MASS PRODUCTION OF CONIFER HYBRIDS

Project 3223

Report Fifteen
A Progress Report
to
MEMBERS OF THE INSTITUTE OF PAPER CHEMISTRY

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SUMMARY

Emphasis in 1987 was concentrated more on our target species than in earlier years. Having found initiation in Norway spruce, our model system, rather straightforward, increased attention was given to obtaining embryogenesis in the target species. Greater effort was also devoted to embryo development/maturation and conversion. Work in the biochemistry area even more toward direct support of cell and tissue culture activities.

INITIATION OF EMBRYOGENIC CALLUS

The year was a landmark one for initiation. Our first embryogenic loblolly pine cultures were obtained early in the year. They have been maintained without great difficulty, and others have since been initiated. Overall frequencies have been low, but several open-pollinated families proved quite responsive and the best averaged five percent across treatments and lines. All told, embryogenic lines were obtained from 7 of the 10 families used in the experiments. Viewed another way, initiation occurred with explants from three separate collections, including those made in the U.S. during summer, 1986, from South America in winter, 1987, and from the U.S. in summer, 1987. Our protocol thus yielded reproducible results, and worked with explants from a number of genetic backgrounds. We were also able to identify the optimal explant developmental stage. This more precise definition of explant stage will increase efficiency of future work on initiation - fewer collections, lightened workloads, and more time for experimentation on other fronts.
The year also saw initiation of embryogenic callus in pitch X loblolly pine hybrids. To our knowledge, this is the first such report for a conifer hybrid. Mass cloning from even hybrid embryos would have considerable value because of the difficulty and expense of producing commercial quantities of hybrid seed by conventional means.

One hybrid line is highly vigorous, producing numerous early stage embryos with clear potential for development. Once this and the most active loblolly lines have been increased, we will use them in experiments on embryo development/maturation. At that time, we also would be pleased to offer samples to member companies for research in their own laboratories. In the meantime, we are using the results to frame new ways to increase initiation frequencies.

Efforts to induce embryogenesis in Douglas-fir intensified in 1987, with particular emphasis on explant developmental stage and media composition. Several test media produced translucent white callus, phenotypically similar to embryogenic spruce callus. Interestingly, such callus originated from different tissues depending upon explant developmental stage. Some calli produced cell types and proembryos resembling those in spruce, and results of biochemical assays were similar to those for embryogenic callus of other species. All cultures have since turned green or brown, and reproducible embryogenesis still eludes us, at least for the present. That is, some green cultures may yet prove embryogenic. One such culture, initiated in 1985, became embryogenic about 18 months later. Viewed as a whole, these results suggest that we are close to having the proper explant, and to triggering its potential. Further protocol refinements are needed, and we are considering the differential origin and sprucelike behavior in designing new experiments.
In keeping with near-term plans, efforts to develop additional biochemical markers have ceased, and results have been summarized for publication. Assays of the six species for which we have embryogenic and non-embryogenic callus show that most available markers clearly separate the two callus types. Indeed, one marker, total reductants, successfully predicted embryogenic potential of loblolly and pond pine cultures before appearance of somatic embryos. Similarity of responses among species suggests that embryogenesis is governed by a common set of metabolic reactions, and that the greatest utility of our markers is yet to be realized - provision of clues to critical mechanisms. Developing an appreciation for these may enable us to start or stop the correct ones at appropriate times.

DEVELOPMENT/MATURATION

The exciting news for the year about initiation was supplemented by modest progress toward assured and predictable embryo development. Considerable effort was devoted to empirical as well as biochemical studies of development. Included were several experiments evaluating factors leading to improved development in white spruce, and their impact on development in loblolly pine. A combination of abscisic acid and higher sucrose concentration clearly stimulated embryo development in both species. The two substances had complementary effects, and their utility varied with when they were applied and how long they were retained in the media.

At the time of treatment, white spruce calli were further advanced than pine, and the best media moved the former much further along. Indeed, embryogenesis was completed in white spruce; many embryos reached the cotyledon stage and one seedling was transferred to soil. Pine development stopped before the cotyledon stage, but nevertheless did progress farther than in earlier work.
Different callus lines varied in response to treatment, and no one treatment affected all lines the same. This outcome may be a genetic effect, but more likely results from individual calli not initially being at the same stage of development. To offset this, we are working to improve our understanding of such variation, of what stages are receptive to manipulation, of how long each stage remains responsive to treatment, and of what treatments are needed when embryos move to yet another stage.

In another approach to development, an attempt was made to develop immature zygotic embryos of loblolly pine in culture and convert them to seedlings. Immature embryos at several stages of development were cultured on different media, with and without various nutrients and extracts of female gametophytes. Results showed that precotyledonary embryos could not be grown into plants, and only half or less reached the cotyledon stage. Development stopped even on our best media. Combining leads from these experiments with results from earlier work on somatic embryos nevertheless provided much information about the stage at which development stops and about the kinds of media changes that are needed. In contrast, zygotic embryos just beyond the cotyledonary stage readily elongated, regardless of culture conditions, and many were rooted and transferred to soil. Thus, techniques available for conversion to seedlings, though not optimal, are reasonably workable. Improving their efficiency should be relatively straightforward, and easier than perfecting the methods needed for early development.

To aid and abet the empirical approaches described above, we are also endeavoring to understand the physiology and biochemistry of embryo development. Typical approaches involve monitoring appearance and/or disappearance of proteins, lipids, and other compounds during development of zygotic and somatic
embryos. Knowing patterns normal for zygotic embryos may enable us to better steer somatic embryo development.

Along these lines, methods for examining protein patterns were refined for application to conifer systems, and used to follow protein accumulation in developing zygotic embryos of loblolly pine. Embryos in six different stages of development were available from cones collected for initiation experiments. A number of proteins were conspicuous by virtue of gradual accumulation over the summer, several were found to appear suddenly at later stages, and a few appeared during mid-development and disappeared later. Results mirrored those observed in a number of Angiosperms, where both protein patterns and development have been linked to changes in concentration of abscisic acid. Such findings underscore the merits of using tools, such as abscisic acid and media osmolarity, to further development of somatic embryos.

Earlier observation of differences in the number and size of lipid bodies in electron micrographs of zygotic and somatic embryos prompted work on patterns of lipid accumulation in embryogenic calli, developing embryos, and fully developed seeds. Preliminary results indicate that both embryos and female gametophytes from fully developed seed have similar lipid composition. Of greater interest, a polar lipid compound was found in embryogenic spruce callus, but not in nonembryogenic callus. This same or a similar lipid was later found in early stage zygotic embryos of loblolly pine. Detailed comparisons of developing zygotic and somatic embryos are underway to determine if this or related compounds can be used to track or promote development.

In a similar vein, an effort was made to capitalize on earlier demonstration of parallels between glutathione biosynthesis and total reductants
content and development of zygotic embryos of red and white pines. These parameters along with activity of the enzyme, glutathione reductase, were examined in developing zygotic embryos and female gametophytes of Douglas-fir and loblolly pine. Changes in glutathione and total reductants content of Douglas-fir varied much as in the earlier pine work. Observations in loblolly differed somewhat, possibly as a result of delayed seed development occasioned by drought. Patterns of enzyme activity in loblolly paralleled those of glutathione, suggesting a role in regulating reduced glutathione content. Such findings are helping determine when and how to use tools, such as inhibitors of glutathione metabolism, to foster development of somatic embryos.

CONVERSION AND FIDELITY/PERFORMANCE

"Somatic seedlings" produced in 1986 figured in several experiments this year and some new "seedlings" are being readied for transfer to the greenhouse. Results to date indicate that Norway spruce lines used in the experiments produced mature embryos at frequencies varying from 1 to 15 percent. With our best treatments, 56 percent of mature embryos initiated root growth, and 29 percent of these survived transfer to and growth in the greenhouse. Though overall recovery was low, the experiments improved our understanding of the individual process steps and provided several useful leads.

Surviving plants are phenotypically similar to and behave like regular spruce seedlings. They set buds, went dormant over winter, and initiated spring growth in synchrony with their zygotic counterparts. Growth during 1987 roughly equalled that of controls. This is the first demonstration of normal growth and behavior of such plants across seasons.
Some preliminary work on genetic fidelity was also done, mainly in the interest of testing and refining isozyme analyses. First results confirmed, as expected, that somatic "seedlings" of the same clone and developmental status had identical isozyme patterns. Zygotic seedlings, in contrast, had variable patterns. Perhaps the most significant outcome was enhanced sensitivity of the techniques. Isozyme patterns can be detected for single needles and even individual embryos. Since some isozyme patterns vary with developmental stage, the techniques should be useful for tracking development of zygotic and somatic embryos, and identifying ways to improve protocols for development.

EXPLORATORY RESEARCH

Efficiency of mass cloning would be increased by systems that produce large numbers of somatic embryos rapidly and with minimal labor. Cell suspensions are an attractive alternative in these regards, and exploratory work with Norway spruce suspensions paid significant dividends in 1987. Embryo proliferation was rapid, with embryo numbers peaking at roughly 100 per mL of suspension after only 14 days. Proliferation continued when subculturing was done at 10 to 12 day intervals. Manipulations attempted to date, regrettably, did not foster complete embryo development, but several promising tacks, including sequential changes in composition of suspension media, are being pursued.

Protoplast cultures could provide an ideal system for mass cloning, genetic transformation, and generation of novel variants. Exploratory work aimed at developing a workable protoplast system continued this year with cells from our successful Norway spruce suspension cultures. A variety of methods for protoplast isolation and culture were tested. The best gave significant yields of viable protoplasts, with roughly 50 percent remaining viable for use in
further experiments. About five percent divided at least once, and a small fraction continued dividing to form small cell clusters. Given this degree of progress, we are continuing to explore means for fostering division and obtaining callus.

Also on the exploratory front, availability of sweetgum shoot cultures provided an opportunity for attempting genetic transformation. The work was successful and marks the first introduction and expression of foreign genes in a commercially important southern hardwood species. In addition, methods used to confirm transformation will be useful in our main effort on somatic embryogenesis.
INTRODUCTION

Advances in cell and tissue culture of forest trees were considerable in 1987. In many ways, the year was a landmark one at the Institute. Much progress was made on a variety of fronts, ranging from initiating embryogenic callus through enhancing embryo development to planning for the future.

The most significant accomplishment occurred in our work on loblolly pine. We were successful in initiating embryogenic callus and repeated the feat on several occasions with explants from a number of different genetic backgrounds. Optimal explant developmental stage was also identified. Access to cones from South America permitted experimentation during both summer and winter months and was responsible in part for our being able to test a variety of protocols and more quickly determine the best. We are quite confident of our protocol, as it works well with a number of pines. Along the way, we achieved yet another first - embryogenic callus in the hybrid of pitch and loblolly pine, a hybrid of much commercial interest.

Work on development and maturation of somatic embryos, and their conversion to "somatic seedlings," regrettably, did not progress as well as anticipated. A number of new protocols, involving different growth regulators, media osmolarity, and nitrogen sources were tested. These produced only a very few new "seedlings," but did foster embryo development in some species to stages beyond those typically seen in earlier work. As a result, we acquired an improved understanding of these important steps and developed new hypotheses and approaches for testing in 1988.

Difficulties on these important fronts are such, however, that we will add appropriate new talent in the coming year and further alter the focus of
our biochemistry efforts. Though consistently supportive of tissue culture efforts over the past year, our biochemistry workers will become even more involved in improving our abilities to develop, mature, and recover seedlings.

Efforts to increase visibility of our work also intensified and have begun to pay a variety of dividends. Roughly half the individual contributions in this report have been accepted by or submitted to technical journals or conference proceedings, or are being readied for submission to such outlets. Well-known visitors and invitations to national and international conferences are also part of the growing returns. Several visitors during 1987 brought us new ideas and opportunities. For example, a national tissue culture conference will contain a session on forest trees as a result of interaction between us and the conference organizing committee. With continued effort on these fronts, we also expect to become more attractive to both the industry and government agencies from the standpoint of contracts and/or grants.

The year brought another noteworthy, though quite unexpected, development. An unusually large number of entering students elected to take their research experience in the Forest Biology Division. The majority will work on cell and tissue culture problems, thereby adding significant leverage to staff efforts.

This report summarizes work during the past year, and the considerable progress made during that time. It should also be viewed, however, as the foundation for intensified work in traditional areas and for new work on yet other fronts. We will devote more effort to raising initiation frequencies in our target species and to refining protocols for embryo development, maturation, and conversion in both our model system and target species. Continued refocusing
of our biochemistry should contribute much to these efforts. In addition, we expect to open research on mass cloning of selected hardwoods in response to enlarged member company interest. The road ahead may be difficult, but we remain enthusiastic about making somatic embryogenesis a usable technology and look forward to taking on new challenges.
INTRODUCTION

The first successful reports of embryogenesis in conifers utilized immature embryos of Norway spruce (Hakman et al. 1985) and female gametophytes of European larch (Nagmani et al. 1985). By optimization of media components it has been possible to extend the "window" of initiation to mature embryos of spruce (von Arnold 1987, and Report Fourteen; see also section on somatic embryogenesis in white spruce in this report). Our current efforts to initiate embryogenic cultures in Pinus have concentrated mostly on immature embryo explants. In order to avoid initiating cultures over a broad time span, which may include nonresponsive stages of embryo development, there is a need to identify useful markers or indices of embryo development that easily identify the optimum stage. Fertilization has served as a useful explant developmental marker in numerous herbaceous plants. For example in cereals, where controlled pollinations can easily be made, the optimum stage of embryo explant development can be identified by the number of days postpollination (Kamo et al. 1985).

It is difficult to efficiently and precisely determine the time of fertilization in conifers because of the extended and species dependent variable lag in time between pollination and fertilization. Our previous results (Report Fourteen) with Norway and white spruce have shown that the optimum stage of immature embryo development for initiation of embryogenic callus was post-cotyledonary. Our initial results with pond and white pine suggested that the optimum window was at a much earlier stage, i.e., from the proembryo to just prior to the cotyledonary stage of development (Report Fourteen).
Results presented here summarize our efforts to initiate embryogenic callus from loblolly pine and pitch x loblolly pine hybrid immature embryos. In order to determine the optimum window for initiation, the frequency of initiation as a function of embryo development has been determined and comparisons have been made to initiation in spruce. Several clones of loblolly pine with differing genetic background were included in this study in order to evaluate clonal effects on initiation.

MATERIALS AND METHODS

Plant Materials

Refer to the appendix for a list of the loblolly pine clones and one pitch x loblolly pine hybrid included in this study. Results are presented for three separate cone collections of loblolly pine: 1) cones collected during the summer of 1986 and held in cold (4°C) for approximately 2 months prior to initiation, 2) cones collected in Brazil and shipped to IPC during January and February, 1987, and 3) cones collected during the summer of 1987 and cultured fresh upon receipt at IPC. The pitch x loblolly hybrid cones were collected during the summer of 1987 and cultured fresh upon receipt.

Seeds were removed from cones and surface sterilized in 20% commercial bleach for 15 min followed by three rinses in sterile distilled water. Seed coats were aseptically removed and the following explants were cultured: 1) the immature embryo enclosed in the intact female gametophyte (hereafter referred to as "whole female gametophyte" explants), 2) as above, but the female gametophyte cut open longitudinally ("split female gametophyte"), and 3) isolated immature embryos.
Media and Culture Initiation

The basal media used (see appendix for complete list of components) were MSG, MSCG, DCR, and DZL. The MSG is similar to the BLG medium used by Amerson et al. 1985. MSG is devoid of ammonium nitrogen. MSG medium used according to the "Smith protocol" also contained 1% activated charcoal (Smith et al. 1985). MSCG is a modified MSG containing casein hydrolyzate. DCR medium contains about one-fourth the level of ammonium nitrogen as basal MS (Gupta and Durzan, 1985). DZL medium was the same as that reported by Gupta and Durzan (1986) with the exception that the inorganic nitrogen components ($\text{NH}_4\text{NO}_3$ and $\text{KNO}_3$) were used at full strength. Media contained auxin ($2,4$-$\text{D}$) at levels from 0 to 11 mg/L and cytokinin (BA) from 0 to 1 mg/L. Hereafter, medium coded as MSG 2/1 refers to the basal medium (MSG) and the auxin/cytokinon levels (2/1) in mg/L. The DZL medium also contained 5 mg/L of the cytokinin, kinetin, and is coded DZL 11/4,5. All components of the media were autoclaved at 121 psi for 15 min, with the exception of glutamine, which was filter sterilized and added to warm (≈ 50°C) medium.

Five explants were cultured in each 50 mm plastic petri plate containing 6-8 mL of medium. Cultures were incubated in the dark at 23°C. Four weeks after initiation, explants were transferred to a new position on the same plate. Two to four weeks later, explants which formed embryogenic callus (thereafter identified by an individual line no., refer to appendix for code) were transferred to fresh medium. Subsequently the embryogenic callus was subcultured to fresh medium at 2-3 week intervals.

Data Collection and Analysis

At the time of culture initiation, embryo length and the presence or absence of cotyledonary primordia was determined on randomly selected subsamples
(n = 10-12) from clones F through P and the pitch x loblolly hybrid. Length measurements were made on a dissecting microscope with an eye-piece micrometer. Six to eight weeks after culture initiation the number of explants per plate which formed embryogenic callus was recorded. In one experiment the number of explants which formed nonembryogenic callus was also recorded. Data are presented as mean values among replicated experimental units (e.g., the percentage of embryogenic explants per culture plate).

RESULTS AND DISCUSSION

Stage of Explant Development

The stages of loblolly, pitch, and pitch x loblolly pine embryo development from cones collected during the summer of 1986 and 1987 are shown in Fig. 1. Note the similarities in the time course of embryo development between the two years. In the subsequent discussions considerable attention will be given to the optimum stage of embryo development for initiation of embryogenic callus. In particular, cotyledonary primordia formation is examined as an index of optimum embryogenic potential. In loblolly pine, embryos collected prior to about August 1 were predominantly precotyledonary, whereas subsequent collections contained mostly cotyledonary embryos (Fig. 1). Similarly, this transition from precotyledonary to cotyledonary development occurred about July 1 in both pitch and pitch x loblolly pine.

Census of Cultures

Over 27,000 explants of loblolly and pitch x loblolly pine were cultured and 69 embryogenic callus lines were initiated (Table 1). The results of specific experiments on initiation of the embryogenic callus are discussed in the following sections according to each cone collection time listed in Table 1.
Figure 1. Time course of embryo development in loblolly, pitch, and pitch x loblolly pine in 1986 (open circles) and 1987 (closed circles). A) Embryo length, B) Percentage of embryos with cotyledonary primordia.
Table 1. Census of loblolly and pitch x loblolly pine explants cultured and embryogenic callus lines initiated.

<table>
<thead>
<tr>
<th>Pine Species</th>
<th>Explant Collection</th>
<th>Number of</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Explants</td>
<td>EC Lines</td>
<td></td>
</tr>
<tr>
<td>Loblolly</td>
<td>GA &amp; SC</td>
<td>July, 86</td>
<td>1,775</td>
<td>9</td>
</tr>
<tr>
<td></td>
<td>Brazil</td>
<td>Jan., 87</td>
<td>8,991</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>GA &amp; SC</td>
<td>July, 87</td>
<td>15,667</td>
<td>48</td>
</tr>
<tr>
<td>Pitch x loblolly</td>
<td>SC</td>
<td>June, 87</td>
<td>885</td>
<td>6</td>
</tr>
<tr>
<td>TOTALS</td>
<td></td>
<td></td>
<td>27,318</td>
<td>69</td>
</tr>
</tbody>
</table>

aGA = Rincon, Georgia, SC = Summerville, South Carolina, and Brazil = Tres Barras, Santa Catarina.

Cold Stored Cones from July 1986 Collection

During the summer of 1986 over 15,000 explants of loblolly pine were cultured. A white mucilaginous callus with embryogenic potential was initiated from these cultures, but it could not be maintained (refer to Report Fourteen). The experiments described in this section utilized the remaining loblolly pine cones collected during the summer of 1986 which were held in cold storage (Table 2). The clones are ranked according to the number of explants that were cultured. The number of cones available in cold storage varied considerably from clone to clone. Therefore, the number of explants varied considerably, from 450 for clone H to only 9 from clone C. Most of the experiments were conducted with explants from clones H, J, G, and F; and of these, clones H and F were responsive. One embryogenic callus line was derived from explants of clone B, in an experiment with considerably fewer explants. It is not possible to draw conclusions solely from this data with regard to clonal differences in initiation, due to the large differences in the number of explants cultured per clone.
Table 2. Summary of initiation of embryogenic callus from 1986 cold stored cones of loblolly pine.

<table>
<thead>
<tr>
<th>Clone</th>
<th>Total No. Explants</th>
<th>Number EC Line</th>
<th>EC Initiation, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>H</td>
<td>450</td>
<td>3</td>
<td>0.7</td>
</tr>
<tr>
<td>J</td>
<td>325</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>G</td>
<td>280</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>F</td>
<td>275</td>
<td>5</td>
<td>1.8</td>
</tr>
<tr>
<td>I</td>
<td>160</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>E</td>
<td>137</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>A</td>
<td>114</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>B</td>
<td>50</td>
<td>1</td>
<td>1.0</td>
</tr>
<tr>
<td>D</td>
<td>25</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>C</td>
<td>9</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

aExplants were both isolated immature embryos and whole female gametophytes. Explants were derived from July 14, July 21, July 28, and Aug. 4 collections and immature embryos were mostly precotyledonary. Data derived from experiments: RP488, 501, 506, and 524.

Isolated immature embryos were cultured on four medium treatments (Table 3). These preliminary results suggested that low levels of 2,4-D (2-3 mg/L) were effective, whereas a higher level (10 mg/L) was not. The results also suggested that precotyledonary embryos less than 0.3 mm were more effective than later stage embryos.

Table 3. Effect of stage of explant development and media modifications on initiation of embryogenic callus from immature embryo explants of loblolly pine.

<table>
<thead>
<tr>
<th>Stage of Explant Developmenta</th>
<th>Frequency (%) of Embryogenic Lines Maintained</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Basal Medium.</td>
</tr>
<tr>
<td></td>
<td>DCR 10/0.5        DCR 3/0.5        MSG 10/1  MSG 2/1</td>
</tr>
<tr>
<td>Embryo Size, Embryos with Cotyledons, %</td>
<td>N</td>
</tr>
<tr>
<td>mm</td>
<td></td>
</tr>
<tr>
<td>0.1-0.3</td>
<td>0</td>
</tr>
<tr>
<td>0.7-1.2</td>
<td>37</td>
</tr>
</tbody>
</table>


bN = number of explants cultured per each medium treatment.
The explants and media on which the nine embryogenic callus lines were initiated are summarized in Table 4. These were the first cell lines of loblolly pine with the embryogenic phenotype that were successfully maintained by the IPC tissue culture team. Lines (LP13F)5 and (LP12H)1 have shown the best somatic embryo developmental potential (refer to section in this report on pine somatic embryo development). The cell lines were derived from either the culture of female gametophytes with the developing immature embryo intact (whole female gametophyte) (Fig. 2A) or from the culture of isolated immature embryos (Fig. 2B). In both cases the embryogenic callus originated from the suspensor region of the immature embryos. We have referred to the white, mucilaginous callus formed at the archegonial end of the female gametophyte as "extruded callus" (Fig. 2A). On maintenance medium the lines were composed of mostly elongated suspensor like cells and preembryonal masses ("PEM's").

