
<td>10(NS1)7</td>
<td>22d</td>
<td>438c</td>
<td>3</td>
</tr>
<tr>
<td>10(NS1)8</td>
<td>32d</td>
<td>535b</td>
<td>3</td>
</tr>
<tr>
<td>5(NS4)6</td>
<td>56d</td>
<td>1250a</td>
<td>4</td>
</tr>
</tbody>
</table>

### TOTAL REDUCTANTS TEST

**Reductants, A760/g fr. wt. ± S.D.**

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Embryogenic Callus</th>
<th>Nonembryogenic Callus</th>
<th>N</th>
</tr>
</thead>
<tbody>
<tr>
<td>10(NS2)5</td>
<td>31 ± 4</td>
<td>n.a.</td>
<td>3</td>
</tr>
<tr>
<td>7(NS2)4</td>
<td>n.a.</td>
<td>366 ± 67</td>
<td>4</td>
</tr>
<tr>
<td>7(NS1)13</td>
<td>63 ± 8</td>
<td>n.a.</td>
<td>3</td>
</tr>
<tr>
<td>5(NS3)17</td>
<td>n.a.</td>
<td>266 ± 73</td>
<td>3</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>White Callus</th>
<th>Green Callus</th>
</tr>
</thead>
<tbody>
<tr>
<td>5(DF9)E1</td>
<td>12</td>
</tr>
</tbody>
</table>

n.a. = not applicable
SCREENING PINE SOURCES FOR MORPHOGENIC POTENTIAL

Purpose: Identify pine seed sources with high organogenic potential.

Application: Obtain explant material from these seed sources for evaluation of embryogenic potential.

Seed sources for organogenic survey:

I. Loblolly pine (LP) full-sib crosses

1) LP 11 - 1135 x 18 - 1206
2) LP 11 - 1135 x 11 - 1017
3) LP 11 - 1052 x 11 - 1011
4) LP 11 - 297 x 11 - 283
5) LP 11 - 297 x 11 - 274
6) LP 11 - 1049 x 8 - 1003
7) LP 11 - 268 x 11 - 297
8) LP 11 - 268 x 11 - 283
9) LP 11 - 268 x 11 - 229
10) LP 11 - 268 x 11 - 274

II. Pitch pine (PP) half-sib clones

1) PP 62
2) PP 65
3) PP 71
4) PP 75
5) PP 80
Loblolly and pitch pine cotyledon regeneration protocol

1. Germinate nicked seeds in 1% H₂O₂, 30°C, 4 days, cool white fluorescent light, 24 μEm⁻²sec⁻¹.

2. Sterilize gametophyte 5 minutes with 15% clorox.

3. Excise embryos and place cotyledons on BLG medium with 10 mg/L BAP.

4. Incubate for 17 days, 23°C, cool white fluorescent, 24 μEm⁻²sec⁻¹ and incandescent, 14 μEm⁻²sec⁻¹.

5. Determine % green cotyledons.

6. Transfer explants to 1/2 GD medium with charcoal. Incubate 28 days as in step 4.

7. Determine % organogenic cotyledons.

Percent organogenic cotyledons from mature embryos of full-sib loblolly pine lines

<table>
<thead>
<tr>
<th>Full-sib seed source</th>
<th>mean % organogenic cotyledons per embryo</th>
<th>N</th>
</tr>
</thead>
<tbody>
<tr>
<td>LP 268 x 229</td>
<td>96 A</td>
<td>30</td>
</tr>
<tr>
<td>LP 268 x 297</td>
<td>83 AB</td>
<td>30</td>
</tr>
<tr>
<td>LP 297 x 274</td>
<td>82 AB</td>
<td>29</td>
</tr>
<tr>
<td>LP 268 x 283</td>
<td>72 ABC</td>
<td>27</td>
</tr>
<tr>
<td>LP 1052 x 1011</td>
<td>71 ABC</td>
<td>29</td>
</tr>
<tr>
<td>LP 268 x 274</td>
<td>63 BC</td>
<td>24</td>
</tr>
<tr>
<td>LP 297 x 283</td>
<td>55 BCD</td>
<td>28</td>
</tr>
<tr>
<td>LP 1049 x 1003</td>
<td>45 CD</td>
<td>25</td>
</tr>
<tr>
<td>LP 1135 x 1017</td>
<td>44 CD</td>
<td>29</td>
</tr>
<tr>
<td>LP 1135 x 1206</td>
<td>31 D</td>
<td>26</td>
</tr>
</tbody>
</table>

aDetermined after 17 days on induction medium (BLG with 10 mg/L BAP) and an additional 28 days on 1/2 GD with charcoal.