Table 4. Summary of embryogenic callus lines initiated from explants derived from 1986 cold stored cones of loblolly pine.

<table>
<thead>
<tr>
<th>Explant</th>
<th>Initiation Medium</th>
<th>Research Plan (RP) No.</th>
<th>EC Line No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Whole female gametophyte</td>
<td>MSG 0/0</td>
<td>506</td>
<td>(LP11H)20</td>
</tr>
<tr>
<td></td>
<td>MSG 0/0</td>
<td>506</td>
<td>(LP11H)21</td>
</tr>
<tr>
<td>Immature embryo</td>
<td>DCR 3/0.5</td>
<td>488</td>
<td>(LP13B)1</td>
</tr>
<tr>
<td></td>
<td>MSG 2/1</td>
<td>501</td>
<td>(LP12F)1</td>
</tr>
<tr>
<td>Immature embryo</td>
<td>DCR 3/0.5</td>
<td>501</td>
<td>(LP12F)2</td>
</tr>
<tr>
<td>Immature embryo</td>
<td>MSG 2/1</td>
<td>501</td>
<td>(LP12F)3</td>
</tr>
<tr>
<td>Immature embryo</td>
<td>DCR 3/0.5</td>
<td>501</td>
<td>(LP13F)5</td>
</tr>
<tr>
<td>Immature embryo</td>
<td>DCR 3/0.5</td>
<td>501</td>
<td>(LP13F)6</td>
</tr>
<tr>
<td>Immature embryo</td>
<td>DCR 3/0.5</td>
<td>524</td>
<td>(LP12H)1</td>
</tr>
</tbody>
</table>
Brazilian Cones

Three collections of loblolly pine cones were received from Brazil (Table 5). The three collections spanned the desired range of early embryo development; from the proembryo stage on the January 6 collection, to the beginning of cotyledon development (clones K and P) on the February 10 collection.

Table 5. Stage of embryo development of loblolly pine cones received from Brazil in 1987.

<table>
<thead>
<tr>
<th>Collection No.</th>
<th>Date</th>
<th>Mean Embryo Length, a mm (cotyledonary, %)</th>
<th>Clones,</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>K</td>
<td>L</td>
</tr>
<tr>
<td>1</td>
<td>Jan. 6</td>
<td>pe(0)</td>
<td>pe(0)</td>
</tr>
<tr>
<td>2</td>
<td>Feb. 3</td>
<td>0.8(30)</td>
<td>0.6(20)</td>
</tr>
<tr>
<td>3</td>
<td>Feb. 10</td>
<td>1.4(90)</td>
<td>0.2(0)</td>
</tr>
</tbody>
</table>

\(^{a}pe = \) proembryo stage; too small to measure on dissecting microscope. ND = not determined.
The results of four experiments using whole female gametophyte explants (with intact developing embryos) are summarized in Table 6. Three embryogenic callus lines were established on only the Smith protocol (MSG 0/0) from over 5000 explants cultured. The results of one experiment (IV = RP555) comparing the culture of whole versus split female gametophytes was inconclusive because embryogenic callus was not initiated on any of the media or treatments tested. It should also be noted that the three embryogenic callus lines obtained, LP1K-2, LP1L-1, and LP10-1, were all derived from cone collections which contained only early stage proembryos (refer to Table 5). This stage of embryo development preceded cotyledonary development by several weeks.

Table 6. Summary of embryogenic callus (EC) lines established from Brazilian loblolly pine whole female gametophyte (with embryos intact) explants.

<table>
<thead>
<tr>
<th>Initiation Medium</th>
<th>Experimentsa</th>
<th>Number of Explants</th>
<th>EC Lines</th>
</tr>
</thead>
<tbody>
<tr>
<td>MSG 0/0</td>
<td>I, II, and III</td>
<td>2500</td>
<td>3</td>
</tr>
<tr>
<td>DZL 11/4,5</td>
<td>III and IV</td>
<td>1528</td>
<td>0</td>
</tr>
<tr>
<td>MSG 2/1</td>
<td>IV</td>
<td>1040</td>
<td>0</td>
</tr>
</tbody>
</table>

\(a\) I = RP537, II = RP543, III = RP545, and IV = RP555.

Three embryogenic callus lines were also established from the culture of isolated immature embryos (Table 7). It should also be noted that these three embryogenic callus lines, LP2N-3, LP2N-4, and LP3L-1, were all derived from collections which contained precotyledonary embryos (refer to Table 5 for the stage of embryo development).

In summary, all of the embryogenic callus lines derived from the Brazilian cones were from precotyledonary embryos or from whole female gametophytes which contained precotyledonary embryos.
Table 7. Summary of embryogenic callus established from Brazilian loblolly pine immature embryo explants.

<table>
<thead>
<tr>
<th>Collection Date</th>
<th>Developmental Stage</th>
<th>Number of EC Lines, a</th>
</tr>
</thead>
<tbody>
<tr>
<td>2/3/87</td>
<td>Precot.</td>
<td>0 0 2</td>
</tr>
<tr>
<td>2/10/87</td>
<td>Mostly precot.</td>
<td>0 0 1</td>
</tr>
</tbody>
</table>

a400 to 500 explants from five seed families cultured per each treatment and date.

Summer 1987 Collections

The three initiation protocols used were: 1) whole female gametophytes cultured on MSG 0/0, 2) whole female gametophytes cultured on MSCG 5/0, and 3) isolated immature embryos cultured on DCR 3/0.5. Embryogenic callus was initiated on all three protocols, albeit at very low frequencies. The results presented in Fig. 3 are initiation frequencies pooled across clones. Note that the initiation window of the embryogenic callus extruded from whole female gametophytes was from June 22 through July 20, prior to the time when isolated embryos could be cultured. Thus it appears that the female gametophyte may serve to protect the delicate embryos or act as a nurse tissue enabling initiation at an earlier stage of development. The highest initiation of embryogenic callus, about 1.3%, occurred on July 27 from embryos cultured on DCR 3/0.5 (Fig. 3). The mean length of embryo explants collected July 27 was about 0.5 mm and nearly all embryos were precotyledonary (refer to Fig. 1).

The level of differentiation of the embryogenic callus of loblolly pine varied considerably among cell lines derived from individual explants. Some embryogenic callus lines were composed of PEM's and elongated suspensorlike cells when maintained on proliferation medium (Fig. 4A). Other cell lines also
contained somatic embryos while growing on proliferation medium (Fig. 4B). An example of a loblolly pine embryogenic callus line with numerous well formed somatic embryos is shown in Fig. 4C. The level of differentiation of this embryogenic loblolly pine line is similar to Norway spruce, in that numerous somatic embryos were visible microscopically on the surface of the callus.

\[ I = \text{MSG 0/0} \]
\[ A = \text{MSCG 5/0} \]
\[ B = \text{DCR 3/0.5} \]

Figure 3. The frequency of initiation of embryogenic callus as a function of time in loblolly pine using three protocols. A, solid bars = whole female gametophyte explants on MSG 0/0. B, shaded bars = whole female gametophyte explants on MSCG 5/0. C, open bars = immature embryos on DCR 3/0.5.

A summary of initiation among the ten clones surveyed is presented in Fig. 5. The only data included in the Fig. 5 comparison were from collection.
dates July 6 through August 3 where an equal number of explants (about 1000) were cultured of all ten clones. Earlier and later collections did not contain all ten clones (for instance, the June 22 and 29 collections contained only clones F, G, H, J, and R) and were not included in the Fig. 5 comparison in order to avoid any clonal bias.

Figure 4. Differences in the level of differentiation among embryogenic callus lines of loblolly pine while grown on proliferative conditions. A: Micrograph of embryogenic callus composed of pre-embryonal masses (pem's) and elongated suspensorlike cells. Line LP2H-4 initiated on MSCG 5/0. B: Micrograph of embryogenic callus with well formed somatic embryo (se). Line LP6F-2 initiated on DCR 3/0.5. C: Somatic embryos visible microscopically on the surface of embryogenic callus. Line LP3C-1 initiated on DCR 3/0.5. Scale bars: A = 100 μm, B = 250 μm, C = 500 μm.
Embryogenic callus was initiated in seven of the ten clones surveyed (Fig. 5). The most responsive clones (F, H, and D) accounted for 26 of the 35 (74%) embryogenic callus lines. It should be noted that initiation of embryogenic callus in other experiments not included in the Fig. 5 comparison was only from the responsive clones F, G, and H. Thus all other experiments conducted were consistent with the results in Fig. 5. This is the first time that the IPC tissue culture team has been able to make a large scale comparison of initiation among clones tested together on an equivalent basis in terms of replications and media treatments. It strongly suggests that certain clones are more responsive than others. Furthermore, these results are consistent with previously described results obtained from the 1986 cold stored cones, where clones F and H were the most responsive. Our results are also consistent with studies in numerous other plant species, as in maize and barley for example (Hodges et al. 1986, Luhrs and Lorz 1987), where highly embryogenic genotypes have been identified.

In a separate experiment we also compared initiation of embryogenic callus on the Smith protocol and a modification of the medium described by Gupta and Durzan (1986). As indicated previously, the modified medium, DZL, was the same as that reported by Gupta and Durzan (1986) with the exception that the inorganic nitrogen components (NH₄NO₃ and KNO₃) were used at full strength. Only the most responsive clones, F and H, were used in this study and the results are summarized in Table 8. Embryogenic callus was only initiated on the Smith protocol, during late June from clone H and during mid-July from clone F. Initiation at an earlier date from clone H is consistent with the fact that embryo development of clone H preceded clone F by one to two weeks (data not shown). This result further emphasizes the importance of the stage of embryo development for initiation. The lack of initiation with the modified Gupta and Durzan medium (DZL) is difficult
to interpret precisely. It is possible that the full strength nitrogen we used was inhibitory to initiation. In fact, excessive secretions from the whole female gametophyte explants into the medium were observed and may have been due to the high levels of nitrate (53 mM) in the DZL medium. Further tests are planned to evaluate initiation on the half-strength medium of Gupta and Durzan.

<table>
<thead>
<tr>
<th>IPC CODE</th>
<th>FAMILY</th>
<th>0</th>
<th>0.2</th>
<th>0.4</th>
<th>0.6</th>
<th>0.8</th>
<th>1.0</th>
</tr>
</thead>
<tbody>
<tr>
<td>F</td>
<td>7-34</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>●</td>
</tr>
<tr>
<td>H</td>
<td>11-9</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>●</td>
<td></td>
</tr>
<tr>
<td>D</td>
<td>10-1018</td>
<td></td>
<td></td>
<td></td>
<td>●</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>10-1003</td>
<td></td>
<td></td>
<td>●</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>10-1011</td>
<td></td>
<td>●</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>G</td>
<td>7-56</td>
<td></td>
<td></td>
<td></td>
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<td>B</td>
<td>10-1007</td>
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<td></td>
<td>●</td>
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<td>E</td>
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</tr>
<tr>
<td>J</td>
<td>11-16</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>●</td>
</tr>
<tr>
<td>R</td>
<td>11-25</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>●</td>
</tr>
</tbody>
</table>

**Figure 5.** Comparison of initiation frequency of embryogenic callus among ten families of loblolly pine. Each data point includes initiation events from two of the following protocols: MSG 0/0, MSCR 5/0, and DCR 3/0.5. Approximately 1000 explants cultured per clone.

The highest initiation frequency obtained on a per treatment basis was 3 to 5% from immature embryos of clones H and F cultured on DCR 3/0.5 medium (Fig. 6). This high level of initiation occurred on July 27 and dropped to much lower levels within two weeks (Fig. 6A). Initiation of embryogenic callus was nearly mutually exclusive with initiation of nonembryogenic callus (compare Fig. 6A and 6C). That is, during the optimum precotyledonary stage of embryo development, July 27, very little nonembryogenic callus was initiated. In contrast, when the
embryos were mostly cotyledonary, August 10, little embryogenic callus was initiated
but nonembryogenic callus was initiated very efficiently. This agrees with our
previous results (refer to Report Fourteen) that have shown that nonembryogenic
callus can easily be initiated from *Pinus* embryos during late (postcotyledonary)
development. It also illustrates the fact that identification of embryogenic
callus can be made quite unambiguously during the optimum precotyledonary stage,
because it is essentially the only type of persistent cell division that occurs.
The results in Fig. 6B also show that nearly 50% of clone F embryos on July 27
initiated cell division in the suspensor region. This division of cells in the
suspensor region occurred transiently during the first 7 to 14 days in culture.
Only about 10% of the embryos maintained cell division activity and formed embryo-
genic callus (compare Figs. 6A and 6B). This finding suggests that it may be
possible to significantly increase initiation frequencies of embryogenic callus
in loblolly pine by optimization of the medium components in order to maintain a
higher frequency of persistent cell division activity of the suspensor region.

Table 8. Comparison of the frequency of initiation of embryogenic callus
in loblolly pine on the Smith protocol (MSG 0/0) and a modification
of the medium described by Gupta and Durzan (DZL 11/4,5).

<table>
<thead>
<tr>
<th>(Month/date)</th>
<th>Clone F</th>
<th>Clone H</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MSG 0/0</td>
<td>DZL 11/4,5</td>
</tr>
<tr>
<td>6/15</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>6/22</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>6/29</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>7/6</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>7/13</td>
<td>0.8</td>
<td>0</td>
</tr>
<tr>
<td>7/20</td>
<td>0.8</td>
<td>0</td>
</tr>
<tr>
<td>7/27</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>8/3</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>8/10</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

*Postfertilization female gametophyte explants, 75 to 120, cultured per each clone, medium treatment, and date.*
Figure 6. The time course of three morphogenic responses of immature embryos of loblolly pine cultured on DCR3/0.5 medium. A: Initiation frequency of embryogenic callus. B: Frequency of transient division of cells in the suspensor region. C: Initiation frequency of nonembryogenic callus. The stage of embryo development is shown on the top. Solid bars = clone F and open bars = clone H.

Initiation in Pinus vs. Picea

Our previous results with spruce have shown that immature embryos at the postcotyledonary stage initiate the highest frequency of embryogenic callus (Report Fourteen and Becwar et al. 1988). A comparison of initiation of embryogenic
callus in white spruce and loblolly pine is shown in Fig. 7. The results emphasize differences in the optimum initiation windows in spruce and pine. Unlike spruce, the optimum stage in loblolly pine occurs at a much earlier stage of embryo development, during the precotyledonary stage. Note also the differences in initiation frequency between spruce and pine (Fig. 7). The highest initiation frequency in spruce is nearly 10-fold higher than pine. This difference between spruce and pine has been consistent with several species; white pine (Wann et al. 1988) and pond pine (Report Fourteen) have also been very low, whereas Norway spruce has been very high (Report Thirteen and Becwar et al. 1987). The site of origin of the embryogenic callus is also different between spruce and pine. Spruce embryogenic callus originates from the hypocotyl (Nagmani et al. 1987) and less frequently from cotyledons (Krogstrup 1986, Lelu et al. 1987). In contrast, pine embryogenic callus originates from the suspensor region. As indicated previously, postcotyledonary pine embryos produce only nonembryogenic callus.

Pitch x Loblolly Hybrid Initiation

Embryogenic callus has also been initiated in the pitch x loblolly hybrid (Table 9). All media tested were effective, albeit at low frequencies. Initiation was similar in all respects to loblolly pine; embryogenic callus originated from the suspensor region of precotyledonary embryos. Even though the July 6 collection of embryos were predominantly cotyledonary (refer to Fig. 1), the two embryogenic calli initiated from this collection were solely from precotyledonary embryos. The pitch x loblolly embryogenic callus line, PL5-1, has proven to be highly vigorous and efficiently produces early stage somatic embryos (Fig. 8). The level of differentiation of the embryogenic callus of pitch x loblolly hybrid is affected by the basal medium formulation used for maintenance. When line PL5-1 is maintained on DCR 3/0.5, few early stage somatic embryos are formed (Fig. 8A). Upon transfer to a basal medium devoid of ammonium nitrogen, MSG, numerous early stage
somatic embryos differentiate within 14 days (Fig. 8B). These cultures have shown further development potential upon transfer to development medium devoid of auxin (Fig. 8C). Further experiments are in progress to use somatic embryos from this highly embryogenic line for studies aimed at improving maturation.

Figure 7. The relationship of the development stage of immature embryo explants (top graphs) to the initiation frequency (bottom graphs) in white spruce and loblolly pine. The shaded region represents > 50% cotyledonary development. Open bars are isolated immature embryo explants and solid bars are immature embryos with intact female gametophytes.
### Table 9. Initiation of embryogenic callus in pitch x loblolly pine hybrid.

<table>
<thead>
<tr>
<th>Explant</th>
<th>Collection (Month/Date)</th>
<th>MSG 0/0</th>
<th>MSG 2/1</th>
<th>DCR 3/0.5</th>
<th>MSCG 5/0</th>
</tr>
</thead>
<tbody>
<tr>
<td>Postfertilization</td>
<td>6/8</td>
<td>0</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>F. gametophyte</td>
<td>6/15</td>
<td>0.9</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td></td>
<td>6/22</td>
<td>0</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>Embryo</td>
<td>6/29</td>
<td>--</td>
<td>1.1</td>
<td>2.1</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>7/6</td>
<td>--</td>
<td>0</td>
<td>1.0</td>
<td>1.0</td>
</tr>
</tbody>
</table>

*85 to 110 explants cultured per treatment.*

**CONCLUSIONS**

The results described in this section detailed the first successful experiments in which the IPC tissue culture team initiated and maintained embryogenic callus in the target species loblolly pine. Experiments with explants derived from three separate collections of immature cones established the optimum stage of embryo development. Early stage embryos, prior to cotyledonary development, are optimum in loblolly pine, and the embryogenic callus originates from the suspensor region. By comparison, postcotyledonary embryos were optimum in spruce. Defining the optimum window of initiation will streamline future experiments on initiation and avoid the necessity of collecting cones over extended periods of time. The optimum window of initiation of embryogenic callus in loblolly pine was July 21 to 28 for immature cones collected during both 1986 and 1987. Embryogenic callus was also initiated from precotyledonary embryos of cones received from Rigos in Brazil.
Figure 8. Embryogenic callus of the pitch x loblolly pine hybrid line PL5-1. 
A: Micrograph of the dispersed embryogenic callus maintained on DCR 
3/0.5 medium. Note the predominance of PEM's and suspensorlike 
cells, but few well formed somatic embryos. B: Typical somatic 
embryo from the same line after transfer to MSG 2/1. C: Further 
development of somatic embryos after transfer to MSG with 2.6 mg/L 
ABA and 6% sucrose. Scale bars: 500 μm.

In order for somatic embryogenesis to have broad potential utility for 
mass propagation, it will need to be applicable to numerous clones of differing 
genetic background. Our results are encouraging in this regard, as the survey of 
embryogenic potential among clones showed 7 of 10 responsive. With this high level 
of responsiveness among clones and the fact that refinements in initiation proto-
cols have decreased genotype specificity in other plant species, we feel that 
initiation in loblolly pine can be refined to similarly lack clonal specificity.
Two loblolly pine clones, F (7-34) and H (11-9), have shown the highest efficiency for initiation of embryogenic callus. Even so, with the highest initiation frequency of about 5%, there is a need to significantly increase the initiation frequency. Another clone, C (10-1011), has initiated embryogenic callus lines which appear similar to spruce embryogenic callus lines, in that numerous somatic embryos covered the callus surface. Increasing the initiation frequency will increase the probability of obtaining highly embryogenic lines.

Embryogenic callus has also been initiated in the pitch x loblolly pine hybrid. To our knowledge, this is the first example of somatic embryogenesis in a conifer hybrid. Cloning from hybrid embryo explants is a prime example of a potential "spin-off" application for somatic embryogenesis.
INTRODUCTION

Our efforts to induce somatic embryogenesis in callus cultures of Douglas-fir are on the increase, particularly during the past two years since the reports of somatic embryogenesis in Norway spruce (Hakman, et al., 1985), European larch (Nagmnaï and Bonga, 1985), sugar pine and loblolly pine (Gupta and Durzan, 1986 and 1987) and radiata pine (Dale Smith, 1987 personal communication) were made. Additionally, our own research efforts at IPC concentrated on the standardization and refinement of experimental protocols for regular production of somatic embryos in Norway spruce (model species) and in loblolly pine (the other target species). These studies have helped us to identify some factors that could be critical for the initiation and development of somatic embryos in conifer species in general. Accordingly, when we designed several experiments on the initiation of somatic embryos in Douglas-fir, we considered various factors such as the nitrogen source, choice of explant material, environmental factors (light and dark) and, finally, a wide variety of genotypes.

MATERIALS AND METHODS

Plant Material

Developing female cones of Douglas-fir were provided by Weyerhaeuser (Federal Way, WA) during June through August. Table 10 summarizes the number and variety of clones (genotypes) and the collection dates of the cones provided to us during the last three years. Explants were harvested from these cones within 24-48 hrs of arrival, and unused cones were stored in kraft bags for up to 6 weeks at 4°C. Seeds were removed from the cones and sterilized by treatment with 25% commercial bleach (Hilex) for 15 min followed by 3 rinses with sterile...
water. Mature seeds were also supplied by Weyerhaeuser and stored frozen or refrigerated until used. Prior to excision of the embryos, the seeds were surface sterilized in 30% \( H_2O_2 \) for 45 min and rinsed three times with sterile water. The seeds were inbribed overnight in a final rinse of water before their use for excision of embryos.


<table>
<thead>
<tr>
<th>Cone Sources</th>
<th>Date/Year</th>
<th>Time Interval (Weekly Interval)</th>
<th>Explants Cultured</th>
</tr>
</thead>
<tbody>
<tr>
<td>WTC - 1022, 1026, 1027, 1054, and 1070</td>
<td>1985</td>
<td>June 25-August 30</td>
<td>Fertilized ovules immature embryos [at precotyledonary and cotyledonary stages]</td>
</tr>
<tr>
<td>WTC - 167-171; 195, 196, 205, 207</td>
<td>1986</td>
<td>April 18-August 20</td>
<td>Fertilized ovules immature embryos [at precotyledonary and cotyledonary stages]</td>
</tr>
<tr>
<td>WTC - 357-361</td>
<td>1987</td>
<td>July 2-August 6</td>
<td>Fertilized ovules immature embryos [at precotyledonary and cotyledonary stages]</td>
</tr>
</tbody>
</table>

**Culture Initiation**

All cultures were initiated on agar-solidified nutrient media (Bacto, Difco 0.8%) with growth hormones as indicated in Table 11. In addition to the combinations of the growth regulators used as in Table 11, combinations of 2,4-D and BA on a factorial basis were also tried (Report Fourteen). Fertilized ovules excised from the cones collected between June 25-July 17 were placed
directly on the medium, in accordance with the Smith protocol. The isolated embryos were excised under aseptic conditions and placed on nutrient media. All cultures were initiated in 5 cm diameter plastic petri dishes containing five explants/dish. Five petri dishes/treatment were used for a total of 25 explants per medium-growth regulator combination. Table 11 shows the total number of fertilized ovules and embryos cultured per year (1987) on a variety of nutrient media. Previous annual reports (Thirteen and Fourteen) have indicated the total number of cultures raised during 1986 and 1985.


<table>
<thead>
<tr>
<th>Total No. of Cone Collections Made</th>
<th>Total No. of Clones Used</th>
<th>Total No. of Explants Useda</th>
<th>Environment Light/Day</th>
</tr>
</thead>
<tbody>
<tr>
<td>19 (1985-1987)</td>
<td>7 or 13 Collections/Clone (1985-1987)</td>
<td>Fertilized Ovules=1529</td>
<td>MSG + 1% activated charcoal 16 hr light/8 hr dark 23 ± 1°C</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Embryos=3865</td>
<td>MSG 2, 2,4-D 1 BA</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>DCR 3, 2,4-D 0.5 BA</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>1/2 BLG + 2, 2,4-D 0.2 BA</td>
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<td></td>
<td></td>
<td></td>
<td>1/2 BLG + 2.0 NAA 1.0 BA</td>
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<td>FMSb 2.0, 2,4-D 0.2 BA</td>
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<td></td>
<td></td>
<td></td>
<td>MSCG 5 2,4-D 0 BA</td>
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<td></td>
<td>HM 2, 2,4-D 1 BA</td>
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<td></td>
<td>HM 1, 2,4-D 0 BA</td>
</tr>
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<td></td>
<td></td>
<td></td>
<td>HMCc 5, 2,4-D 0 BA</td>
</tr>
</tbody>
</table>

aTotal no. of explants cultured in 1987; for 1985 and 1986, refer to Annual Reports Thirteen and Fourteen, for media treatments tried.

bFMS = MS medium without NH₄NO₃, but with Gln 5 μM + 1/2 FeEDTA.

cHMC = HM + casein hydrolyzate 1g/L.
Culture, Maintenance and Evaluation

After four weeks, all the treatments were scored as a percentage of explant responding by callus formation, whether embryogenic or nonembryogenic. Callus cultures exhibiting the embryogenic phenotype were subcultured every two weeks by transfer to medium of the same composition. Some callus samples were also subjected to biochemical analysis (for estimation of GSH, total reductants and protein). Additionally, some squash preparations of callus were made for histological examinations under the light microscope.

RESULTS AND DISCUSSION

None of the fertilized ovules cultured on MSG medium responded favorably to the culture medium. However, the response of the isolated embryos to the other culture media (Table 11) was rather encouraging, at least in the beginning. After two weeks of culture, a translucent, white mass of callus was observed, originating both from the suspensor and the embryonal head of the explant on DCR3/0.5 and MSCG 5/0 media (Fig. 9, 10). Immature embryos at the cotyledonary stage of development (explant) also responded by producing white callus (exhibiting the same phenotype as embryogenic callus) from the hypocotyl and the cotyledons (Fig. 11,12). Some of the callus cultures that were initiated in light (16 hr light and 8 hr dark) turned green and are being maintained that way. Some of the callus lines that were initiated in the dark initially produced white granular callus which subsequently turned yellow or green upon transfer to light conditions.

Out of the several nonembryogenic green callus lines maintained through 1985, a mass of mucilaginous, white callus also was observed in association with nonembryogenic green callus in one callus line. This result was observed approximately after 18 months of initiation (Fig. 13,14); it was initiated on HM
Figure 9-14. 9-10. Immature zygotic embryo explants at precotyledonary stage. Note the initiation of white callus (wc) from both suspensor (s) and embryonal head (eh). Scale bar = 1 mm. 11-12. Zygotic embryo explants at cotyledonary stage. Note the initiation of callus from hypocotyl (hy) and cotyledons (c). Scale bar = 1 mm. 13-14. Embryogenic callus (ec) in association with nonembryogenic callus (ne). Scale bar = 1 mm.
Figure 15-17. 15-16. Somatic proembryos with compact, dense embryonal heads (eh) and long suspensors (s). Scale bar = 100 μm. 17. Two-celled early proembryo. Scale bar = 100 μm.
with 1 and 2 mg/L 2,4-D and was maintained on the medium of the same composition. Squash preparations of this callus sample revealed several somatic proembryos with distinct embryonal heads subtended by suspensors (Fig. 15-16). This callus line is being maintained and some of it was transferred to different media for further development of these somatic embryos.

The same experimental protocol followed during the subsequent 2 years (1986 and 1987) to obtain embryogenic callus lines did not result in similar success. However, some of the callus lines which phenotypically resembled spruce embryogenic callus lines initiated in 1987 also showed cell types characteristic of spruce embryogenic callus (Fig. 13 and 14). Occasionally, in some samples, early organization of proembryos at the 2-celled stage was also observed (Fig. 17). However, all of these callus lines that had the potential of becoming embryogenic eventually turned brown and could not be maintained.

Biochemical analysis of some of the above callus samples for total reductants ranged between 65-100 (average of 87 ± 19) \( A_{760} \) nm/g fwt, characteristic of embryogenic callus lines of other conifer species (Annual Report Fourteen, 1987). Furthermore, glutathione analysis on an earlier promising line with a total reductants value of 8 showed 62 ± 18 nmoles GSH/g fwt, also encouraging.

CONCLUSIONS

The above experimental results (with different clones representing different genotypes and variety of media formulations) indicate that some of the callus lines initiated showed the potential of becoming embryogenic initially, although they could not survive. Some of those callus lines which turned green, might still give rise to embryogenic callus, if maintained over a period of time, as observed in one of the callus lines in 1985. There appear to be no
clonal differences among 19 clones tested as far as the response is concerned, and no definite conclusion could be reached as far as explant choice is concerned. However, occasional appearance of white mucilaginous callus in association with green callus indicates that Douglas-fir behaves like spruce. No conclusions can be reached yet regarding the effect of media formulations including the nitrogen source or growth regulators and the environmental conditions such as light or dark. These findings, however, leave us with broad scope for further experimentation to improve the initiation frequencies of embryogenic callus (as observed in 1985) to develop methods to maintain the callus lines which phenotypically resemble embryogenic callus, and to standardize the procedures.
SOMATIC EMBRYO DEVELOPMENT – WHITE SPRUCE AND LOBLOLLY PINE

BACKGROUND

While the conditions for initiation and maintenance of embryogenic callus of spruce and pine are now well established, the further development of somatic embryos into plantlets remains largely an uncontrolled event. The difference between somatic embryo development in spruce compared to pine appears to be largely a matter of degree, with somatic embryos of pine abandoning a developmental sequence at an earlier stage than spruce. If a certain commonality exists in embryo development among conifers, then uncovering factors that influence development in spruce (where sufficient quantities of somatic embryos are available) should be applicable to loblolly pine. This is shown to be the case in this section. Factors that contributed to embryo development in white spruce, when applied to loblolly pine, stimulated embryo development in a similar manner. Indeed, the results presented in this section also indicate that factors effecting embryo development (both somatic and zygotic) are common among a wide variety of species, including angiosperms. Although this conclusion is not surprising, it is encouraging from the standpoint that the considerable amount of work done previously with embryo culture of angiosperms will be applicable to conifers.

In this section the influence that ABA and the osmotic environment exert on the development of proembryos in spruce or pine is demonstrated. At first glance it would seem that ABA and the osmotic environment of the culture could be categorized as hormonal and physical factors, respectively, that influence embryo development. However, it has been shown that control of precocious germination, storage protein synthesis (Quatrano, 1986) and even
enhancement of the initiation of embryogenic callus (Close and Ludeman, 1987) can be achieved by either treatment with ABA or an elevated level of osmoticum. Therefore it can not be overlooked that ABA and elevated osmolarity exert their effects on the same pathways, especially given the role ABA plays in water relations in plants.

In the culture of immature zygotic embryos, the addition of ABA inhibited precocious germination in cotton, wheat, rape and soybean (see references contained within Quatrano, 1986). In the culture of somatic embryos, ABA has been successfully used to complete somatic embryo development in Umbelliferous plants (Ammirato, 1974; Kamada and Harada, 1981), soybean (Ranch et al., 1985), cucumber (Ziv and Badesh, 1986), and most recently spruce (Becwar et al., 1987) and Douglas-fir (Durzan and Gupta, 1987). Levels typically in the range of 0.1-100 μM ABA have allowed for successful completion of embryogenesis. Without ABA, zygotic proembryos may skip the later stages of embryogenesis and germinate directly to produce spindly, abnormal seedlings (precocious germination). In the presence of ABA, abnormalities like secondary embryogenesis and embryo dedifferentiation are reduced. In effect, ABA allows for successful completion of the later stages of embryogenesis and produces a morphologically balanced embryo.

Artificial increases in the osmolarity of the culture medium by addition of sucrose, mannitol or sodium chloride has allowed for the successful culture of immature zygotic embryos of many species (for review of early literature, see Raghavan, 1976). The use of elevated osmolarity in embryo culture is an example where analysis of liquid endosperm has provided an important clue to modification of the \textit{in vitro} environment. Elevated osmolarity has also
assisted in somatic embryo development in Umbelliferous plants (Ammirato, 1985), jojoba (Wang and Janick, 1986) and papaya (Litz and Conover, 1982). Elevated osmolarity is typically achieved by adding sucrose in a range of 6-21%-levels beyond what is considered required as a carbon source (1-3%). Again, the resulting enhancement of embryo development by increased osmolarity is similar to that obtained with ABA-inhibition of precocious germination, a reduction in embryo dedifferentiation and an embryo that has, overall, a more normal appearance.

Taken together, the combination of ABA and elevated osmolarity have enhanced development in white spruce, allowing for completion of the cloning cycle in this species. In loblolly pine, the combination of the two treatments have resulted in our first precotyledonary stage embryos in this species. In this section, we describe for the first time the enhanced embryo development of a conifer by a combination of ABA and elevated osmolarity.

SOMATIC EMBRYO DEVELOPMENT IN WHITE SPRUCE

Introduction

In the spring of 1986, the IPC tissue culture team became aware of Dr. Inger Hakman's success in initiating embryogenic callus in white spruce (Hakman and Fowke, 1987). During the summer of 1986, the team initiated embryogenic callus lines from both immature and mature embryos of white spruce in order to establish a possible additional model species to Norway spruce. The initiation protocol used was that reported by Hakman and Fowke (1987). Embryo development could only be achieved under elevated levels of sucrose (3 vs. 6%) and ABA (10-15 μM).
Materials and Methods

1. Initiation

White spruce seeds were obtained from the U.S. Forest Service Oconto River Seed Orchard and stored at 2-4°C. Seeds were sterilized in a commercial bleach solution (Hilex, 20%, v/v) containing 1% Tween 20 for 20 minutes, then in 70% ethanol for 2 minutes, and rinsed 3 times in sterile water. Embryos were excised under aseptic conditions and cultured on the basal medium given by Hakman (see Appendix) containing 2 mg/L 2,4-D and 1 mg/L BA. Cultures were incubated under a 16 h photoperiod of 2000 lux (800 incandescent) and 22°C. Embryogenic callus was also initiated from immature embryos of white spruce by this same procedure (see Progress Report Fourteen).

After initiation all cultures were maintained under the environmental conditions described above on Hakman basal medium containing 2 mg/L 2,4-D and 1 mg/L BA and transferred at regular intervals.

2. Plantlet Development

Several culture regimes were tested to stimulate somatic embryos to develop into plantlets. Cultures were transferred to media containing increasing ABA levels (1-15 μM), at two sucrose levels (3 or 6%), and the addition of BSO (10^-5 M). Included in these investigations was the method for plantlet development in Norway spruce reported by Becwar et al. (1987).

The protocol to induce development of white spruce somatic embryos is outlined in Fig. 18. Briefly, embryogenic callus was transferred to medium containing 10-15 μM ABA and 6% sucrose. Callus was transferred to fresh media at 2 week intervals and somatic embryos developed directly on the callus. For
the development of somatic embryos in embryogenic callus derived from mature embryos, BSO (10⁻⁵M) was included in the media.

\[
\text{HM} + 2 \text{ mg/l } 2,4\text{-D} \\
1 \text{ mg/l BA} \\
\downarrow \\
\text{HM} + 10-15 \mu\text{M ABA} \\
3-6\% \text{ SUCROSE} \\
\downarrow \\
\text{SUBCULTURE FOR SOMATIC EMBRYO DEVELOPMENT} \\
\downarrow \\
\frac{1}{2} \text{HM} + 10\% (\text{v/v}) \text{ COCONUT WATER; 1.5\% SUCROSE; NO GROWTH REGULATORS FOR FURTHER MATURATION}
\]

Figure 18. Protocol for somatic embryo development in white spruce.

In both systems, embryos were removed from the callus when the hypocotyl region showed elongation of 2-3 mm and were laid on half strength basal media without growth regulators containing 10\% (v/v) coconut water for further development.

When primary roots developed, somatic embryo plantlets were transferred to sterilized vermiculite in Magenta GA7-3 plastic pots and placed in a growth chamber under a 16 h photoperiod of 7500 lux and 22°C.

RESULTS AND DISCUSSION

The IPC tissue culture team was successful in producing embryogenic callus from mature seed of white spruce, albeit at a lower frequency (0.8\%) than that obtained from immature seed of white spruce (40\%) (see Report Fourteen).
Undoubtedly, this decline in initiation frequency reflects to some extent the increase in maturity of the explant. However, since the mature and immature had a different genetic background, the difference in initiation may not be completely due to differences in explant maturity. When somatic embryo development was attempted in embryogenic callus from both immature and mature embryos of white spruce using the Norway spruce protocol, somatic embryos failed to develop. Embryos embarked upon a normal course of development, but a rapid dedifferentiation of the embryonal head ensued after 2 weeks. Further attempts to induce development were aimed at the reduction of this callusing response. Changes in the development media led to removal of the IBA and an increase in both the ABA and the sucrose level. A range of ABA levels from 10-15 μM proved optimum for reducing callusing of developing somatic embryos. The exact level of ABA required appears to be genotype dependent. While some callus lines showed development over the entire range of ABA levels, other lines showed development only at specific levels. In combination with the elevated ABA, an increased sucrose level (6%) produced a further reduction in the callusing effect (Fig. 19). Enhancement of embryo development by BSO was not seen in the immature white spruce lines tested but was evident in the mature white spruce. However, as only one mature line was tested, it is not possible to conclude from this whether this effect was due to tissue source or genetic differences. Results from the effects of BSO on embryo development in Norway spruce suggest the latter (see Report Fourteen).

After 6-8 weeks, well-developed somatic embryos were transferred to half-strength media containing coconut water and 1.5% sucrose (Fig. 20). Continued culture of somatic embryo plantlets in the presence of elevated sucrose (6%) inhibited the growth of the plantlets and led to necrosis. Reducing the sucrose level eliminated this effect. To date one somatic embryo plantlet has survived transfer to soil (Fig. 21).
Figure 19. Effect of elevated osmolarity on somatic embryo development in white spruce (HM medium 10 μM ABA 10 μM BSO). 3% sucrose (top) 6% sucrose (bottom).
Figure 20. Somatic embryos in white spruce from callus initiated from mature (top) and immature (bottom) embryos.
Figure 21. Somatic embryo-derived plantlet established in soil.

LOBLOLLY PINE SOMATIC EMBRYO DEVELOPMENT

Materials and Methods

Embryogenic callus of loblolly pine was initiated from immature embryos on DCR basal medium (see Appendix) supplemented with 2,4-D (3 mg/L), BA (0.5 mg/L), and glutamine (250 mg/L). The callus were maintained in the dark at 22°C on a 14 day subculture schedule. All media were solidified with Difco Bacto Agar in DCR medium at a concentration of 0.6% (w/v) and in MSG medium at a concentration of 0.8% (w/v). Abscisic acid was filter sterilized prior to addition to the autoclaved media. Callus was essentially undifferentiated on maintenance media.

For development of somatic embryos the calli were subcultured to MSG basal medium lacking auxin and cytokinin, but supplemented with various concentrations of ABA and sucrose. Concentrations of ABA tested were 0, 2, 5, 10, and 15 μM. Concentrations of sucrose tested were 3% and 6% (w/v). Seven callus
pieces (250-500 mg ea) were used per treatment. When tested on various concentrations of ABA and sucrose, the cultures were moved to room light (13-15 μE m⁻² s⁻¹) and approximately 28°C.

RESULTS AND DISCUSSION

In order to develop loblolly pine somatic embryos, it was necessary to transfer the embryogenic tissue from DCR supplemented with auxin and cytokinin to MSG lacking auxin and cytokinin but supplemented with abscisic acid and an increased concentration of sucrose. Direct transfer of callus to DCR with ABA and sucrose failed to induce somatic embryo development.

Table 12 shows the effect of sucrose and ABA concentrations on somatic embryo development. The best treatment was 10 μM ABA with 6% sucrose. In Table 12, an equivalency in response to ABA and elevated sucrose is suggested by the observation that 6% sucrose at 5 μM ABA gives the same level of embryogenesis as 3% sucrose and 10 μM ABA. However, increasing ABA at lower sucrose levels was not beneficial.

Table 12. Effect of ABA and sucrose on somatic embryo development.

<table>
<thead>
<tr>
<th>% Sucrose</th>
<th>ABA, μM</th>
<th>0</th>
<th>2</th>
<th>5</th>
<th>10</th>
<th>15</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1-3</td>
<td>0</td>
</tr>
<tr>
<td>6</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1-3</td>
<td>7-10</td>
<td>1-3</td>
</tr>
</tbody>
</table>

Number of somatic embryos observed per callus.

The subcultured embryogenic callus was white and mucilaginous with very few aspects of organization (Fig. 22). After 4-5 weeks on 10 μM ABA and 6% sucrose, dense white nodules appeared (Fig. 23) very similar to preembryogenic
masses seen in white pine embryogenic callus (Fig. 23; Wann et al., 1987). The nodules continued to develop into distinct embryos with long suspensor cells (Fig. 24). These precotyledonary somatic embryos always appeared at the callus surface. Once the somatic embryos reached this point in development, it was necessary to remove them from the callus. If the somatic embryos remained in the callus, the somatic embryo heads callused over and turned brown.

Figure 22. Embryogenic loblolly pine callus on maintenance medium (DCR 3/0.5).

Figure 23. Embryonal masses in loblolly pine embryogenic callus (MSG 10 μM ABA and 6% sucrose, 5-6 wks).
Figure 24. Well-formed loblolly pine somatic embryo.

By removing the precotyledonary somatic embryo from the callus, callusing over of the embryo itself was delayed but not completely repressed. Development beyond this point has not been achieved.

After the above procedure was successfully repeated, 15 other embryogenic callus lines were tested. These lines were initiated under various protocols. Somatic embryos had been observed occasionally, but not reliably in six of these 15 lines. Using the procedure described above, all 15 lines failed to develop somatic embryos. Only the callus line used in the original studies (LP 501-12H)1 was able to reproducibly differentiate somatic embryos using this procedure.

CONCLUSIONS

White Spruce and Loblolly Pine Somatic Embryo Development

A combination of ABA and elevated osmolarity has been shown for the first time to stimulate embryo development in conifers. Although the mode of
action of ABA and increased osmolarity may be the same, the two substances are not strictly interchangable, but complementary. For example, elevated osmolarity may allow more embryos to remain at a stage that will be receptive to ABA or vice versa. Although application of elevated osmolarity and ABA to white spruce and loblolly pine cultures allowed for embryo development, the initial state that the cultures were in was different in the two species. In white spruce, well-defined embryo initials (with suspensors) were present at the time the treatments were applied. In loblolly pine, only preembryogenic masses (lacking suspensors) were present.

The final state that embryos of these two species attained under these treatments was also different. Elevated osmolarity and ABA allowed for successful completion of somatic embryogenesis in white spruce. In white spruce it became apparent that ABA and elevated osmolarity at some point becomes inhibitory and must be removed in order to successfully complete embryo development. In loblolly pine the developmental sequence was abandoned much earlier, even before cotyledon development. It seems unlikely that ABA and elevated osmolarity would become inhibitory at this point, and work with zygotic embryos suggests that the medium formulation that supports embryo differentiation does not support embryo development.

Common to both species as well was the observation that different callus lines required different treatments such that no one treatment stimulated development to the same extent in all lines. In white spruce, different lines required different levels of ABA, and in some, BSO was stimulatory. In loblolly pine, only several lines of many responded to the treatment outlined. Since many of the callus lines contain embryos in different stages of development when
grown proliferatively, these apparent genetic differences may be traced back to the maintenance conditions. Upon removal of 2,4-D, the percentage of somatic embryos (or PEMs in loblolly pine) that are of the requisite stage of development to respond to ABA and elevated osmolarity may be quite different. Therefore, in order to achieve a unified developmental treatment, it may be necessary to devise a proliferative treatment (or timing thereof) that can put the greatest percentage of embryos in all lines at the same stage of development. To achieve this would require a more complete understanding of what stages in embryo development are receptive to a given treatment and for how long. The distinct possibility exists that treatments we have devised are functioning at maximum efficiency, but due to the very low percentage of somatic embryos or PEMs that are capable of responding to these treatments, development frequencies appear very low.

The investigations outlined here represent first steps in unlocking embryo development. Although empirical, they successfully allowed completion of embryo development in white spruce. In loblolly pine, once media considerations are attended to it is likely that embryo development will proceed to a further stage. However, enhanced frequencies of development may require a more accurate knowledge of treatment timing and which development stages are in a state receptive to *in vitro* manipulations.
EMBRYO CULTURE OF LOBLOLLY PINE

INTRODUCTION

Embryo culture of coniferous species has attracted attention for several decades. The ability to successfully rear immature zygotic embryos into plants would have many potential applications for tree improvement. For example, embryo culture may permit (1) embryo rescue in intra- and interspecific hybrids that are typically abortive, (2) assessment of embryo performance at an early stage free from the influence of gametophyte, and (3) potential for gene transfer by injection of the zygote followed by embryo rescue. In the context of Project 3223, attempts to rear immature embryos of loblolly pine into plants are expected to provide valuable information on the nutritional, hormonal and environmental conditions necessary to rear somatic embryos into plantlets. Of course the tacit assumption is that zygotic embryos are equivalent to somatic embryos in their response to culture conditions. To date, this has not been proven, and therefore the relevancy of using zygotic embryos to model somatic embryo culture is still in question. For our purposes, we are concerned with cultures of zygotic embryos 2-3 weeks (precotyledonary) after fertilization. Culture of embryos younger than this does not appear necessary, since we can reproducibly (albeit at a low frequency) carry somatic embryos from at least one callus line to this stage before they dedifferentiate and abandon an embryogenic sequence.

The culture of immature zygotic embryos could also be expected to reveal whether the culture environment itself exerts an influence on the quality of the plants produced. Morphological "artifacts" can be introduced into the plants that have been reared from immature embryos in vitro. This observation has been best documented in corn. In corn, unusual characteristics of somatic plantlets (e.g.,
reduced size, terminal silks, reduced male fertility) were once thought to be somaclonal variations. However, when plantlets were reared in vitro from immature zygotic embryos cultured on the same medium but lacking growth regulators, these same characteristics showed up in the plants (Earle, 1985). In our studies of conifer somatic embryogenesis, we would like to know which, if any, characteristics exhibited by somatic embryos are merely a consequence of the in vitro environment and which characteristics may be indicative of stable genetic changes.