bN = Number of embryos from which cotyledons were excised, 5-6 cotyledons per embryo.
Comparison of the frequency of green loblolly pine explants after 17 days in culture to the frequency of organogenesis.

<table>
<thead>
<tr>
<th>Full-sib seed source</th>
<th>mean % cotyledons per embryo</th>
<th>green&lt;sup&gt;a&lt;/sup&gt;</th>
<th>organogenic&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>LP 268 x 229</td>
<td>94</td>
<td>96 A</td>
<td></td>
</tr>
<tr>
<td>LP 268 x 297</td>
<td>88</td>
<td>83 AB</td>
<td></td>
</tr>
<tr>
<td>LP 297 x 274</td>
<td>87</td>
<td>82 AB</td>
<td></td>
</tr>
<tr>
<td>LP 268 x 283</td>
<td>82</td>
<td>72 ABC</td>
<td></td>
</tr>
<tr>
<td>LP 1052 x 1011</td>
<td>84</td>
<td>71 ABC</td>
<td></td>
</tr>
<tr>
<td>LP 268 x 274</td>
<td>70</td>
<td>63 BC</td>
<td></td>
</tr>
<tr>
<td>LP 297 x 283</td>
<td>72</td>
<td>55 BCD</td>
<td></td>
</tr>
<tr>
<td>LP 1049 x 1003</td>
<td>67</td>
<td>45 CD</td>
<td></td>
</tr>
<tr>
<td>LP 1135 x 1017</td>
<td>66</td>
<td>44 CD</td>
<td></td>
</tr>
<tr>
<td>LP 1135 x 1206</td>
<td>61</td>
<td>31 D</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup>Determined after 17 days on induction medium, BLG with 10 mg/l BAP.

<sup>b</sup>Determined after 17 days on induction medium and an additional 28 days on 1/2 GD with charcoal.

Percent organogenic cotyledons from mature embryos of half-sib pitch pine clones.

<table>
<thead>
<tr>
<th>Half-sib seed source</th>
<th>mean % organogenic cotyledons per embryo&lt;sup&gt;a&lt;/sup&gt;</th>
<th>N&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>PP 62</td>
<td>85 A</td>
<td>17</td>
</tr>
<tr>
<td>PP 65</td>
<td>72 AB</td>
<td>30</td>
</tr>
<tr>
<td>PP 75</td>
<td>49 B</td>
<td>27</td>
</tr>
<tr>
<td>PP 80</td>
<td>49 B</td>
<td>30</td>
</tr>
<tr>
<td>PP 71</td>
<td>45 B</td>
<td>28</td>
</tr>
</tbody>
</table>

<sup>a</sup>Determined after 17 days on induction medium (BLG with 10 mg/L BAP) and an additional 28 days on 1/2 GD with charcoal.

<sup>b</sup>N = Number of embryos from which cotyledons were excised, 4-6 cotyledons per embryo.
Comparison of the frequency of green pitch pine explants after 17 days in culture to the frequency of organogenesis.

<table>
<thead>
<tr>
<th>Half-sib seed source</th>
<th>mean % cotyledons per embryo</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>green&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>PP 62</td>
<td>90 A</td>
</tr>
<tr>
<td>PP 65</td>
<td>77 AB</td>
</tr>
<tr>
<td>PP 75</td>
<td>64 B</td>
</tr>
<tr>
<td>PP 80</td>
<td>61 B</td>
</tr>
<tr>
<td>PP 71</td>
<td>57 B</td>
</tr>
</tbody>
</table>

<sup>a</sup>Determined after 17 days on induction medium BLG with 10 mg/l BAP.

<sup>b</sup>Determined after 17 days on induction medium and an additional 28 days on ½ GD with charcoal.