MATERIALS AND METHODS

Immature seeds were sterilized and embryos aseptically removed as previously described (Report Fourteen, p. 27). Embryos were cultured on a variety of media that were either liquid with cheesecloth supports or solidified with agar. For each treatment, 30 immature embryos were cultured in 5 cm petri dishes (5 embryos/replication). All embryos (except those noted below) were cultured in the dark at 22°C. This formed the basis for statistical analysis. Care was taken that comparisons were made with embryos at the same initial stage of development. After 4-6 weeks, treatments were scored as either (1) a "germinative" response (elongation of the embryo, cotyledon formation) or (2) a "callus" response (generalized swelling, granular appearance of the embryo owing to dedifferentiation). The remaining embryos either exhibited no response, or gradually turned brown.

In one experiment, postcotyledonary (3rd week August) embryos were cultured under several conditions in both the light and dark. Except for a few embryos that were obviously damaged, the majority of these embryos readily elongated and were transferred to agar-solidified 1/2 MS (macro- and microelements) or MSG. On these media, the embryos were either placed on slants or the cotyledons were embedded in medium and inverted (hanging embryo culture). After
several weeks, some of the embryos had formed roots, and these were transferred to a soilless potting medium (equal parts sand:peat:perlite) and maintained in the fog chambers (7500 lux, 22°C, 100% RH) until shoot growth was observed.

For treatments examining the effect of water-soluble gametophytic extracts, the natural extracts were prepared from loblolly pine ovules removed from the immature cones of the various 1987 summer collections. Ovules were homogenized in RO water in a Ten Broeck homogenizer (50 mg fresh weight per mL). The supernatant obtained after centrifugation at 27,000 x g for 15 min at 5°C was freeze-dried for use in tissue culture experiments. The pellet was further processed with alkali, but none of these alkaline extracts have been used yet. The water extract used in the experiments reported here originated from collection LP7R. On 7-31-87 110 mg (freeze-dried weight) were obtained from 940 mg ovules (fresh weight); of this, 58 mg were given to tissue culture personnel on 8-31-87 for incorporation into medium evaluations.

RESULTS AND DISCUSSION

To date, no precotyledonary zygotic embryos have been successfully grown into plants. A small percentage of these embryos did proceed to the cotyledonary stage before cessation of growth. In contrast, embryos cultured just after the stage of cotyledon formation readily elongated under all conditions examined and many could be rooted and transferred to soil.

Owing to the negative results in rearing plants from precotyledonary embryos, conclusions on the effect of various media formulations are provisional at best. However, it is possible to gain some insight into what are, at least, inappropriate conditions for the growth and development of precotyledonary embryos. In Table 13 the treatments that gave the best response in terms of
callus formation (dedifferentiation) of the immature embryos are an example. Note that the media formulation that is currently considered the best for embryo formation from loblolly pine callus line (LP 524-12H)1, (MSG containing 6% sucrose and 2.6 mg/L ABA), resulted in a high frequency of callus formation. Again, it should be recalled that this is not meaningful callus formation. Rather, it is more an overall disintegration of the embryo into parenchymatous cells and not the production of a callus type that could be proliferated through subculture.

All MSG-based treatments resulted in excessive callus formation irrespective of the presence of ABA or elevated osmolarity. Correspondingly, somatic embryos, soon after they were formed also responded to these treatments in a manner indistinguishable from zygotic embryos. Although negative, the similar responses of somatic and zygotic embryos to these treatments at least does not rule out the relevancy of using zygotic embryo development to model the somatic process. Table 13 even suggests that conditions present within embryogenic callus cultured on the best development medium are not conducive to development. When zygotic embryos were placed on embryogenic callus growing on MSG containing 6% sucrose and 2.6 mg/L ABA, the zygotic embryo almost quantitatively responded by callus formation.

The reason for this strong tendency for callus formation is unknown. However, MSG can be regarded as a formulation that can easily support the growth of conifer callus when supplemented with auxin and cytokinin. In particular, the high glutamine levels (1450 mg/L) seem especially promotive toward a callus response. Apparently, this ability to foster callus growth is dominant in these experiments to the extent that the effects of ABA and elevated osmolarity are masked, if they occur at all.
Table 13. Embryo culture in loblolly pine. Treatments stimulating a callus response in precotyledonary embryos (Clone B; collected 7/28/87).

<table>
<thead>
<tr>
<th>Media</th>
<th>Addenda</th>
<th>Callus Formation</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>Frequency, %</td>
</tr>
<tr>
<td>MSG</td>
<td>2.6 mg/L ABA</td>
<td>72 ± 10</td>
</tr>
<tr>
<td></td>
<td>2.6 mg/L ABA, 6% sucrose</td>
<td>68 ± 22</td>
</tr>
<tr>
<td></td>
<td>2.6 mg/L ABA, 6% sucrose 20% CW</td>
<td>59 ± 30</td>
</tr>
<tr>
<td></td>
<td>Embryos placed on EC</td>
<td>~ 100</td>
</tr>
<tr>
<td></td>
<td>Cultured on 2.6 mg/L ABA, 6% sucrose</td>
<td></td>
</tr>
<tr>
<td>1/4MS</td>
<td>400 mg/L NH₄NO₃</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>400 mg/L NH₄NO₃, 2.6 mg/L ABA</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>100 mg/L NH₄NO₃, 50 Gln</td>
<td>32 ± 17</td>
</tr>
<tr>
<td></td>
<td>100 mg/L NH₄NO₃</td>
<td>20 ± 24</td>
</tr>
<tr>
<td></td>
<td>0 NH₄NO₃ 0 Gln</td>
<td>4 ± 9</td>
</tr>
<tr>
<td></td>
<td>50 mg/L Gln</td>
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</tbody>
</table>

Given the tendency toward callus formation on MSG, consideration was directed toward media commonly used for development, including 1/4 MS and White's medium (WH). The use of 1/4 MS, at NH₄NO₃ level of 400 mg/L resulted in embryo necrosis within 1-2 weeks of culture, regardless of the presence of ABA. When the NH₄NO₃ level was reduced to 100 mg/L, necrosis was not observed, although this level did not foster development. When reduced nitrogen was eliminated from the medium or only supplied as 50 mg/L glutamine, very little dedifferentiation was seen in the embryos. From these experiments, then, it was tentatively concluded that high salt media like MSG or even 1/4 MS tended to favor callus formation. In particular, ammonium ion appeared to be quite toxic unless reduced from 400 mg/L. Thereafter, another series of experiments was conducted.
with White's medium, an extremely low salt medium. This medium was also tested
with various levels of aqueous extracts of female gametophytes.

White's medium fostered embryo development to the cotyledonary stage in
less than 20% of the embryos collected in late July (see Tables 13 and 14). For
embryos collected the percentage was closer to 40% (see Table 15). In both
cases, embryo development was arrested at the cotyledonary stage. Neither the
presence of glutamine, gametophytic extracts, elevated osmolarity, coconut water
nor ABA enhanced development when compared to the basal medium (WH + 2% sucrose
+ 0 mg/L reduced nitrogen) (see Tables 14 and 15). The failure of the above men-
tioned substances to promote zygotic embryo development in a manner similar to
their promotion of somatic embryo development could be attributed to a medium
formulation drastically out of line with embryo requirements. In particular,
the results with gametophytic extracts are perplexing. If mineral or hormonal
factors were important, it might be expected that aqueous gametophyte extracts
could provide the missing supplements to a simple artificial medium. However,
this was not the case, and it adds the possibility that water-insoluble factors
may also have to be considered along with alternative media formulations.

In those infrequent cases where embryo development proceeded to the
cotyledonary stage, many of their morphological features were quite dissimilar to
the equivalent stage in ovulo. For example, in vitro reared embryos (1) were
decisively green, even when cultured in the dark, (2) had a reduced number of
cotyledons, apparently due to fusion of the cotyledons (see Fig. 25 and 26), and
(3) showed precocious development of the cotyledons with very little elongation
of the embryo proper (see Fig. 26). While these morphological discrepancies were
observed between zygotic embryos in vitro and in their natural conditions, many
of these same morphological features were exhibited by somatic embryos. In Norway
spruce cotyledonary fusion has been observed (see Fig. 27) as well as an incomplete development of the cotyledonary ring (see Fig. 28). In Norway spruce difficulty is also encountered in stimulating embryo elongation, although cotyledon development proceeds in an apparently normal fashion. These observations provide strong evidence that many seemingly abnormal morphological features of somatic embryo development may not be a result of genetic changes brought on by the somatic embryogenesis process, but are rather a consequence of the in vitro environment. It may well be that the process of somatic embryogenesis is more normal than we think, and these observations also reinforce the notion of using zygotic embryo development as a model.

Table 14. Embryo culture in loblolly pine. Effect of gametophytic extracts on the development of precotyledonary embryos (WH medium; 4-6 weeks).

<table>
<thead>
<tr>
<th>Clone</th>
<th>Collection Date</th>
<th>Addenda</th>
<th>Extract Conc., mg/L</th>
<th>Development, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>B</td>
<td>7/28/87</td>
<td></td>
<td>0</td>
<td>20 ± 13</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>10</td>
<td>17 ± 15</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>100</td>
<td>13 ± 10</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>1000</td>
<td>17 ± 23</td>
</tr>
<tr>
<td></td>
<td></td>
<td>-Gln</td>
<td>0</td>
<td>10 ± 11</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>10</td>
<td>15 ± 13</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>100</td>
<td>23 ± 15</td>
</tr>
<tr>
<td></td>
<td></td>
<td>10% CW; -Gln</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>10</td>
<td>3 ± 10</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>100</td>
<td>13 ± 9</td>
</tr>
<tr>
<td>A</td>
<td>-Gln</td>
<td></td>
<td>0</td>
<td>33 ± 20</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>50</td>
<td>40 ± 13</td>
</tr>
<tr>
<td>B</td>
<td>-Gln</td>
<td></td>
<td>0</td>
<td>18 ± 22</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>50</td>
<td>10 ± 16</td>
</tr>
</tbody>
</table>
Table 15. Embryo culture in loblolly pine. Effect of ABA and sucrose concentration on the development of precotyledonary embryos (Clone B, WH medium 4-5 weeks).

<table>
<thead>
<tr>
<th>Collection Date</th>
<th>Sucrose, %</th>
<th>ABA, mg/L</th>
<th>Development, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>8/6/87</td>
<td>2</td>
<td>0</td>
<td>54 ± 10</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>2.6</td>
<td>20 ± 13</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>0</td>
<td>17 ± 17</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>2.6</td>
<td>30 ± 13</td>
</tr>
</tbody>
</table>

Whereas precotyledonary embryos underwent very little development in response to any of the culture conditions examined, cotyledonary embryo elongated at a frequency greater than 95% on all treatments (WH medium 2 or 6% sucrose ± 2.6 mg/L ABA, light or dark). Amazingly, only 2-3 weeks developmentally separated the precotyledonary embryos from the cotyledonary embryos. Consistent with generally accepted theory of embryo development, it could be that during this two week period a switch from heterotrophic to autotrophic embryo growth occurs. Under heterotrophic growth the embryo relies on nutritional resources of the gametophyte and has very little reserves or the biosynthetic capacity to handle or produce specialized nutritional substances. However, at a certain point the embryo becomes more nearly autotrophic, having developed sufficient reserves and biosynthetic capability for it to be cultured \textit{in vitro} under simple defined conditions (Raghavan, 1976). The lack of stringency on the culture conditions for cotyledonary zygotic embryos suggests that this stage is the cut off point for using zygotic embryogenesis as a model. The female gametophyte has, by this time, established within the zygotic embryos self sufficiency to function on their own given appropriate conditions whereas the above might not be true for somatic embryos. The \textit{in vitro} environment in no way prepares the cotyledonary somatic embryo for the germinative type of growth, and somatic embryos likely remain heterotrophic well past the cotyledonary stage.
Figure 25. Examples of precotyledonary embryo culture in loblolly pine. Embryo culture proceeded from a precotyledonary stage to the above stages before cessation of growth. Note reduced number of cotyledons and lack of shoot apex development (WH medium + 10 mg/L extract; 6 wks).
Figure 26. Precocious cotyledonary development in embryo culture of loblolly pine. Note lack of development in the embryo proper.

Figure 27. SEM micrograph of a Norway spruce somatic embryo. Arrow indicates furrow between apparently fused cotyledons.
Figure 28. Typical somatic embryo development in Norway spruce callus. Arrows indicate incomplete development of cotyledonary ring.

Although zygotic embryos may be heterotrophic, there is no replacement for the promotive effect of the gametophyte. While embryo elongation was nearly quantitative and media-independent, the subsequent "germination" or root development was not. Primary roots formed on embryos cultured hanging or on slants, but the rooting frequency was poor and media-dependent (25% on MSG; 8% on 1/2 MS). Furthermore, the rate of root growth was approximately 10 times slower than for germinating zygotic embryos. Despite the reduced rate of root growth, 12 of 19 plants (63%) have survived transfer to soil and have exhibited shoot growth. Taken together, the reduced conversion frequency (rooting efficiency x plant transfer efficiency) even for zygotic embryos implies that this step in the embryogenesis process will likewise demand considerable attention. A demonstration that it can be accomplished even at a low frequency with zygotic embryos suggests that this step will be easier than the maturation step (precotyledonary \textarrow{\rightarrow} cotyledonary embryo).
MATURATION AND GERMINATION OF NORWAY SPRUCE
SOMATIC EMBRYOS AND CONVERSION TO PLANTS

INTRODUCTION

Reports on somatic embryogenesis in conifers have emphasized initiation of cultures and in several cases have verified in vitro developmental potential of the somatic embryos (Hakman et al. 1985, Hakman and von Arnold 1985, Nagmani and Bonga 1985, von Arnold and Hakman 1986, Krogstrup 1986, Gupta and Durzan 1986a and b, Gupta and Durzan 1987, Hakman and Fowke 1987, Nagmani et al. 1987, Becwar et al. 1987a and b, Lu and Thorpe 1987, von Arnold 1987, Lelu et al. 1987, Durzan and Gupta 1987). Although transfer of conifer somatic embryo plantlets to nonaxenic conditions has been achieved (Nagmani and Bonga 1985, Gupta and Durzan 1987, Becwar et al. 1987b, Durzan and Gupta 1987), the continued growth and development of these plantlets has not been verified. In order for in vitro embryogenesis to serve as a viable mass propagation system in conifers it will be necessary to develop efficient systems for the maturation and conversion of somatic embryos to plants and to verify the uniformity and "true-to-type" nature of these plants. Because of the long life cycle of trees it is essential to verify at an early stage that the regenerated plants are normal.

The regeneration process can be divided into three stages of development: (1) the "maturation" of immature somatic embryos to the cotyledonary stage, (2) primary root development or "germination," and (3) plant survival or "conversion" and growth in soil. Here the term conversion specifically refers to the process of germinant-to-plant development (Redenbaugh et al. 1986 and 1987). There is little quantitative information on the frequency at which conifer somatic embryos undergo these stages of development. The only reported figures are for Norway spruce where 80 (mature) somatic embryos per gram of
callus yielded 4-8 plantlets (Gupta and Durzan 1986a) and for loblolly pine where 1-5 plantlets were obtained per gram of cell mass (Gupta and Durzan 1987).

This report provides detailed quantitative information on maturation and germination frequencies of Norway spruce somatic embryos, and the conversion of a limited number of somatic embryos to plants. This is also the first report of continued growth of conifer somatic embryo plants, including overwintering and renewed vegetative growth from resting buds.

MATERIALS AND METHODS

Initiation and Development

Embryogenic callus lines were initiated from immature embryos of Norway spruce (Picea abies as previously described (Becwar et al. 1986a and b) using a modified MS basal medium containing 3.4% sucrose and 0.5% agar (pH 5.5) (von Arnold and Eriksson 1981). For initiation and maintenance the basal medium was supplemented with 2,4-D (2 mg/L) and BA (1 mg/L). To induce development of somatic embryos, the following protocol was used, unless noted. Pieces of embryogenic callus (~500 mg) were transferred to basal medium supplemented with 1% activated charcoal for 7 days and then to basal medium with 1 μM each of ABA and IBA for 14 days (Becwar et al. 1986a and b). Thereafter, callus pieces were transferred to fresh medium with ABA and IBA every 21 days, until development of somatic embryos was completed. In one experiment, embryogenic callus pieces were also transferred from the basal medium with activated charcoal to basal medium with no growth regulators. For both initiation and development, cultures were maintained at 23°C with 16 hr irradiance from cool-white fluorescent and incandescent lights.

Maturation

Maturation frequency, the number of somatic embryos which developed to the cotyledonary (mature) stage relative to the number of immature stage somatic
embryos, was calculated as follows. First, the number of somatic embryos per unit weight of callus was determined after 21 days on the development medium as previously described (Becwar et al. 1986). Briefly, replicate (> 4) subsamples (= 100 mg) of embryogenic callus were aseptically weighed, dispersed in liquid, and plated in a thin layer of medium solidified with low melting point agarose (No. A4018, Type VII, Sigma Chem.). The number of somatic embryos per subsample was counted by viewing the dispersed embryogenic callus through a dissecting microscope at 15X. Subsequently, the number of mature somatic embryos on the remaining preweighed pieces of embryogenic callus (= 400 mg) were counted. Maturation frequency = (number of mature somatic embryos per gram of callus/ number of immature somatic embryos per gram of callus) x 100.

Germination

At maturity, somatic embryos were removed from callus pieces, and aseptically placed in 30 mL glass vials (one per vial) which contained 10 mL of quarter strength basal medium with no growth regulators. Equal numbers of somatic embryos were placed in each of three germination treatments (see Fig. 29). The percentage of somatic embryos with primary root development, "germination," and the length of the primary root were determined after 21 days. Culture conditions for germination were 23°C with 16 hr irradiance from cool-white fluorescent and incandescent lights.

Conversion

Germinating somatic embryos with primary roots > 1 cm (hereafter referred to as plantlets) were transferred to either Jiffy soil mix or Jiffy soil mix: Perlite (5:1). In one experiment, somatic plantlets and seedling plantlets at an equivalent stage of development were transferred to soil mix in Magenta GA-3 vessels. The seedlings were derived from in vitro germinated zygotic embryos.
High relative humidity was maintained by controlling the tightness of the lids on GA7-3 vessels. The somatic and seedling plantlets were grown in a growth chamber (environmental conditions as described above for germination) for two months before transfer to the greenhouse. Somatic plantlets were also transferred directly from in vitro to greenhouse conditions in plastic planter trays containing soil mix. High relative humidity was maintained by enclosing the planter tray in clear plastic bags. Plants were fertilized weekly (1% solution of 14-14-14) and grown under ambient September to October light supplemented with fluorescent light (16 hr, 100 μE m$^{-2}$ sec$^{-1}$) at 20 to 25°C. Plants which set resting terminal buds during October to November were transferred to lower temperatures (5-15°C) and ambient light. The dormant plants overwintered at -5 to 10°C and ambient December to February light. On March 1 they were returned to greenhouse conditions (20-25°C) with ambient light.

Figure 29. Three somatic embryo germination treatments: (1) control = somatic embryo upright with radicle immersed in medium, (2) slant = somatic embryo on surface of medium poured on slant, and (3) inverted = cotyledons of somatic embryo immersed medium and vial inverted.
Data Analysis

Data are presented as mean values among replicated experimental units (e.g., culture plates or germination vials). Where appropriate, an analysis of variance was conducted followed by Duncan's New Multiple Range Test for multiple comparisons of means. Mean values followed by like letters were not significantly different \( (P = 0.05) \).

RESULTS

The efficiency of three stages of regeneration from Norway spruce somatic embryos was quantified: (1) maturation, development of immature somatic embryos to the cotyledonary stage (Fig. 30a, 30b), (2) germination, growth of the primary root (Fig. 30c), and (3) conversion, establishment and growth of plantlet in soil (Fig. 30d).

Maturation

The frequency at which immature somatic embryos of Norway spruce developed to the mature cotyledonary stage is given in Table 16. Induction of development on medium with the growth regulators IBA and ABA (control development protocol) resulted in both a higher density of immature somatic embryos and higher maturation frequency than development on medium lacking the growth regulators (experiment No. 1). Therefore, in subsequent experiments only the control developmental protocol was used. There were considerable differences in maturation frequency among the three embryogenic callus lines tested. Each callus line was derived from an individual immature embryo explant. Line NS1-5 had consistently low maturation frequencies, 1 to 4%, whereas both lines NS1-8 and -13 had higher maturation frequencies, about 14%. 
Figure 30. Regeneration of Norway spruce plants from embryogenic callus (A–E) and comparisons of somatic and seedling plants derived from zygotic embryos (F–H). A: Immature somatic embryos after 21 days on development medium. The density of somatic embryos was determined at this time. B: Mature (cotyledonary) somatic embryos. C: Somatic embryo germination – primary root (pr) growth. D: Conversion – survival and growth of somatic plant in soil. E: Somatic plant with dormant bud (db) set prior to overwintering. Photo taken 9-26-87. F: Renewed vegetative growth (arrow) of control seedling plant (ze). G: Renewed vegetative growth of somatic plant (se). Both F and G photos taken 3-20-87. H: Control seedling plant (left) and somatic plant (right). Photo taken 7-9-87. Scale bars: A–C = 1 mm, D–G = 1 cm, H = 5 cm.
Table 16. Maturation frequency of Norway spruce somatic embryos derived from three embryogenic callus (EC) lines.a

<table>
<thead>
<tr>
<th>Experiment No.</th>
<th>EC Line No.</th>
<th>Developmental Protocol</th>
<th>Somatic Embryos</th>
<th></th>
<th>Maturation Frequency, %</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Immature, mean no./gr.</td>
<td>Mature, mean no./gr.</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>NS1-5</td>
<td>Control</td>
<td>(4) 580^a</td>
<td>(12) 16.6^a</td>
<td>2.9</td>
</tr>
<tr>
<td>1</td>
<td>NS1-5</td>
<td>-gr. reg.</td>
<td>(4) 230^b</td>
<td>(12) 0.2^b</td>
<td>0.1</td>
</tr>
<tr>
<td>2</td>
<td>NS1-5</td>
<td>Control</td>
<td>(17) 640</td>
<td>(17) 23.3</td>
<td>3.6</td>
</tr>
<tr>
<td>3</td>
<td>NS1-5</td>
<td>Control</td>
<td>(10) 880</td>
<td>(19) 12.0</td>
<td>1.4</td>
</tr>
<tr>
<td>1</td>
<td>NS1-13</td>
<td>Control</td>
<td>(4) 150^b</td>
<td>(12) 22.2^a</td>
<td>14.8</td>
</tr>
<tr>
<td>1</td>
<td>NS1-13</td>
<td>-gr. reg.</td>
<td>(4) 90^b</td>
<td>(12) 0.6^b</td>
<td>0.7</td>
</tr>
<tr>
<td>4</td>
<td>NS1-8</td>
<td>Control</td>
<td>(14) 390</td>
<td>(14) 53.1</td>
<td>13.5</td>
</tr>
</tbody>
</table>

^aControl developmental protocol, as described in materials and methods, contained the growth regulators, IBA and ABA. The -gr. reg. developmental protocol was the same as the control without the growth regulators. N = number of pieces of embryogenic callus sampled. Means within columns of experiment No. 1 followed by like letters are not significantly different.

Germination

The effect of three culture treatments (see Fig. 29) on germination of somatic embryos is summarized in Table 17. The percentage of somatic embryos which germinated was approximately doubled on culture treatments which avoided immersion of the radicle in the solidified medium relative to the control treatment where the radicle was immersed in the solidified medium. Germination frequencies were as high as 82% on individual replicate trials of both the slant and inverted treatments. Statistically, only the mean germination frequency of the inverted treatment was significantly higher than the control treatment. The rate of root growth, as measured by the root length at 21 days, was significantly
higher on the slant treatment (Table 17). It should be noted that the root development of somatic embryos was growth of the primary root rather than development of adventitious roots. Qualitative observations (data not shown) of somatic embryos on the three culture treatments revealed that (1) excessive callusing occurred at the radicle region of somatic embryos immersed in solid medium (control treatment), (2) somatic embryos germinated on the slant treatment had the most vigorous primary roots, and (3) primary roots of somatic embryos germinated on the inverted treatment had more root hairs than either the control or slant treatments.

Table 17. Germination of Norway spruce somatic embryos on three culture treatments.a

<table>
<thead>
<tr>
<th>Treatment</th>
<th>N</th>
<th>Germination, %</th>
<th>Root Length, mm</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>mean [range]</td>
<td>mean [range]</td>
</tr>
<tr>
<td>Control</td>
<td>60</td>
<td>27a [0-46]</td>
<td>2.3a [0.5-4.0]</td>
</tr>
<tr>
<td>Slant</td>
<td>58</td>
<td>45ab [20-82]</td>
<td>5.9b [1.0-8.5]</td>
</tr>
<tr>
<td>Inverted</td>
<td>58</td>
<td>56b [25-82]</td>
<td>2.0a [1.0-5.5]</td>
</tr>
</tbody>
</table>

aSomatic embryos derived from line NS1-5. Refer to materials and methods and Fig. 29 for complete description of treatments. N = total number of somatic embryos per treatment, with an average of 9 per replicate germination trial.

Conversion and Continued Plant Growth

A total of 31 somatic embryo plantlets were transferred to the greenhouse. Nine (29%) survived transfer to greenhouse conditions and set resting terminal buds (Fig. 30E). The nine somatic plants survived overwintering (to -5°C) and renewed vegetative growth synchronously with control seedlings grown and overwintered in the same conditions (compare Fig. 30F and 30G). Six of the nine somatic plants continued growth and appeared phenotypically normal relative
to seedling derived plants. Figure 30H shows a somatic plant and a seedling control. A further description of the growth characteristics of the six somatic plants is given in the report section on somatic plant fidelity.

DISCUSSION

Using a method to quantify the level of somatic embryogenesis in Norway spruce cultures (Becwar et al. 1987b), we have been able to determine the efficiency of embryo maturation. Our results showed that maturation was the least efficient of the three stages of regeneration studied—maturation, germination, and conversion. The highest maturation frequencies obtained were 14-15%, whereas the highest embryo germination levels ranged from 45 to 56%, and 29% of the germinants were converted to plants.

The differences observed in maturation frequency among the three embryogenic callus lines may be due to genetic differences in embryo development capacity. Whereas about 14% of the embryos of line NS1-8 and -13 matured, only 1-4% of line NS1-5 embryos matured. In several crop plants somatic embryogenesis and regeneration have been shown to be genotype specific (Reisch and Bingham 1980, Hodges et al. 1986, Luhrs and Lorz 1987). It may be possible to select embryogenic spruce lines with increased embryo maturation efficiency. We cannot rule out the possibility that differences in the density of somatic embryos and competition among developing embryos may have also affected maturation frequencies. The majority of somatic embryos which did not develop reverted to callus formation.

Our initial results showed the inclusion of low levels of IBA and ABA improved somatic embryo development. Several developmental protocols for conifer somatic embryos have included greatly reduced levels of 2,4-D, e.g., 10
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to 100-fold lower levels than during initiation and maintenance (Gupta and Durzan 1986a, Gupta and Durzan 1987, Lu and Thorpe 1987). The beneficial effect we observed with the auxin, IBA, may be similar to the beneficial effect of sequential reduction of 2,4-D. The addition of ABA to a somatic embryo development medium of angiosperms was shown to prevent precocious germination, inhibit accessory embryo formation, and decrease the frequency of abnormal embryos (Ammirato 1974 and 1983). The first report of the use of ABA in conifer somatic embryo development was with Norway spruce (Becwar et al. 1987a and b). Several reports have indicated that ABA did not improve development of conifer somatic embryos (Hakman and von Arnold 1985, Lu and Thorpe 1987). Recent reports indicated ABA levels as high as 9 μM were optimal for maturation of Norway spruce somatic embryos (von Arnold and Hakman 1988) and that ABA was beneficial for inhibition of cleavage of Douglas-fir somatic embryos (Durzan and Gupta 1987). These observed differences in the efficacy of ABA in conifer somatic embryo development may in part relate to differences in media and developmental protocols used by various workers. Regardless, our current results are consistent with numerous reports that suggest ABA improves somatic embryo maturation.

Our results also demonstrated that the highest frequencies of germination of Norway spruce somatic embryos were obtained on treatments which avoided immersion of the radicle in agar medium. The inverted culture, in which the apical meristem and cotyledons of the embryo are immersed in medium and the vial inverted, was first shown to increase root growth of zygotic embryos of sugar pine (Brown and Gifford 1958, Berlyn and Miksche 1965). In the present study with somatic embryos, although the frequency of germination was significantly higher on the inverted treatment, the root length was not different from control embryos germinated with the radicle immersed in agar. The fact that
only somatic embryos germinated on the surface of the agar medium (slant treatment) had significantly longer roots, suggests that the root growth of the inverted embryos may have been inhibited by desiccation stress. The results of Brown and Gifford (1958) with zygotic embryos also suggested that the uptake of sucrose via the cotyledons (inverted treatment) improved both the rate and duration of root growth relative to when it was supplied to the embryo directly via the root. This is similar to \textit{in vivo} embryo germination where carbohydrates are supplied to the embryo via the cotyledons from the female gametophyte. Thus, our results suggest that the somatic embryos of Norway spruce respond similarly to changes in conditions of germination as do zygotic conifer embryos.

In order for low-cost, high-volume propagation of conifers via somatic embryogenesis to be realized, it will be necessary, as recently emphasized for horticultural crops (Redenbaugh 1986a), to place more emphasis on the embryogeny of conifer somatic embryos (i.e., development, maturation, and conversion). Much progress has been made in conifer somatic embryogenesis since the first reports in Norway spruce (Hakman \textit{et al.} 1985) and European larch (Nagmani and Bonga 1985). This progress has been mainly in the area of early embryogenesis - initiation, embryo formation, and verification of the morphological similarity of somatic and zygotic embryos. Emphasis needs to be placed on the efficient production of high quality somatic embryos - those that can be efficiently converted to vigorous plants that are phenotypically normal and genotypically identical to the explant tissue.

Redenbaugh \textit{et al.} (1986b) have provided impressive evidence for the utility of an empirical approach to improve somatic embryo conversion frequencies by establishing conditions which produced high quality somatic embryos. They found that each stage of the regeneration process, including initiation,
induction of development, and maturation affected embryo quality. For instance, maintenance of alfalfa callus for longer than three months or subculturing the callus at infrequent intervals led to a decline in embryo conversion. Using an optimized protocol for production of high quality somatic embryos, they were able to raise the conversion frequency from 0.5 to 32% (Redenbaugh et al. 1986b).

A decrease in regeneration potential with increasing time in culture has frequently been reported for plant tissue cultures. It should be noted that we have observed similar effects with our spruce somatic embryo regeneration system. The results reported here were conducted on cultures that were 6 to 12 months old. Since that time, the density of somatic embryos has remained high (i.e., the number of somatic embryos per gram of callus on developmental medium), but the maturation, germination, and conversion frequencies have dropped. Thus the overall regeneration efficiency may be affected by factors prior to the maturation-conversion process and in particular it may be necessary to regularly initiate new embryogenic cell lines in order to maintain a high plant recovery frequency.

The potential efficiency of the entire regeneration process, i.e., the plant recovery frequency, was 2.4%, based on the highest maturation level (15%) attained with line NS1-8. The overall efficiency of regeneration for line NS1-5, where maturation frequencies were considerably lower (< 4%), was 0.6%. Thus, only about 1-2% of the somatic embryos induced to develop were converted to plants. Certainly, these levels will have to be raised significantly, probably by an order of magnitude, for regeneration via somatic embryogenesis to be of commercial use in mass propagation of conifers. Our results indicate that maturation is the key "bottleneck" in attaining a higher plant recovery
frequency. Even though germination frequency was the highest of the three steps, it is likely that further refinements in germination techniques will result in significant improvements in germination. It should be noted that preliminary studies resulted in germination frequencies of about 11% (refer to p. 80-81, Project 3223 Annual Report Fourteen. Note also that the terms germination and conversion were used equivalently in Report Fourteen). We, therefore, have improved germination by reaching a 56% optimum level reported in the current experiments.

To our knowledge, this is the first quantitative analysis of the efficiency of the somatic embryo regeneration process in conifers. The only quantitative data available for comparison is with Norway spruce, where Gupta and Durzan (1986a) reported 4-8 plantlets per gram of embryogenic callus. It is important to note that this value is for the production of \textit{in vitro} plantlets (equivalent to germinants in our current study) and gives no information about the overall plant recovery frequency. Combining our maturation (Table 16) and germination (Table 17) data for comparative purposes indicates that we obtained about 10 plantlets per gram of embryogenic callus from line NS1-5. A potentially higher number of plantlets could be obtained from line NS1-8, 30 per gram of callus, if germination frequencies are similar between lines (Table 17 germination results are for line NS1-5). Thus, our results with line NS1-5 agree with or are slightly higher than the previously reported value. Our results with line NS1-8 suggest the potential for further improvements in the number of \textit{in vitro} plantlets recovered per gram of callus.

A conversion frequency of only 29% (obtained in this study) suggests that the \textit{in vitro} derived plantlets (germinants) are extremely delicate and sensitive to environmental changes that occur during the transfer to soil. Here
again, further refinements in techniques of plantlet transfer from in vitro to nonaxenic conditions are needed. Our observations suggested that allowing the somatic germinants to acclimatize to the transfer to soil under growth chamber conditions was superior to direct in vitro greenhouse transfer. Furthermore, strict control of the relative humidity was essential during the initial growth of the plantlet in soil. It seems likely, therefore, that refinements in techniques alone can significantly improve both the germination and conversion frequency. The area of maturation warrants a concentrated research effort, both basic and empirical, in order to improve maturation frequencies.

CONCLUSION

Our results have provided quantitative information on the efficiency of the regeneration process from somatic embryos of Norway spruce. This study is the first to examine all stages in the conifer regeneration process - maturation, germination, and conversion. Although the overall plant recovery frequency was low, 1-2%, our results clearly demonstrate that it is possible to regenerate phenotypically normal plants from Norway spruce somatic embryos. Previous reports have emphasized the production of plantlets under in vitro conditions. Our results provide essential information on the continued growth of the somatic plantlets. In terms of physiological response to changing environment, the somatic plants have responded (set dormant buds, overwintered, and renewed vegetative growth) strikingly similar to control seedlings. This is the first demonstration of overwintering and renewed vegetative growth from dormant buds of conifer somatic embryo plants.
BIOCHEMISTRY OF SOMATIC EMBRYOGENESIS AND EMBRYO DEVELOPMENT

In the recent past, molecular level investigations in this project were focused primarily on markers to characterize embryogenic vs. nonembryogenic calli (e.g., Report Fourteen, pp. 83-). Given that the visual and tactile indicators of conifer embryogenic callus have held up well for all conifer species investigated, the need for molecular markers has lessened considerably. Nevertheless, the latter have been and continue to be useful in sorting doubtful cases and were found to be predictive in some instances as noted below.

The shift is now toward utilizing the molecular information associated with the markers to promote somatic embryo development. Consequently, this section consists of both a summary of molecular marker results for embryogenic vs. nonembryogenic calli for the various conifer species examined and some initial results wherein some molecular parameters were monitored over the course of zygotic embryo development to provide guideposts by which to monitor somatic embryo development. Sandwiched between these topics is an early report from exploratory research on lipid analysis, yielding another marker and potential guidepost for somatic embryo development.

BIOCHEMICAL DIFFERENCES BETWEEN EMBRYOGENIC AND NONEMBRYOGENIC CALLI OF CONIFERS

Introduction

Morphological differences between embryogenic and nonembryogenic callus phenotypes are well known and provide a basis for selection of cultures with a high efficiency for plant regeneration (Nabors et al. 1983). Embryogenic conifer callus is no exception in this respect, exhibiting a clear to translucent,
mucilaginous phenotype that is easily recognized. Although embryogenic conifer callus has been described morphologically and histologically (Hakman et al. 1985; Gupta and Durzan 1987; Hakman et al. 1987), little is known about this tissue on the molecular level. Only recently has some data of this nature begun to be reported (Wann et al. 1987). Comparative biochemical analyses of these two callus phenotypes could lead to rapid and definitive tests for embryogenic potential in addition to identification of metabolic features important for the initiation and development of somatic embryos.

In this laboratory, biochemical analyses of embryogenic and nonembryogenic calli of Norway spruce recently revealed marked differences in rates of ethylene evolution and apparent protein synthesis as well as in glutathione (GSH) and total reductant content (Wann et al. 1987). In this report it is shown that the biochemical differences noted between embryogenic and nonembryogenic Norway spruce calli are also characteristic of Pinus and Larix species, implying metabolic similarity among all of these conifers. Additionally, useful means to recognize embryogenic calli were also found in isozyme and total protein patterns.

MATERIALS AND METHODS

**Initiation of Embryogenic and Nonembryogenic Calli**

Embryogenic calli of *Pinus taeda* L., *Pinus strobus* L., *Picea abies* (L.) Karst, and *Picea glauca* (Moench) Voss were initiated from immature embryos. In *Picea* spp., initiation of embryogenic callus was by the method of Hakman et al. (1985). In *Pinus taeda* and *Pinus strobus*, initiation occurred on a wide variety of media in the dark, in some cases similar to conditions described by Gupta and
Durzan (1986). In *Pinus serotina* Michx., embryogenic callus was extruded from the archegonial end of cultured intact ovules, similar to the method described by Smith *et al.* (1985). Embryogenic cultures of *Larix decidua* Mill were generously provided by J. M. Bonga and were initiated as described by Nagmani and Bonga (1985). Some of the details of the initiation processes for the various conifers studied are depicted in Table 18. Nonembryogenic callus was obtained under most of the conditions listed above by the culture of embryos of much later stages of development than are required for the initiation of embryogenic callus.

Table 18. Summary of procedures used to initiate embryogenic calli in various spruce and pine species.

<table>
<thead>
<tr>
<th>Species</th>
<th>Explant</th>
<th>Medium</th>
<th>Growth Regulators, mg/L</th>
<th>Culture</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Picea abies</em></td>
<td>Immature cotyledonary embryos</td>
<td>Lpa</td>
<td>2 2,4-D; 1 BA</td>
<td>Light</td>
<td>Hakman <em>et al.</em> 1985</td>
</tr>
<tr>
<td><em>Picea glauca</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Pinus taeda</em></td>
<td>Immature precotyledonary embryos</td>
<td>MSGb</td>
<td>2 2,4-D; 1 BA</td>
<td>Dark</td>
<td>Gupta and Durzan, 1986</td>
</tr>
<tr>
<td><em>Pinus strobus</em></td>
<td>Immature precotyledonary embryos</td>
<td>DCRc</td>
<td>3 2,4-D; 0.5 BA</td>
<td>Dark</td>
<td>Gupta and Durzan, 1986</td>
</tr>
<tr>
<td><em>Pinus serotina</em></td>
<td>Fertilized ovules</td>
<td>MSGb</td>
<td>None</td>
<td>Light, 1% char coal</td>
<td>Smith <em>et al.</em> 1985</td>
</tr>
</tbody>
</table>

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dMSG medium supplemented with casein hydrolyzate 1000 mg/L and glutamine 500 mg/L (R. Nagmani, unpublished).
Biochemical Assays

Biochemical analyses of embryogenic and nonembryogenic calli were conducted as previously described (Wann et al. 1986, 1987). Briefly, ethylene was determined by gas chromatography; apparent protein synthesis was measured by tritiated leucine incorporation into trichloroacetic acid precipitable protein; GSH was measured by the rereduction of oxidized GSH (GSSG) by commercial GSSG reductase; and total reductants were measured by ferric ion reduction. In *Picea* spp., embryogenic and nonembryogenic calli of the same genotype and age cultured under the same nutritional, environmental and hormonal conditions were assayed. Isogenic analyses of embryogenic and nonembryogenic calli were not conducted in *Pinus* and *Larix*, although the conditions used to initiate both phenotypes were similar.

The extracts used for the isozyme analyses were prepared by the freeze/thaw technique of Berger et al. (1985). The isoperoxidases were resolved by isoelectric focusing (IEF) for 35 min at 25 watts on a pH 3.5-9.5 agarose gradient using an LKB Multiphor 2117. The stain was a modification of that used by Gove and Hoyle (1975) and consisted of 45 mM guaiacol, and 3 mM H₂O in 0.2M acetate buffer, pH 5.0. Some bands are subject to fading as is well known for the guaiacol reaction (Maehly and Chance 1954). This is not a major problem in this agarose IEF system, although any photographs should be taken without undue delay. For the SDS-PAGE analyses, soluble proteins were extracted from embryogenic or nonembryogenic calli by homogenizing the tissue in cold 50 mM HEPES pH 7.5 containing 1 mM PMSF (phenylmethylsulfonyl fluoride). After a five minute centrifugation in an Eppendorf microfuge, protein in the supernatant was quantified by the Bradford procedure (Bradford 1976) and loaded onto standard
7.5-15% gradient SDS-polyacrylamide gels (Laemmli 1970). Equal amounts of protein (2-3 μg) were loaded onto each lane. Visualization was by a modified silver stain (Oakley et al. 1980).

Results and Discussion

Biochemical characterization of embryogenic vs. nonembryogenic calli of several conifers is presented in Table 19. All but the larch embryogenic callus were initiated in this laboratory, and that was started by one of the authors before she joined this laboratory. Data of this nature for Norway spruce have been published previously, but some is presented here for reference. As can be noted in Table 19, for Norway spruce and white spruce we have had the luxury of comparing embryogenic and nonembryogenic calli that originated from a single explant. Only one comparison of an embryogenic with a nonembryogenic callus is presented for each species. Although more data are extant for additional cell lines of each species, that presented is typical of calli with established embryogenic competence or incompetence.

The differences between embryogenic and nonembryogenic calli that were observed for these biochemical parameters were of sufficient magnitude that it can be stated that they are indicative of embryogenic competence in conifer calli. Relative to the other conifers, white spruce can be difficult to sort by these tests, but it does not constitute an exception to the generalization just made. Although interspecific differences were detected and the magnitude of these differences may vary with cell line within species, the trends were always such that relative to nonembryogenic calli of the same species, embryogenic conifer calli (1) evolve ethylene at a lower rate, (2) contain lower amounts of GSH and other nonspecified reductants, and (3) exhibit a greater rate of net leucine incorporation into protein.
Table 19. Biochemical differences between embryogenic (E) and nonembryogenic (NE) conifer callus.

<table>
<thead>
<tr>
<th>Species</th>
<th>Cell Line</th>
<th>Embryogenic?</th>
<th>Protein Synthesis, a cpm/ug</th>
<th>Total Reductants, a A700/g fwt</th>
<th>GSH, a nmol/g fwt</th>
<th>Ethylene, a nL/g fwt</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Pinus taeda</em></td>
<td>(LP12F)1</td>
<td>Yes</td>
<td>6474 ± 220</td>
<td>7 ± 1</td>
<td>59 ± 10</td>
<td>2 ± 2</td>
</tr>
<tr>
<td>(loblolly pine)</td>
<td>(LP10DE)</td>
<td>No</td>
<td>193 ± 28</td>
<td>28 ± 2</td>
<td>510 ± 40</td>
<td>689 ± 151</td>
</tr>
<tr>
<td><em>P. strobus</em></td>
<td>(WP2II)1</td>
<td>Yes</td>
<td>8172 ± 853</td>
<td>13 ± 2</td>
<td>140 ± 19</td>
<td>6 ± 6</td>
</tr>
<tr>
<td>(E. white pine)</td>
<td>(WP5B)3</td>
<td>No</td>
<td>186 ± 45</td>
<td>172 ± 32</td>
<td>378 ± 43</td>
<td>759 ± 61</td>
</tr>
<tr>
<td><em>P. serotina</em></td>
<td>(PO12Ao)1</td>
<td>Yes</td>
<td>7684 ± 878</td>
<td>8 ± 1</td>
<td>88 ± 16</td>
<td>8 ± 1</td>
</tr>
<tr>
<td>(pond pine)</td>
<td>(PO10Ag)3</td>
<td>No</td>
<td>2997 ± 2642</td>
<td>83 ± 22</td>
<td>704 ± 86</td>
<td>111 ± 35</td>
</tr>
<tr>
<td><em>Picea abies</em></td>
<td>(NS1)8</td>
<td>Yes</td>
<td>5061 ± 1614</td>
<td>32 ± 2</td>
<td>120 ± 28</td>
<td>0.15 ± 0.04</td>
</tr>
<tr>
<td>(Norway spruce)</td>
<td>(NS1)8</td>
<td>No</td>
<td>157 ± 77</td>
<td>535 ± 10</td>
<td>325 ± 40</td>
<td>1.75 ± 0.57</td>
</tr>
<tr>
<td><em>P. glauca</em></td>
<td>(WS5B)8</td>
<td>Yes</td>
<td>4960 ± 861</td>
<td>119 ± 14</td>
<td>434 ± 21</td>
<td>252 ± 87</td>
</tr>
<tr>
<td>(white spruce)</td>
<td>(WS5B)8</td>
<td>No</td>
<td>2067 ± 874</td>
<td>210 ± 78</td>
<td>524 ± 116</td>
<td>606 ± 301</td>
</tr>
<tr>
<td><em>Larix decidua</em></td>
<td>(L1-18)5</td>
<td>Yes</td>
<td>1859</td>
<td>85 ± 37</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
<tr>
<td>(European larch)</td>
<td>(L253-2)12</td>
<td>No</td>
<td>53</td>
<td>848 ± 206</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
</tbody>
</table>

N.D. = Not determined.
aMeans ± S.D.; N = 3.

Whereas the full significance of the foregoing observations is not yet appreciated, several important statements can be made about them. For these biochemical parameters, the embryogenic state in conifers is the same irrespective of species. The embryogenic condition in conifers may also be described as a state function, i.e., it is independent of the path by which it is reached. "Path" in this context includes explant, culture conditions and even ploidy level (the *Larix* embryogenic callus is haploid, others are diploid). Biochemical
characterization of the embryogenic state in conifers takes on increased impor-
tance because, morphologically, the embryogenic condition in the calli of these
trees is only nominally similar and is path dependent (Fig. 31). For example,
when the embryogenic calli are grown under proliferative conditions, the extent
of somatic embryo development varies with species. In pines, embryogenic callus
may be dominated by preembryogenic masses or very early stage embryos. However,
in spruce and larch the somatic embryos reach a fairly advanced state of devel-
opment without removal of the embryogenic calli from proliferation media.

The magnitudes of the biochemical differences between the two pheno-
types were such that the assays could be utilized as markers for embryogenic
callus in conifers. This was demonstrated with the total reductants assay which
predicted embryogenic potential months in advance of the appearance of somatic
embryos in white pine and pond pine. In these species, embryogenic potential
was predicted at a time when the calli were dominated by what turned out to be
preembryogenic masses (Fig. 32).

Whereas the foregoing conclusions apply to all of the conifers that were
investigated, white spruce \((Picea glauca)\) stands out as not exhibiting clear
biochemical differences in these parameters between embryogenic and nonembryogenic
callus. The lack of significant differences in total reductants can be easily
understood in light of the observation that, even when growing under prolifera-
tion conditions with 2,4-D, white spruce embryogenic calli sometimes exhibit a
pink coloration indicative of anthocyanins which can reduce ferric ion in the
test. In other words, unlike the other conifers, white spruce somatic embryos
occasionally show precocious secondary product accumulation. The lack of signi-
ficant differences in the other markers for white spruce might be related to the
observation that it takes several subculture intervals to separate the two
Figure 31. Typical level of somatic embryo development attained in embryogenic callus without transfer from proliferation medium: (a) Norway spruce, (b) white spruce, (c) loblolly pine, (d) white pine, (e) pond pine, (f) European larch. Scale bar = 100 μm.
callus phenotypes of spruce (Wann et al. 1987). Thus, for a period, apparently nonembryogenic callus will continue to contain localized regions where embryogenic tissue will emerge. It is quite possible that when the two callus phenotypes of white spruce were assayed, complete segregation of embryogenic and nonembryogenic callus had not yet been completed even though visibly so. It should be noted that the trends in each of these parameters for the two phenotypes are the same in white spruce as for the other conifers. It is likely that with greater replication white spruce would also show statistically significant differences in these parameters for the two phenotypes.

Figure 32. Preembryogenic masses in (a) white pine and (b) pond pine embryogenic callus. Scale bar = 100 μm.

Both isozyme patterns and SDS-PAGE separations of soluble proteins have been useful in distinguishing embryogenic from nonembryogenic calli as well. Fig. 33 shows that three different Norway spruce cell lines each exhibit distinctive guaiacol isoperoxidase banding patterns for embryogenic versus nonembryogenic callus extracts. Isozyme patterns of other enzymes such as acid phosphatase (not shown) are also capable of sorting the two phenotypes. The proteins separated and visualized by SDS-PAGE showed clear differences between embryogenic and
nonembryogenic calli with the exception of white spruce (Fig. 34). White spruce presents difficulties for the other markers also as discussed above. The most obvious difference observed on the protein gels is the prominence of a protein of approximately 18-20 kd in nonembryogenic tissues of all but the white spruce. The use of isozymes and total protein patterns to identify embryogenic tissue is becoming common, e.g., see Everett et al. (1985) and Sung and Okimoto (1981).

Figure 33. Peroxidase isozyme patterns in duplicate of extracts of embryogenic (E) and nonembryogenic (NE) calli of the three distinct Norway spruce cell lines labeled 1, 5, and 8. The pH gradient runs from 3.5 (bottom) to 9.5 (top).

The biochemical assays utilized here are all relatively rapid, convenient, and require small amounts of tissue. In the case of ethylene evolution, the assay is nondestructive, enabling reuse of the tissue in other experiments. These attributes make these assays attractive candidates for markers of embryogenic potential. Nevertheless, biochemical markers have limited utility when conifer embryogenic callus has such a striking phenotype that usually it can be visually
recognized. Not all nonembryogenic conifer callus is green, however, and the markers can be used to rogue this material. It seems likely that biochemical assays such as these and others will find greater utility in future attempts to identify key metabolic steps in the growth and development of somatic embryos into plants. For example, inhibition of GSH biosynthesis by buthionine sulfoximine in embryogenic callus of Norway spruce has been observed to cause a doubling of the maturation frequency of somatic embryos (unpublished, this laboratory), similar to effects noted for wild carrot somatic embryos (Earnshaw and Johnson 1985).

![Figure 34. SDS-PAGE of soluble proteins extracted from white spruce (WS), pond pine (PP), white pine (WP) and loblolly pine (LP) embryogenic (E) and nonembryogenic (NE) calli.](image)

**Conclusions**

Embryogenic and nonembryogenic calli of loblolly pine (Pinus taeda), Eastern white pine (P. strobus), pond pine (P. serotina), white spruce (Picea glauca), and European larch (Larix decidua) were analyzed for biochemical parameters previously shown to be indicative of an embryogenic state in Norway spruce.
Concentrations of glutathione and total reductants as well as rates of ethylene evolution and incorporation of radioactive leucine into protein in the two callus types were consistent with the Norway spruce observations. Embryogenic potential of loblolly pine and pond pine callus was predicted by biochemical analysis in advance of the appearance of somatic embryos. Other parameters such as isozyme patterns and SDS-PAGE of soluble proteins could also be used to distinguish embryogenic from nonembryogenic conifer callus. Among the species investigated, white spruce was the most difficult to sort by these methods.

LIPIDS — ANOTHER POSSIBLE BIOCHEMICAL MARKER OF EMBRYOGENIC CALLI AND AID IN MATURATION STUDIES?

Introduction

In the course of preparing extracts of zygotic tissue in various stages of development (from the 1987 summer cone collections), it was noted that Douglas-fir aqueous extracts prepared in July were quite turbid, whereas analogous loblolly pine extracts were not. Since this turbidity is indicative of lipids and lipid bodies were abundant in zygotic pine embryos studied previously in conjunction with chloroplast development research, we began to wonder why little turbidity was evident in the pine extracts. However, turbidity did appear in the pine extracts in August. Apparently, dry weather in the South during early summer may have slowed loblolly pine ovule development in summer 1987. Nevertheless, given the importance of lipids in membranes and as energy sources, impetus was provided to examine lipids as another possible parameter distinguishing embryogenic from nonembryogenic callus. To see if lipid changes might be associated with embryo development, analysis of embryos and gametophytes from mature conifer
seeds was also started. No research had been conducted on lipids since the very early days of this project, long before the advent of embryogenic callus.

**Materials and methods**

After some exploratory investigations on extraction and thin layer chromatography, most extractions were conducted with a single phase solvent consisting of isopropanol/chloroform/methanol (3/2/1, v/v). Likewise, filtered extracts were chromatographed mainly on 250 μm silica gel with a two stage development. The first solvent, chloroform/methanol/acetic acid (98/2/1, v/v) (Bitman et al. 1981), was used to move neutral lipids out near the solvent front. Therefore, this solvent was run to the end of the plate, then followed after drying with a solvent to move polar lipids. The latter, chloroform/methanol/acetic acid/water (170/30/20/7, v/v) (Harborne 1973), was stopped at 15 cm. After drying, lipids were detected with iodine vapor.

**Results and Discussion**

From the very first chromatograms of Norway spruce callus extracts, it was evident that the chlorophyll and carotenoid pigments found in nonembryogenic callus (only) would complicate the desired comparisons between the phenotypes. Therefore, since both phenotypes had become available for white spruce wherein the nonembryogenic callus was not green, the comparison was easier there. A temporary switch in species appeared justifiable on the grounds that extracts of mature seeds of Norway spruce, white spruce, loblolly pine, and Douglas-fir yielded very similar chromatograms across these four species (Fig. 35).

Results to date may be summarized as follows. In addition to the just mentioned similarity of mature seed lipids across species, for every species the gametophyte components were nearly identical to the embryo components except for
quantitative differences. The latter was most notable for the neutral lipids which were quantitatively dominant in all extracts as might be expected. However, the most exciting finding was a polar lipid spot in embryogenic white spruce callus that was missing in the nonembryogenic callus (Fig. 36). This also appears to be the case for Norway spruce, considering the pigment interference (see above). This polar lipid, which is likely to be a phospholipid, also seems to have a counterpart in extracts of early (only) stage loblolly pine ovules. Since this component is associated with embryogenic rather than nonembryogenic callus and not vice-versa, it is hoped that this finding will be useful in future studies of somatic embryo development.

![Figure 35](image1.png) ![Figure 36](image2.png)

Figure 35. Thin layer chromatograms of lipids extracted from mature seeds of loblolly pine (LP), Douglas-fir (DF), white spruce (WS) and Norway spruce (NS). Neutral lipid spots are at the very top; other spots are polar lipids.

Figure 36. Thin layer chromatograms of lipids extracted from embryogenic (E) and non-embryogenic (NE) white spruce calli. A polar lipid apparently unique to E callus is marked by the arrow.

Conclusion and Future Directions

The lipid research reported here still is too incomplete to permit its value to be ascertained. Further characterization of the polar lipid is
desirable and perhaps essential before exploitation of this finding in somatic embryo development can be attempted. Also, whether what is now a similar $R_f$ spot on chromatograms is really an identical compound across conifer species needs attention. Following this, the appearance/disappearance of this substance at various stages of zygotic embryo development needs to be verified and somatic embryo development examined in the same respect. If somatic embryo development appears to be on a different course in this regard, the stage could be set for intervention to promote development.

PROTEIN SYNTHESIS DURING LOBLOLLY PINE ZYGOTIC EMBRYO DEVELOPMENT

Introduction

As is evident in other sections of this report, one of the key problems facing the research team is the maturation and/or conversion of somatic embryos into plantlets. While the successful initiation and maintenance of embryogenic conifer calli involved a tissue culture approach, stimulating further development of the embryos may require an understanding of the physiological and biochemical aspects of embryo development. One approach by which to better understand and document the development of embryos, either somatic or zygotic, is to monitor gene expression in the developing embryos. This is most easily done by looking at the proteins present in the tissues by SDS-PAGE (polyacrylamide gel electrophoresis). Using this technique, proteins or protein subunits are separated on the basis of size; smaller proteins migrate through the gel faster than larger proteins. A goal of this type of work is to compare the proteins present (gene expression) in developing zygotic embryos to those found in somatic embryos. We may be able to determine, for example, that protein synthesis in the early development of the somatic embryos mirrors that in zygotic embryos, but at later stages the pattern deviates from that in the natural system. This might help us identify,
in a biochemical or molecular sense, when the development of the somatic embryos becomes abnormal.

Gene expression, as visualized by SDS-PAGE, has been studied in developing embryos of a number of species. In a recent review, Quatrano describes work of this type that has been carried out with crop species including cotton, rapeseed, wheat and soybean (Quatrano, 1986). Cotton seed/embryo development has received the most attention. In work done by Dure's lab, sets of proteins that are only expressed during certain stages of cotton embryo development have been identified. Groups of proteins were found to be synthesized during early, mid or late embryogenesis or during early germination. Some proteins were found to be synthesized during more than one developmental stage, while others are synthesized more or less continuously (Dure, 1985). In other work relevant to our efforts, proteins present in developing somatic embryos were compared to those in zygotic embryos (Crouch, 1982; Crouch and Sussex, 1981). Many proteins found in developing zygotic embryos did appear in the somatic embryos, but the proteins did not appear in the somatic embryos until they were more developed than the zygotic embryos in which the proteins were found. This led Crouch to conclude that "although the ...proteins are present in both zygotic and nonzygotic embryos, the timing and extent of accumulation differ" (Crouch, 1982). These types of comparisons are planned in our lab.

**Materials and Methods**

Immature embryos were obtained from developing loblolly pine cones collected and shipped to IPC at intervals throughout the summer growing season by Union Camp personnel in Rincon, GA. Cones from clone "E" were used in this study. These cones were also the source of explant material for tissue culture studies. Since a cone collected on a given date contained immature embryos at
various stages of development, samples consisted of immature embryos of an arbitrarily defined stage of development, rather than the date of cone collection. Embryos were separated into six stages of development; stage one being precotyledonary embryos and stage six being embryos, dissected from cones received September 15, that appeared to be fully developed. The embryos comprising the samples were photographed so that embryos of similar stages can be identified in later studies.

Loblolly pine seedlings germinated and grown in either light (normal greenhouse conditions) or total darkness were also analyzed with the intent of using these samples to help in the identification of rubisco (ribulose 1,5-bisphosphate carboxylase/oxygenase) in the embryo samples.

Soluble proteins were extracted by homogenizing the tissue in cold 50 mM HEPES (pH = 7.5) containing 1 mM PMSF (phenylmethylsulfonyl fluoride, Sigma Chemical Company). After centrifuging 5 min in an Eppendorf microfuge, protein in the supernatant was quantified by the Bradford dye binding assay (Bradford, 1976) and loaded onto standard 12% or 7.5-15% gradient SDS-polyacrylamide gels (Laemmli, 1970). Approximately equal amounts of protein (2-3 μg) were loaded onto each lane and visualized in the gel using a modified silver stain technique (Oakley et al., 1980).

Results and Discussion

The results of our first experiment appear in Fig. 37. The numbers above the lanes on the gel correspond to the stage of embryo development, as described above. Upon even casual inspection, the accumulation of a number of prominent proteins can be observed as the embryos develop. The protein identified by the letter "a", for example, accumulates to a relatively high concentration as the immature embryo develops. While proteins such as a and b accumulate gradually over the course of development, the synthesis of other
Figure 37. SDS-PAGE analysis of soluble proteins isolated from loblolly pine (LP) and white pine (WP) seedlings germinated in the light (L) or complete darkness (D). Lanes 1-6 contain protein present in immature zygotic loblolly pine embryos at different stages of development (see text). Rubisco large subunit (LS) and small subunit (SS) are identified in the seedling samples. Relative sizes (kilodaltons) of proteins are indicated in the two lanes containing protein standards.
proteins, i.e., c, appear to begin abruptly in later stages of development (lanes 5, 6). Other proteins reach their highest level of abundance during the mid stages of development (band d, stage 3 and 4) and then decline as embryo maturation continues. As expected, many proteins are synthesized throughout the course of embryo development (i.e., band e). These results agree with work done with angiosperm species, in which classes of proteins were found to be expressed during different developmental stages (Dure, 1985).

The expression of rubisco, which is abundant in almost all green plant cells, is regulated both by developmental stage and light. Rubisco, composed of two protein subunits having molecular weights of approximately 55 and 14 kd (kilodaltons), is an enzyme responsible for carbon fixation in photosynthesis. In an attempt to identify this protein in the samples of immature embryos, proteins were isolated from dark and light grown seedlings. Subunits of the rubisco enzyme are easily identified on a gel due to their size and abundance in the extract. The large subunit (LS) and small subunit (SS) are prominent bands in the lanes containing proteins extracted from the seedlings (Fig. 1). Using the protein bands in the seedling preparations to identify rubisco subunits in the embryo samples, it is evident that the rubisco large subunit (LS) is not among the proteins that accumulate to a large degree during the early developmental stages included in this study. Rubisco small subunit, on the other hand, appears to accumulate slowly as the embryo matures (band f).

While attempts to identify most of the proteins visualized on these gels would prove futile, some of the more abundant proteins might be tentatively identified as seed storage proteins. It would be expected that the abundant proteins in female gametophyte tissue would be storage proteins. Comparison of
these proteins in the female gametophyte tissue with those in the developing embryo is a crude way to determine if the storage proteins are present in the embryo. These are the types of comparisons that have been made using other species (Crouch and Sussex, 1981). If the storage proteins are found in the zygotic embryos, then it is reasonable to assume that they should also appear in the somatic embryos, if the somatic embryos are following the normal pattern of development. This has also been shown with somatic embryos of angiosperm species including Brassica (Crouch, 1982). Ultrastructural studies of somatic embryos of *Crambe abyssinica* and oil palm have shown that protein bodies and lipid bodies are present in the somatic embryos but not in the nonembryogenic calli (Jones, 1974). It seems that this storage or reserve material plays an important role in the development of somatic embryos of these species.

If our somatic embryos are found to lack storage proteins or don't express a group of proteins that normally appear during zygotic embryogenesis, it is reasonable to assume that we might be able to induce their synthesis by manipulating culture conditions. Factors including growth regulators (especially ABA), osmolarity and cold treatment might be used to change the patterns of gene expression in the somatic embryos. ABA levels are known to rise during zygotic embryo development (Wang *et al.*, 1987) and more importantly have been used to induce the formation of morphologically normal somatic embryos in several species. Ammirato reported that "ABA...produces "clean" embryos, that is, single structures with excellent cotyledons, unexpanded radicles, and axes free of any proliferations" (Ammirato, 1974).

**Conclusions**

The pattern of protein synthesis or expression was found to change in developing zygotic embryos of loblolly pine. Specific proteins are found to accumulate as the embryo matures from precotyledonary through fully developed
embryo. This information will serve as a baseline by which to judge the biochemical/molecular development of our somatic embryos. If proteins in the somatic embryos are found not to resemble those in the zygotic embryos, cultural factors such as the growth regulator ABA will be used to stimulate the expression of the correct set of proteins in the somatic embryos. This type of work and analysis of proteins found in early stages of seed/embryo germination are currently under-way.

TOTAL REDUCTANTS, GLUTATHIONE LEVELS AND GLUTATHIONE REDUCTASE ACTIVITY DURING THE COURSE OF ZYGOTIC EMBRYO DEVELOPMENT

Introduction

The total reductants test and glutathione (GSH) analysis have both been useful in the sorting of embryogenic from nonembryogenic conifer calli. Since GSH is known to be one of many reducing agents capable of response in the total reductants test, GSH analysis might be viewed as a specific case of the simpler general test. Given that changes in GSH and total reductants content have been linked to development previously, including white and red pine zygotic embryogenesis (Johnson et al., 1987), these parameters were examined here in the developing ovules of the target species. The enzyme, glutathione (GSSG, oxidized form of GSH) reductase, was examined also as it is one of the factors controlling the level of GSH in living cells.

Materials and Methods

Glutathione analyses and total reductants tests were conducted as previously described (Earnshaw and Johnson, 1985; Johnson et al., 1987). The GSSG reductase extraction and analysis were adapted from Earnshaw and Johnson (1987). The enzyme was extracted from developing ovules (embryos and gametophytes in some late stage collections) of Douglas-fir or loblolly pine with 10 mM
potassium phosphate buffer, pH 7.0. Triplicates of three ovules each were extracted with 2.0 mL of cold buffer and centrifuged at 17,000 x g for 15 min to yield supernatants for analysis. Both the sample and reference cuvettes contained 0.4 μmoles NADPH, 1.7 mg BSA, and 4.5 μmoles EDTA, all in buffer with additional buffer to bring the total volume to 1.50 mL in the reference cuvette but leaving room for extract and GSSG in the sample cuvette. Extract in buffer was added first to the sample to check for nonspecific reductase activity, and then 1.0 μmole GSSG was added to start the main reaction which was followed by monitoring NADPH oxidation at 340 nm.

Results and Discussion

Fluctuations in the content of reductants in general and glutathione in particular in loblolly pine and Douglas-fir developing ovules are shown in Fig. 38 and 39. In late stages of development, segregation of the zygotic embryo from the gametophyte showed GSH and reducing power in general to be more concentrated in the embryos than in the gametophytes. This agrees with previous findings for white pine and red pine (Johnson et al., 1987). Although the fluctuations shown in Fig. 38 and 39 also resembled analogous data for white pine and red pine in the case of Douglas-fir, this was less so for loblolly pine. Note in particular that the loblolly pine exhibited no peak of glutathione in July as did Douglas-fir but rather just a steady increase well into August. Whether this is really a species difference needs verification since, as noted earlier above for lipid analysis, climatic conditions in the South may have slowed loblolly pine ovule development in the summer of 1987. Also shown in Fig. 39 is the GSSG reductase activity in developing loblolly pine ovules over the same period. The strong parallel between this activity and the glutathione curve suggests that this enzyme could be expected to play a significant role in regulating the GSH content during development. On the other hand, analysis of dissected ovules of
loblolly pine collected on 8/10/87 showed that the enzyme was about five times more active in the gametophyte than in the zygotic embryo at that time. Additional factors such as glutathione biosynthesis and degradation need examination to get a more complete picture.

Figure 38. Total reductants in developing ovules of loblolly pine and Douglas-fir. The plotted zero development time is June 16, but the actual fertilization date was not determined.

Figure 39. Glutathione in developing ovules of loblolly pine and Douglas-fir. The plotted zero development time is June 16, but the actual fertilization date was not determined. Also plotted is the corresponding glutathione reductase activity in loblolly pine ovules. Glutathione units are nmoles/g fresh weight and reductase units are ΔA340nm/min/kg fresh weight.
Conclusions and Future Directions

The data on reductants and an associated enzyme presented here suggest that timing of metabolic events in loblolly pine ovule development may be different than in the other conifers that have been examined. However, the weather conditions prevailing for the developing loblolly pine cones analyzed here make verification essential before any firm conclusions can be drawn. The distribution data for the reductants is solid except for the need to extrapolate back to the early stages of development where, to date, it has not been possible to analyze segregated zygotic embryos.

More analyses of GSH and its metabolism are planned for the developing ovules of the target species and the Norway spruce model to provide a better baseline for what might be expected of developing somatic embryos. HPLC analysis for glutathione is under development to aid this effort.
FIDELITY AND PERFORMANCE OF SOMATIC NORWAY SPRUCE PLANTS

INTRODUCTION

Reported here is the recent growth history of a few Norway spruce somatic plants and control seedlings that were produced in 1986 and are still extant. Isozyme patterns were obtained from single needles, and these signatures were used in preliminary tests for fidelity of the somatic embryogenesis process. Since some isozyme patterns also change with development, the technique for single needles has been extended to single embryos and may find utility in somatic embryo maturation research.

MATERIALS AND METHODS

For the origins and early history of the somatic plants and controls discussed here, refer to Report Fourteen, pp. 68-82. The isoelectric focusing and staining of peroxidase were as described above on p. 85, but individual needles and embryos were extracted. To accomplish this miniaturization of the extraction procedure, small plastic centrifuge tubes equipped with a plastic pestle (Disposable Kontes Pellet Pestle Mixer, Cat. No. K-749520) were used. This allowed extraction of individual needles or embryos to be carried out with 100 μL or less of 10 μM HEPES, pH 7.6, followed by centrifugation without any transfer step.

Acid phosphatase was extracted and isoelectric focused in the same manner with staining by the procedure of Hamaker and Snyder (1973). The twenty Norway spruce seedlings analyzed to show the variation in isoperoxidase patterns were 85 days old at the time of analysis.
RESULTS AND DISCUSSION

Thirty-one Norway spruce somatic plants were produced from the initiation research conducted in the summer of 1986. Nine (not seven as stated on p. 82 of Report Fourteen) of these somatic plants set dormant buds in the fall, overwintered, and resumed growth in the spring of 1987. Although all appeared healthy, three of the nine somatic plants died rather suddenly of unknown causes in midsummer 1987. Seven Norway spruce seedlings that had been started from seed at the same time as the surviving six somatic plants also set dormant buds and overwintered; one of these seedlings also died in midsummer 1987. The twelve plants (six somatic and six seedling controls) were measured and analyzed in late summer to obtain the data presented below. Note that five of the six somatic plants originated from embryogenic calli initiated from a single explant.

The foregoing surviving somatic plants and seedlings are pictured in Fig. 40 as they appeared in late summer 1987. Table 20 presents the height growth and branching data for these plants and seedlings. Although it is recognized that this is too small a sampling to begin drawing conclusions, the fact that five of the six somatic plants originated from a single callus line (NS384-1)5 provided the opportunity to try a fidelity check. If no somaclonal variation had been introduced by the in vitro regeneration process, five of these six plants should be similar not only in appearance but also at the molecular level, since they along with the control seedlings had been kept under identical conditions. The challenge was to miniaturize one of our biochemical marker procedures so that substantial molecular information could be obtained from a very small portion of each plant.
Figure 40. Norway spruce somatic plants (left) and control seedlings (right) started in 1986 as they appeared in later summer 1987. All of the somatic plants except T1 originated from the same embryogenic callus source. Somatic plants, left to right are: J1, D1, L1, D2, L2, T1. Control seedlings, left to right are: Z2, Z3, Z1, Z4, Z5, Z7.

Table 20. Comparison of growth characteristics of Norway spruce somatic embryo plants (SE) and control seedlings grown from zygotic embryos (ZE). Data recorded 8/21/87.

<table>
<thead>
<tr>
<th>Plant Origin and Code</th>
<th>Embryogenic Callus Line No.</th>
<th>Plant Height, cm</th>
<th>Increase in Height, %</th>
<th>No. of Laterals with Active Growth</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>SE</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>J1</td>
<td>NS1-5</td>
<td>29</td>
<td>87</td>
<td>3</td>
</tr>
<tr>
<td>D1</td>
<td>NS1-5</td>
<td>23</td>
<td>229</td>
<td>4</td>
</tr>
<tr>
<td>L1</td>
<td>NS1-5</td>
<td>19</td>
<td>280</td>
<td>6</td>
</tr>
<tr>
<td>D2</td>
<td>NS1-5</td>
<td>16</td>
<td>220</td>
<td>3</td>
</tr>
<tr>
<td>L2</td>
<td>NS1-5</td>
<td>13</td>
<td>117</td>
<td>10</td>
</tr>
<tr>
<td>T1</td>
<td>NS1-9</td>
<td>8</td>
<td>23</td>
<td>0</td>
</tr>
<tr>
<td><strong>ZE</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Z2</td>
<td>--</td>
<td>37</td>
<td>95</td>
<td>13</td>
</tr>
<tr>
<td>Z3</td>
<td>--</td>
<td>36</td>
<td>125</td>
<td>7</td>
</tr>
<tr>
<td>Z1</td>
<td>--</td>
<td>27</td>
<td>74</td>
<td>7</td>
</tr>
<tr>
<td>Z4</td>
<td>--</td>
<td>26</td>
<td>126</td>
<td>7</td>
</tr>
<tr>
<td>Z5</td>
<td>--</td>
<td>22</td>
<td>175</td>
<td>8</td>
</tr>
<tr>
<td>Z7</td>
<td>--</td>
<td>17</td>
<td>70</td>
<td>6</td>
</tr>
</tbody>
</table>

*Increase in height relative to measurement on 5/22/87.*
After several trials, it became possible to obtain useful isozyme patterns from individual needles, thereby making it possible to acquire the desired information while inflicting very little damage on the plants. Both guaiacol peroxidase and acid phosphatase isozymes could be used in this manner. When this fidelity test was applied to single needles of the six somatic plants, only four of the five plants that originated from the same genotype yielded identical isozyme patterns (Fig. 41). One of the five plants (L2) had apparently undergone some change in the culture process. The sixth plant (T1) was of a different genotype and yielded different isozyme patterns as expected. Furthermore, as can be seen in Fig. 42, these patterns were reproducible so long as needles of the same general age were analyzed.

Figure 41. Acid phosphatase (left) and guaiacol peroxidase (right) isozyme patterns of later summer extracts of single spring needles of the six somatic plants pictured in Fig. 1 and coded in Table 1. The pH gradient runs form 3.5 (bottom) to 9.5 (top).

A word of caution is in order about the T1 isozyme pattern. Beyond the fact the T1 was of a separate genotype, it had already set a dormant bud at the time of analysis after only a short growth period. Therefore, physiological state may have been a factor in the isozyme patterns for T1, particularly
in the case of peroxidase which is known to be so affected (Greppin et al., 1986). On the other hand, plant L2 not only has different isozyme patterns than its clones for both enzymes, but reference to Fig. 40 reveals it to be somewhat different in appearance.

![Figure 42](image)

**Figure 42.** Reproducibility of and needle age influence on the isoperoxidase patterns of somatic plants L1 and L2. On the right are the patterns from five single needle extracts of each plant using mature needles as in Fig. 2. On the left are the analogous single needle extracts from young developing (flushing) needles and from old mature needles produced the previous year. pH gradient as in Fig. 2.

The utility of making these comparisons of isozyme patterns assumes that substantial variability exists in seedling populations. Indeed this is the case, for peroxidase at least, as is evident for 20 seedlings that were analyzed (Fig. 43). Each of these patterns appears to be unique within this group of 20 and different also from the somatic plants.

Single embryos can also be analyzed. In Norway spruce, for an enzyme like peroxidase, the isozyme pattern constantly changes as the somatic embryo matures (Fig. 44). Changes of this kind have also been observed by others (Greppin et al., 1986). This may in fact prove to be a very good way to monitor normal maturation if good baseline patterns can be obtained for zygotic embryos as they mature (not yet in hand).
CONCLUSIONS AND FUTURE DIRECTIONS

The most important outcome of this initial fidelity research was that it showed that valuable information for checking fidelity could be obtained from...
very small samples. Therefore, when many more somatic plants become available and/or when embryogenic callus is obtained from a cotyledon with both the source plant and the resulting somatic plants remaining available for analysis at a later date, these miniaturized analyses could become very useful. Should it turn out that genotypic differences appear mainly in mature needles or are limited to certain portions of the pH gradient with all genotypes undergoing a characteristic (not necessarily identical) change in isozymes with development of embryos, this kind of analysis may become a powerful aid in maturation research.
LIQUID SUSPENSIONS - AN ALTERNATE CULTURE SYSTEM

Introduction

The commercial success of conifer somatic embryogenesis will be dependent upon the efficient mass production of somatic embryos, high frequency maturation and the ultimate production of artificial seeds. Suspension cultures, which require the minimum of individual manipulations, have the potential to meet these requirements as well as provide tissue sources readily adaptable for protoplast, biochemical and genetic improvement studies. The ease of maintenance and rapid growth of liquid culture systems, combined with the recent successes in the area of conifer somatic embryogenesis (Hakman and von Arnold, 1985; Nagmani and Bonga, 1985; Gupta and Durzan, 1987; Lu and Thorpe, 1987) have stimulated renewed interest in continuous cloning via suspensions cultures. Conifer embryogenic suspensions have recently been reported from P. menziesii (Durzan and Gupta, 1987), Pinus lambertiana, (Gupta and Durzan, 1986) and Picea glauca (Hakman and Fowke, 1987), and Picea abies (Hakman et al., 1985). However, quantitative data have not been presented. With the advantages of the suspension system and the availability of the embryogenic Norway spruce callus cultures, we too have begun to reexamine the cell suspensions as an alternate method of obtaining somatic embryogenesis. Using quantitative growth measurements, we can report growth rate and embryo yield of rapidly growing embryogenic suspension cultures derived from cultured Norway spruce mature zygotic embryos.

Methods and Materials

Embryogenic callus was initiated from excised embryos of mature Norway spruce (Picea abies) seeds as described in Report Fourteen. Briefly, Norway
spruce seeds obtained from Quality Tree Seed, Inc., Brewster, N.Y. and stored at 4°C, were surface sterilized in 30% \( \text{H}_2\text{O}_2 \) for 45 minutes, rinsed 3 times and imbibed overnight in sterile \( \text{H}_2\text{O} \). Embryos were excised and cultured on a half-strength modified MS basal medium, 1/2 BLG, (Amerson et al., 1985), supplemented with 2 mg/L NAA and 1 mg/L BA. Cultures were maintained at 23°C with 16 hr irradiance (15-50 \( \mu \text{E} \text{ m}^{-2} \text{ s}^{-1} \)) from cool-white fluorescent and incandescent lights and were subcultured biweekly. Embryogenic callus has been maintained on solid media for up to 1 year prior to transfer to liquid media.

Suspension cultures were initiated by transferring 1-2 agar-grown callus masses to 125 mL Erlenmeyer flasks containing 25 mL of liquid medium of the same composition. Tissue readily dispersed when flasks were placed on a gyratory shaker (about 100 rpm), and suspended cells were maintained by subculture (1:10 dilution) at 10-14 day intervals in the same 16/8 photoperiod of diffuse light.

To quantify cell growth and embryo yield, test tubes containing liquid media were inoculated with stock suspension cultures at approximately 10-15 \( \mu \text{L/mL} \) and rotated at 10 rpm on a roller-drum under the same environmental conditions. Sufficient tubes were prepared to provide 3-4 reps at from 8-10 time periods. Fresh and oven dry weight determinations were obtained by filtering tissue from 3-4 tubes onto preweighed Whatman microfibre filters. Fresh weights were determined immediately and dry weights were obtained after 24 hours at 65°C.

The number of somatic embryos in each time period of a growth trial was determined by pipetting 1 mL samples from each of 3-5 tubes (reps) onto either developmental agar media plates which were counted immediately, or imbedded into a 1% merthiolate/agar plate for later determinations. (Maturation studies were evaluated using the same 1 mL sample method or the entire contents of the
Embryos were counted by placing the plate on a background grid over the lighted clear stage plate of a Nikon dissecting microscope viewed at 10X.

Increased embryo induction and maturation were attempted by varying protocol, media composition and culture conditions. Suspensions were screened to obtain more uniform cell clump size and synchronous development as well as to eliminate possible inhibiting effects from deteriorating embryos and/or plasmolyzed cells. Inoculation densities determined by packed cell volume (centrifugation at 100 x g for 5 min) were varied to determine effect on continued development. To promote maturation, embryos at various stages were transferred en masse or by individual selection to either liquid treatments in roller-drum tubes, agar plates or multiwells containing cheese cloth mesh saturated with liquid media.

Results and Discussion

Embryogenic tissues derived from subcultured mature embryo callus have been maintained as a suspension culture for over 12 months. The relatively fine suspension consists of a complex mixture of aggregates of spherical cells with dense cytoplasm, single elongated, vacuolated cells and somatic embryos in various stages of development or dedifferentiation. Figure 45 illustrates the typical somatic embryo stages observed in proliferative suspension medium. The smooth embryonal heads composed of small, dense meristematic cells are associated with either well-defined or irregular suspensor masses of elongated vacuolated cells similar to the early embryos observed in callus cultures.

Tissue growth, determined by the fresh weights of a 2 month old suspension culture, increased from 2 mg/mL at day 0 to approximately 90 mg/mL at day 14 (Fig. 46). In a second study combining growth rate and embryo yield (Fig. 47),
Figure 45. Proliferating Norway spruce somatic embryos in suspension maintenance medium. Note dense embryonal heads composed of small cytoplasmic cells and suspensors with elongated, vacuolated cells. Bar = 0.5 mm.

Figure 46. Fresh weight growth curve of newly established (2 mo. old) Norway spruce embryogenic suspension culture.

Figure 47. Growth curve (A) and somatic embryo yield (B) of Norway spruce suspension culture grown on 0.5 strength BLG medium with 2 NAA and 1 mg/L BA. Vertical bars in A equal standard deviation among triplicate samples. Means of triplicate counts in B followed by unlike letters are significantly different (p = 0.05).
results confirmed the same growth pattern and correlated this rate with embryo density. Tissue volume doubled within about 48 hr during the linear phase of growth, and embryo yield peaked at approximately 100/mL. Optimum subculture time was determined to be at 10-12 day intervals.

Embryo yield was not increased by any media/protocol variation attempted. Final inoculation density (ID) of 15 μL/mL produced the most consistent results. However, growth and embryo development have been observed at from 5-40 μL/mL. The increase of sucrose from 1 to 3%, as well as increase in vitamins, plus inositol and casein hydrolyzate at levels reported by Gupta and Durzan (1986), produced no increase in embryo yield. The decrease to half-strength concentrations of growth regulators resulted in nonsignificant differences in both growth rate and embryo yield (Table 21). The decline in embryo numbers following the peak yield results from recalling or dedifferentiation of the embryo "heads". This is a major problem in current efforts to obtain embryo maturation and plantlet conversion.

Maturation of the bipolar suspension embryos and the subsequent plantlet conversion studies are on-going. From exploratory research, the most advanced development to date has been obtained from a suspension culture initiated from a mature embryo, maintained 5 months on agar prior to an additional 5 months in liquid medium. Early stage embryos from a screened (> 500 μm) population were selected and transferred (10 embryos x 3 reps) to cheese cloth pads (Fig. 48A) saturated with liquid HM, basal medium described by Hakman et al., 1985, supplemented with 1 μM indole-3-buturic acid (IBA) and 5 μM abscisic acid (ABA). Although the continued development (Fig. 48B,C) is encouraging, the stunted appearance (Fig. 48C) is also indicative of the fundamental problem or "bottleneck" that occurs with development of early callus induced somatic embryos as well as immature zygotic embryos.
Table 21. Norway spruce somatic embryo density (number/mL) yielded by 2 levels of growth regulators (NAA/BA) in suspension culture over time.

<table>
<thead>
<tr>
<th>Time</th>
<th>1/2 BLG Basal Media</th>
<th>1/2 BLG Basal Media</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day</td>
<td>2 NAA/1 BA</td>
<td>1 NAA/0.5 BA</td>
</tr>
<tr>
<td>0</td>
<td>14.6 ± 11.9</td>
<td>14.6 ± 11.9</td>
</tr>
<tr>
<td>1</td>
<td>23.3 ± 4.5</td>
<td>19.0 ± 4.4</td>
</tr>
<tr>
<td>6</td>
<td>41.7 ± 17.0</td>
<td>68.3 ± 14.2</td>
</tr>
<tr>
<td>12</td>
<td>97.3 ± 13.0</td>
<td>79.7 ± 33.7</td>
</tr>
<tr>
<td>18</td>
<td>51.7 ± 36.3</td>
<td>61.7 ± 34.7</td>
</tr>
</tbody>
</table>

Sequential treatments are required to induce somatic embryo maturation from agar grown cultures of *Picea abies* (Becwar et al., 1987) and *Coffea arabica* L. (Sondal and Sharp., 1977), and also suspension cultures of *Pseudotsuga menziesii* (Durzan and Gupta, 1987). Preliminary results from ongoing studies suggest that transfer of somatic embryos to decreased NAA levels in combination with low BAP and kinetin concentrations augment the size and maturation to a more well-defined torpedo stage. Transfer to HM solid media has also induced root development. Further tests are underway to repeat these results and obtain a working developmental protocol.

Suspension cultures have also recently been established from embryogenic loblolly pine cultures initiated from immature seed sources. Very early stage somatic embryos were observed (Fig. 49) following two biweekly subcultures in our standard suspension medium. Further modifications, however, appear to be necessary to maintain the original embryogenic capacity.
Figure 48. Selected bipolar early somatic embryo transferred from liquid proliferating medium 1/2 BLG to maturation medium, HM with 1 μM IBA and 5 μM ABA: A, Embryo at time of transfer; B, Embryo development after 15 days; C, Further development following 3 weeks on maturation medium. Bar = 0.5 mm.

Figure 49. Early loblolly pine somatic embryo derived from suspension culture. Bar = 0.1 mm.
Conclusions

Norway spruce embryogenic suspensions can routinely be established and maintained as rapidly growing long-term cultures. The maintenance medium provides for continuous production of embryonic structures which correlate in density with the total culture growth. Suspension cultures have also been established from embryogenic loblolly pine callus sources, and exploratory studies are continuing. Although still a major obstacle, studies to promote the maturation and conversion of the somatic embryos are being pursued vigorously.

TRANSFORMATION AND FOREIGN GENE EXPRESSION IN MICROPROPAGATED SWEETGUM (LIQUIDAMBAR STYRACIFLUA L.)

Introduction

Assured supplies of hardwood fiber for the U.S. pulp and paper industry require a reliable silvicultural system for establishing and managing hardwood plantations from genetically improved material. Vegetative propagation and modern recombinant DNA techniques may become part of such a system. In the Southeastern United States, sweetgum (Liquidambar styraciflua L.) is a candidate for plantation forestry, as it exhibits fast growth on a wide variety of sites and produces a pulp that blends well with pulp of southern pines. A further demonstration of the utility of this species is its consideration for use in biomass production (Raney et al., 1987) as well as in the horticultural industry.

Sweetgum has been propagated in vitro from shoot cultures established from seedling (Sommer et al., 1985) and mature tree explants (Sutter and Barker, 1985). In addition to serving as a system for the clonal propagation, shoot cultures also provide a convenient source of sterile plant material to explore
gene transfer with *Agrobacterium tumefaciens*. Using micropropagated shoots, we describe the first successful introduction of foreign genes into a commercially important southern hardwood forest species.

**Materials and Methods**

**Establishment of Shoot Cultures**

Small cuttings (8-10 inches) containing dormant lateral buds were harvested in March. Cuttings were obtained from trees selected in 3-year-old progeny tests (G. Hansen, Union Camp Corporation, Franklin, VA). Buds were picked from the cuttings and soaked for 30 minutes in a solution containing a small amount of surfactant (Tween 20, 0.1%). Buds were sterilized with 10% commercial household bleach (Hileo, 0.525%) for 15 minutes. After three rinses with sterile water, the apical meristems (plus 3-4 primordial leaves) were excised and sterilized with 1% Hileo for five minutes. After three additional rinses with sterile water, shoot tips were placed on WPM medium (Lloyd and McCown, 1980) containing 0.05 mg/L NAA and 1.0 mg/L BA solidified with 0.8% agar (pH = 5.6). Following the procedure of Sutter and Barker (1986), shoot tips were transferred to fresh medium (by simply moving the bud to a different part of the Petri dish) every 3-4 days. Within 3-4 months, each shoot tip had proliferated a number of shoots, and by the end of six months stable, rapidly growing shoot cultures were established.

**Plant Transformation**

The *A. tumefaciens* employed is a binary vector system developed and described by An (1986). Oncogenic *A. tumefaciens* strain A281 carrying the binary vector designated pGA515-47, which contains neomycin phosphotransferase (npt) and chloramphenicol transferase (CAT) genes driven by nos promoters, was maintained as described (An, 1986). Twelve shoots, 2-3 cm in length, were excised from the shoot cultures and inoculated on the stem portion. An additional
twelve shoots were inoculated on the second or third leaves down from the shoot apex. Inoculation was performed with an overnight culture of *A. tumefaciens*, using a 22 gage needle to make a small wound into which ca. 10 μL bacterial culture was applied. After 8 weeks a gall was observed on the stem of one of the twelve shoots. Sixteen weeks after inoculation, the gall was dissected free of the shoot and placed onto shoot proliferation medium (WPM medium with 0.05 mg/L NAA and 1.0 mg/L BA) containing carbenicillin (500 μg/mL) and cefotaxime (250 mg/mL) to kill any remaining *Agrobacterium*. A pale white callus proliferated from this gall and has been maintained in culture by monthly transfer to fresh medium (antibiotics not present).

**CAT Analysis**

Chloramphenicol acetyl transferase (CAT) activity was determined by the method of Gorman *et al.* (1982) employing the modifications of An (1986). Additionally, the plant extract was incubated at 65°C for 10 min before use in the assay. Twenty microliters of plant extract was incubated with 100 μL of reaction buffer, to which 0.1 μCi [14C]-chloramphenicol (54 mCi/mmol, Amersham Corp.) was added. After 30 min incubation at 37°C, chloramphenicol and the resulting acetylated derivatives were separated on a silica gel TLC plate. The chloramphenicol and radioactive derivatives were then visualized by autoradiography.

**DNA Isolation and Southern Blot Analysis**

DNA was isolated from transformed and untransformed control callus using the method described by Dellaporta *et al.* (1983). Approximately 3 μg of DNA was digested with Eco RI according to manufacturer's instructions and electrophoresed in a 0.7% agarose gel. Undigested DNA from the transformed plant, as well as Eco RI digested and undigested plasmid DNA (pGA515-47) were also included on the gel. Following electrophoresis, the DNA was transferred
to Zeta-Probe nylon blotting membranes (Bio-Rad Laboratories), using an alkaline blotting technique (Read and Mann, 1985). The membranes were then hybridized with radioactive probes consisting of either the pGA515-47 plasmid or just the npt sequence (Church and Gilbert, 1984). Radioactive probes were prepared by nick translating the entire pGA515-47 plasmid or oligolabeling a Bam HI fragment of pD0421 (which contains only the npt II sequence). Labeling reactions were carried out according to the manufacturer's instructions. After hybridization, the membranes were washed at 65°C (Maniatis et al., 1982) and exposed to Kodak XAR-5 film.

**Results and Discussion**

Approximately 8 weeks after inoculation, a small growth was noted on one of twelve treated shoots. Later, this growth enlarged and exhibited characteristics of a crown gall phenotype. After freeing the cultures of *Agrobacterium*, the callus was transferred to and is now maintained on WPM (NAA and BA at 0.05 and 1.0 mg/L, respectively) containing kanamycin at 100 μg/mL. It should be noted that WPM containing NAA and BA at these levels is not typically conducive to normal callus growth of sweetgum. The callus thus exhibits two characteristics of transformed tissue: those being the ability to grow in the absence or at reduced levels of plant growth regulators and the ability to grow well in the presence of kanamycin, a trait imparted by the nptII gene present in the T-DNA. Untransformed control sweetgum callus proved unable to grow in the presence of kanamycin.

**CAT Activity**

Chloramphenicol acetyl transferase activity assays were carried out to demonstrate the presence and expression of the CAT gene in the transformed tissue.
As shown in Fig. 50, acetylated derivatives of chloramphenicol were formed with extracts of transformed tissue but not untransformed control tissue. Although present, the CAT activity in the transformed tissue appeared to be very low. Higher levels of CAT expression might have been obtained using the 35S cauliflower mosaic virus promotor, which has been reported to be 10-15 fold stronger than the nos promotor (Morelli et al., 1985). Insertion of the CAT gene into a relatively inactive area of the genome or the presence of an inhibitor of CAT activity in extracts of the transformed tissue might also have been responsible for the low CAT activity observed. While not obvious in Fig. 51, it appeared that extracts of nontransformed control tissue reduced the apparent activity of authentic CAT isolated from E. coli (data not shown), suggesting the presence of such an inhibitor.

Figure 50. Results of CAT activity assays of transformed (1) and untransformed (2) sweetgum calli. Control treatments included chloramphenicol incubated without a plant extract (3) and incubated with authentic CAT obtained from E. coli (4). Arrows mark positions of weak radioactive spots representing acetylated chloramphenicol derivatives present in incubated extracts of transformed callus.
Figure 51. Southern blot analysis of Eco RI digested DNA isolated from transformed (lane 1) and untransformed (lane 2) sweetgum calli. Lane 3 contained uncut, high molecular weight DNA isolated from transformed callus. Controls included uncut (lane 5) and Eco RI digested (lane 4) pGA515-47 plasmid. Membranes were probed with nick translated p515-47 (A) and an oligo-labeled nptII gene fragment (B).

Southern Blot Analysis

The most convincing evidence demonstrating transformation of the tissues is provided by Southern blot analysis. When probed with the entire pGA515-47 plasmid, the probe hybridized to several bands in the lane containing DNA isolated from the transformed tissue (Fig. 51a). The lengths of these fragments agree with those expected from an Eco RI digest of the T-DNA of pGA515-47. The probe actually hybridized to more fragments than expected, indicating that the transformed callus may not be of clonal origin. Several transformation events could have occurred, in which full length T-DNA as well as fragments of the T-DNA may have been inserted into different cells. The probe did not bind to any fragment of the DNA isolated from untransformed control callus, providing evidence that the probe did not hybridize to a sequence normally present in the sweetgum genome. To prove that the probe was hybridizing to T-DNA that was
incorporated into the sweetgum genomic DNA, and not to plasmid DNA that might have been present in \textit{Agrobacterium} contaminating in the callus tissue, uncut DNA isolated from transformed tissue was probed (Fig. 51a, lane 3). The probe hybridized to high molecular weight (plant) DNA and no bands characteristic of those obtained when the probe hybridizes to undigested pGA515-47 plasmid DNA were observed. Thus, hybridization signals in the DNA isolated from transformed tissue result from the presence of T-DNA in the sweetgum genomic DNA.

A more specific probe was also used to confirm transformation. Using only the nptII sequence as a probe in Southern blot analysis, the presence of the nptII gene was demonstrated in the DNA of the transformed tissue (Fig. 51b). Several bands are missing in this blot when compared with the results obtained using the entire Ti-plasmid as a probe, presumably due to hybridization of pGA515-47 to fragments containing the CAT gene or T-DNA border sequences. As shown on the previous blot, the probe hybridized with high molecular weight plant DNA in the lane containing the uncut DNA from transformed tissue, and did not give a hybridization pattern expected if \textit{Agrobacterium} was still present in the transformed tissues. This blot again confirms the presence of foreign genes, specifically nptII sequences, in the genome of the transformed sweetgum callus. The apparent ability of the transformed tissue to grow in the presence of kanamycin suggests that the nptII gene is not only present, but is being expressed.

Conclusions

While reports of transformation of agronomic crops are plentiful in the literature, work with forest trees has lagged. Conifer shoots and seedlings have been successfully infected with \textit{A. tumefaciens} and transformed calli have been obtained from these tissues (Dandekar et al., 1987; Sederoff et al., 1986). Work with angiosperm forest tree species has progressed significantly farther.
Transformation as well as regeneration of transformed *Populus* hybrids has been reported (Pythoud et al., 1987; Fillatti et al., 1987; Parsons et al., 1986).

The transfer of foreign genes into sweetgum, a hardwood species commercially important in the southern United States, and the subsequent expression of those genes in the transformed tissue are demonstrated in this report. The presence of the foreign DNA was confirmed by Southern blot analysis, and expression of the transferred genes was demonstrated by tumor formation on the inoculated explant, the ability of the transformed tissue to grow in the presence of kanamycin and measurable levels of CAT activity in extracts of the tissue. This work may lead to studies of gene expression and transfer of economically important traits in this commercially important hardwood tree species.

**PROTOPLAST ISOLATION AND CULTURE FROM EMBRYOGENIC SUSPENSION CULTURES OF NORWAY SPRUCE [PICEA ABIES (L.) KARST]**

**Introduction**

The importance of protoplasts as an excellent experimental system for cell cloning, genetic transformation and somaclonal variation has been realized (Binding, 1986). More importantly, mass propagation of commercially important forest tree species would be feasible if protoplasts could be induced to divide and regenerate plantlets (either via organogenesis or somatic embryogenesis). So far, the isolation, culture and regeneration of plants from cultured protoplasts have met with limited success (Dunstan and Thorpe, 1987). However, more recent reports of somatic embryogenesis from isolated protoplasts of loblolly pine (Gupta and Durzan, 1987) and white spruce (Bekkaoui et al., 1987; Atree et al., 1987) are encouraging.
Our main objective in pursuing our research on protoplast isolation and culture is to define a reliable experimental protocol for obtaining a high yield of viable protoplasts and to induce them to divide and regenerate plantlets on a regular basis. This system, when established successfully, is intended to provide an alternate system for mass propagation of genetically superior or modified species.

Materials and Methods

Establishment of embryogenic callus cultures from mature embryos of Norway spruce has been described previously (Report Fourteen). Subsequently, experimental procedures for obtaining suspension cultures from these embryogenic callus lines were established as outlined earlier in this annual report. These cell suspension cultures were used in our experiments for obtaining protoplasts and consisted of free cells, proembryos and clusters of isodiametric cells.

Isolation of Protoplasts

Fast growing cell suspension cultures from 10-14 day old subcultures were centrifuged at 100 g for 5-10 min to obtain a pellet of cells. Approximately 1 g/fresh wt. of cells or 10 mL (pcv) was incubated in a variety of enzyme mixtures. Four types of enzyme formulations with different osmotica, as described for different tree species (Table 22), were prepared. The pH's of the enzyme solutions were adjusted to 6.0 and they were filter-sterilized using Nalgene filterware. Ten mL of cell samples (pcv) were incubated in 5-20 mL of the enzyme solution contained in 5 cm diameter petri dishes. The Petri dishes were sealed with Parafilm and incubated for 4-18 h in a culture room on a platform shaker (50 rpm) at 23 ± 2°C under both light and dark conditions. The enzyme solutions
Table 22. Effect of enzyme combinations and osmotica on protoplast yield and viability.

<table>
<thead>
<tr>
<th>Procedure/Callus Type</th>
<th>Enzyme 1</th>
<th>Enzyme 2</th>
<th>Enzyme 3</th>
<th>Medium 1</th>
<th>Medium 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Russel and McCown's Populus Leaf Tissue</td>
<td>Cellulase (Cooper Bio-medica) 0.5% (w/v)</td>
<td>Macerozyme 0.2% (w/v)</td>
<td>Dextrase 0.1% (w/v)</td>
<td>BSA 0.1% (w/v)</td>
<td>Sucrose 0.5M</td>
</tr>
<tr>
<td>Vardi et al. Microcitrus Embryogenic Callus</td>
<td>Cellulase R-10 0.3% (w/v)</td>
<td>Macerozyme 0.2% (w/v)</td>
<td>Dextrase 0.1% (w/v)</td>
<td>BSA 0.1% (w/v)</td>
<td>Sucrose 0.5M</td>
</tr>
<tr>
<td>Stricklen et al. Ulmus Hybrid Embryogenic Callus</td>
<td>Cellulysin 1.5% (w/v)</td>
<td>Macerozyme 0.5% (w/v)</td>
<td>Dextrase 0.1% (w/v)</td>
<td>BSA 0.1% (w/v)</td>
<td>Sucrose 0.5M</td>
</tr>
<tr>
<td>Merkle and Sommer Norway Spruce Cell Suspensions from Embryogenic Callus</td>
<td>Cellulysin 2% (w/v)</td>
<td>Macerozyme 1% (w/v)</td>
<td>Dextrase 0.1% (w/v)</td>
<td>BSA 0.1% (w/v)</td>
<td>Sucrose 0.5M</td>
</tr>
<tr>
<td>Merkle and Sommer Yellow Poplar Cell Suspensions from Embryogenic Callus</td>
<td>Cellulysin 2% (w/v)</td>
<td>Macerozyme 1% (w/v)</td>
<td>Dextrase 0.1% (w/v)</td>
<td>BSA 0.1% (w/v)</td>
<td>Sucrose 0.5M</td>
</tr>
<tr>
<td>Merkle and Sommer Ulmus Hybrid Embryogenic Callus</td>
<td>Cellulysin 2% (w/v)</td>
<td>Macerozyme 1% (w/v)</td>
<td>Dextrase 0.1% (w/v)</td>
<td>BSA 0.1% (w/v)</td>
<td>Sucrose 0.5M</td>
</tr>
</tbody>
</table>

Modified media includes:
- MS + Tuker
- B5
- B5/MN
- B5/PM
- B5/PM + Tuker
with cell samples were examined periodically for release of protoplasts. The
digested cell population with enzyme solution was filtered through sterilized
Miracloth (150 μm) to remove debris and undigested cell material and was centri-
fuged at 100 g for 10 min. The resulting pellet was again resuspended in 5 mL
of MS liquid medium containing 0.3M sucrose and 0.3M mannitol (referred to as
washing medium) and centrifuged at 100 g for 10 min. The pellet of protoplasts
was washed two times with the same medium and resuspended in culture medium.
Protoplast yields were quantified with a haemocytometer and protoplast viability
was confirmed with fluorescein diacetate (FDA) staining (Widholm, 1972).

Culture of Protoplasts

Purified protoplasts were cultured at a density of 1.5 or 2.5 x 10^5/mL
in 5 cm diameter plastic Petri dishes or Corning 24-well tissue culture plates.
The culture media tested were Hakman's medium for Norway spruce (Hakman et al.,
1985) and MS (Murashige and Skoog, 1962) medium supplemented with glutamine at
50 mg/L, 2,4-D (2.0 mg/L) and BA (1.0 mg/L). The protoplast suspensions were
diluted with culture medium to a density of approximately 1.6 x 10^5/mL and 0.5
mL of the protoplast suspension was dropped into each well of a Corning 24-well
tissue culture plate. Immediately after plating, an 11 mm polyester screen disc
(no HC7-150, Tetko Inc., Elmsford, NY) was floated on top of the solution in
each well, in accordance with the methods described for Populus (Russel and
McCown, 1986). Protoplast cultures were observed periodically, and at regular
intervals 2 or 3 drops of fresh culture medium were added from a Pasteur pipet to
each well.

Results and Discussion

Preliminary experiments with different enzyme combinations and osmotica
(Table 22) to isolate the protoplasts indicated that the enzyme formulations
proposed by Vardi et al., (1986) for Microcitrus (1986) were the best for obtaining high yields of protoplasts in Norway spruce also. An average yield of $2.5 \times 10^5$/g fresh weight of the suspension cultures or per 10 mL (pcv) was obtained at the end of a 14-18 h incubation period in the enzyme mixture. The viability of the protoplasts tested by the FDA varied between 50-60% and protoplasts varied in size between 40-100 μm (Fig. 52).

Approximately 5% of the cultured protoplasts showed the first division (Fig. 53) on HM 2 2,4-D and 1 BA medium supplemented with 50 mg/L glutamine after a week of culture. Subsequent divisions of the protoplasts were not frequent although about 3-5% of the protoplasts formed cell clusters, as indicated in Fig. 54, after 3 weeks of culture. However, further divisions in these cell colonies to form microcalli could not be observed.

Conclusions

The results of our preliminary experiments on protoplast isolation and culture indicated that we have now standardized the procedures for obtaining a high yield of viable protoplasts on a regular basis in our model species (Norway spruce). Approximately $2.5 \times 10^5$/10 mL (pcv) was obtained and 50-60% of these protoplasts were viable. About 5% divided at least once over a two-week culture period and a small fraction divided further to form cell clusters. Experiments are still underway to maximize the number of dividing protoplasts and to obtain callus formation.
Figure 52-54. Protoplasts from embryogenic suspension cultures of Norway spruce.  
52. Isolated protoplasts; note the difference in sizes. Scale bar = 100 µm.  
53. Protoplasts cultured on nylon screens; white arrow points to the dividing protoplasts. Scale bar = 100 µm.  
54. Same protoplasts cultured on nylon screens; white arrows point to the cell clusters. Scale bar = 100 µm.
Now that embryogenic callus has been initiated in several conifers at the Institute, emphasis must turn to increasing the frequency and reliability of each and every step in the overall process. Improved protocols for initiation in our target species, loblolly pine and Douglas-fir, will therefore continue to receive much attention. For the former species, increased frequency of initiation and extension to a wider array of genotypes are the main goals. For the latter, reproducible initiation of embryogenic callus is still the prime target. Expertise in biochemistry will be used to aid traditional tissue culture approaches.

The major obstacle to commercialization of somatic embryogenesis, regardless of species, is embryo development/maturation. The complexity and difficulty of these steps demands that we allocate major resources, existing and new, to their resolution. Traditional approaches, including tests of growth regulators, nutrients, and other environmental factors, will continue. Dedicated efforts will be made to capitalize on positive findings from recent experiments involving addition of abscisic acid, and sequential changes in media osmolarity. Such work will be supported by investigation of the histological, biochemical, and molecular events underlying development in both Norway spruce and the target species. Once baseline data on events in zygotic and somatic embryogenesis are accumulated, results can be used to identify and manipulate factors limiting development of somatic embryos and their conversion to seedlings. Analyses of protein composition, isozyme patterns, lipids, and related compounds are all yielding data that can be exploited to promote development. Any opportunities to obtain "somatic seedlings," enroute to other goals, will be exploited.
All parties to this research agree that Norway spruce remains the best model species. That we have obtained Norway spruce "seedlings" demonstrates that this species can be used to study all process steps. Given this opportunity, the team will work to secure appropriate material and close gaps in our knowledge of the model system. Beyond the model, attention will be focused on the target species. With few exceptions, effort expended on other species will be minimal.

Prerequisite to commercialization of somatic embryogenesis is demonstration that the process produces normal trees; i.e., faithful genetic copies free of physiological abnormalities. Genetic fidelity was given brief consideration in 1987, but further evaluation, beyond refinement of techniques, awaits availability of larger numbers of seedlings.

Parallel to these efforts will be continued work on alternative culture systems. Recent progress with suspension cultures will be exploited to develop systems capable of producing and maturing somatic embryos in large numbers, rapidly and with minimal labor.

Also of importance is extension of embryogenesis to explants from more mature materials. Aside from specialized situations, such as high value hybrids, the process will be of only marginal value until genotypes, mature enough to have been proven genetically superior, can be efficiently and faithfully reproduced en masse. Thus, some effort will remain devoted to obtaining embryogenic callus from more mature explants. Promising results with Norway spruce cotyledons will be followed by additional experimentation, and investigation of biochemical similarities/differences deserves some attention.
Past exploratory research on topics such as protoplasts, technique development and refinement, and genetic transformation will continue. In these and our main research areas, staff efforts will be leveraged by the substantial number of students now in the Forest Biology Division.

Efforts to raise visibility of our work will continue, with emphasis placed on publication, participation in scientific meetings and conferences, distinguished visitors, and development of opportunities for collaboration. Continued effort on these fronts should also make us more competitive in the grant and contract arena.

Several recent events will affect Project 3223 in coming years. In December, the Institute announced its intent to relocate to Atlanta in 1991. Also, challenges and opportunities, old and new, were debated at a long range project planning session in January. Efforts to integrate the outcome of these events into firm plans for 1988 and ensuing years are underway. First efforts are being concentrated on replacing the two industrial research fellows, whose terms expire in the coming year. In addition, we are seeking to add a staff member with strong skills in the area of embryo development. Expansion into hardwoods is expected following a fall conference on industry needs, species desirabilities, and likely approaches. The new year holds many opportunities, and we look forward to making the best of them.
RELATED STUDENT RESEARCH

COMPLETED IN 1987

Daniel Bunker - M.S., Independent Study, entitled "Change in the structure of loblolly pine latewood during delignification." Advisor was T. E. Conners.

Tyrone Cornbower - M.S., Independent Study, entitled "Response of white spruce to mechanical pulping following hemicellulose hydrolysis." Advisors were T. J. McDonough and M. A. Johnson.

Luke Nealey - Ph.D. Program, Organic chemistry orientation, entitled "Isolation and characterization of xyloglucan from suspension cultured loblolly pine cell medium." Advisors were N. S. Thompson and M. A. Johnson.

IN PROGRESS


Lisa G. Dudek - M.S., Independent Study, entitled "Preliminary experiments on encapsulation of zygotic and somatic embryos of Norway spruce." Advisor is N. Rangaswamy.

Russell Feirer - Ph.D. Program, Biochemical orientation, involving biochemical and molecular studies of plant development. In cooperation with the University of Wisconsin, Madison. Advisor is P. Simon.


Gebran Hammam - M.S., Independent Study, entitled "Comparison of somatic and zygotic cell embryos: Picea abies (Norway spruce) and Pinus taeda (loblolly pine)." Advisor is T. E. Conners.


Lorrain Logsdon - M.S., Independent Study, entitled "Patterns of and changes in gene expression associated with maturing and germinating seed." Advisor is R. J. Dinus.

Mary Kay Lynde-Maas - M.S. Independent Study, entitled, "Fructose utilization by embryogenic and nonembryogenic suspension cultures of Norway spruce." Advisor is M. A. Johnson.

Jong-Moon Park - Special Student, Exploratory Research, entitled, "Improving the properties of recycled fibers by chemical and enzymatic treatments." Advisor is M. A. Johnson.

Colleen Walker - M.S. Independent Study, entitled "Optimization and quantification of somatic embryogenic cultures of several conifer species in bioreactors. Advisor is M. Becwar (R. J. Dinus).
COOPERATIVE INVESTIGATIONS

1. North Carolina State University - Cooperative evaluation with Dr. Ralph Mott and Dr. Henry Amerson of procedures for initiating embryogenic cultures of loblolly pine, Norway spruce, and white spruce.

2. Williams College/Merrell-Dow Pharmaceutical Co. - Cooperative study with Dr. Robert Slocum (Williams) plus Drs. A. Bitonti and P. McCann (Merrell-Dow) of polyamine metabolism, and joint preparation of resultant manuscript.

3. International Forest Seed Company - Supply of "rejuvenated" loblolly pine material by Dr. S. Foster for experiments on initiation of embryogenic callus from mature explants.

4. University of Cincinnati - Joint assay with Dr. J. Caruso of endogenous hormone levels, principally ABA and IAA, in embryogenic and nonembryogenic calli. Arrangements now being finalized.
ACKNOWLEDGMENTS

The authors are indebted to members of the Project Advisory Committee for their help with program direction and guidance. Also acknowledged are the innumerable and timely contributions of Debbie Hanson of the Tissue Culture Laboratory; John Carlson and Jud Conkey of the Biochemistry Laboratory; Terry Conners, Mary Block and Ellen Foxgrover of the Electron Microscopy Laboratory; and Robert Arvey, Egon Humenberger, and Gary Wyckoff from Forest Genetics. Special thanks are also given to the many people who helped type, edit, and print our findings over the year, particularly our division secretary, Sandy Berghuis.

The research team also expresses its appreciation to Marvin Zoerb and George Lowerts of Union Camp Corp., Jerry Pullman of Weyerhaeuser Co., and Lee Handley and Dave Canavera of Westvaco Corp. for their help with cone collections.

The authors wish to thank Dr. David Ow (Plant Gene Expression Center, Albany, CA) for generously supplying the plasmid pD0421, which contains an npt gene. Helpful discussions with Dr. R. Amasino (University of Wisconsin) are also appreciated.
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STATUS OF RECENT PUBLICATIONS

PUBLISHED OR IN PRESS:


SUBMITTED:


OTHERS FOR INFORMATION:


Adventitious - Roots, shoots, embryos, or other organs or tissues developing in an abnormal position.

Agar - Polysaccharide complex extracted from algae. Used as gelling agent in tissue culture medium.

Agarose - A gelling agent derived from agar: the neutral (charge) fraction of agar.

Agrobacterium tumefaciens - Bacterial plant pathogen responsible for crown gall in plants. Harbors a tumor inducing (Ti) plasmid which can be used to transport a foreign gene into a plant cell.

Antibiotic resistance gene - A gene that codes for a protein, which imparts resistance to an antibiotic that allows cells to live in the presence of the drug that would normally kill them.

Archegonium - The flask-shaped container of the ovum (egg cell) of some gymnosperms. The swollen base (venter) contains the egg cell and is surrounded by the neck, with neck canal cells.

Aseptic culture - Surface sterilization of parental explants, free from pathogens, but not necessarily free of internal symbionts.

Asexual reproduction - Reproduction without fertilization. New individuals may develop from vegetative parts such as tubers, bulbs, or rooted stems, or from sexual parts such as unfertilized eggs or other cells in the ovule.

Auxins - A class of plant growth hormones of diverse makeup which cause cell enlargement, apical dominance, and root initiation.

Bacillus thuringiensis - Bacterium which produces a protein having a strong insecticidal activity. Depending upon the strain of the bacteria, the toxin may exhibit specificity toward Lepidopteran, Dipteran or Coleopteran insect groups.

Bacteriophage - A virus that attacks bacteria; also called a phage.

Base (nucleic acid) - A flat, ring compound that forms part of one of the nucleotide links of a nucleic acid chain. The bases are adenine, thymine, guanine, cytosine and uracil (commonly abbreviated A, T, G, C, U).

Base pair - Two bases, one in each strand of a double stranded DNA molecule, which are attracted to each other by weak chemical interactions. Only certain combinations of bases will pair: A-T, G-C and A-U.

Callus culture - Proliferation from a parental explant of many cells in protoplasmic continuity, but having no equivalence with any normal tissue. Same as tissue culture.
Cell differentiation - Internal chemical or ultrastructural changes preceding or accompanying specialization of function.

Cell suspension - Culture of single cells in moving liquid medium, often used to describe suspension cultures of cells and cell aggregates.

Chloroplast - A membrane-enclosed subcellular organelle containing chlorophyll. Chloroplasts are the sites of photosynthesis. They contain DNA and ribosomes and can replicate.

Clonal propagation - Propagation of a group of plants derived from a single individual (ortet) by asexual reproduction. All members (ramets) of a clone have the same genotype and consequently tend to be uniform.

Clone - 1. (verb) to undergo the process of creating a group of identical DNA molecules or genes derived from a single source. 2. (noun) a group of genetically identical cells (plants), all derived from a single ancestor.

Cloning vector - Small plasmid, phage or virus DNA molecules used to transfer a DNA fragment or gene from a test tube to a living cell. Some vectors are capable of multiplying inside living cells (bacteria) to result in the multiplication or cloning of the transferred DNA or gene.

Codon - A group of three nucleotides coding for an amino acid.

Conversion - Development of cotyledonary embryo to rooted plantlet.

Coumarins - A class of phenylpropanoid phenolic compounds of which coumarin itself typifies the structures.

Cotyledon - The leaf formed directly from the embryo of an angiosperm or gymnosperm. There may be one (in monocotyledons), two (in dicotyledons), or several (in gymnosperms). They act as storage organs in nonendospermous seeds and as the first photosynthetic organs in endospermous seeds.

Cytokinins - A class of plant growth hormones associated with cell division, assisting with the transmission of the genetic information from the genes to the proteins.

cDNA (complementary DNA) - DNA synthesized from an RNA template in test tubes using the enzyme reverse transcriptase. The DNA sequence is thus complementary to that of the RNA. cDNA is usually made with radioactive nucleotides and is used as a hybridization probe to detect specific RNA or DNA molecules (genes).

Denature - In reference to DNA, denaturation means conversion of double stranded to single stranded DNA.

2D TLC - Two-dimensional thin-layer chromatography.

Diploid - Having two sets of chromosomes in the nucleus. One-half of the chromosomes are contributed by one parent, one-half by the other parent. Many higher organisms are diploid except for their sex cells and associated tissue.
Electroinjection - Method of transporting naked DNA into a plant cell having a cell wall using a short duration DC electrical pulse (see electroporation).

Electroporation - Method of transporting naked DNA (gene) into a protoplast using a short duration DC electrical pulse.

E. coli (Escherichia coli) - A bacterium commonly found in the digestive tracts of many mammals, including humans.

EM - Electron microscope.

Embryo - The young plant developing in the megagametophyte from the fertilization of an egg cell, or without fertilization. In aseptic cultures, adventitious embryos show polarization followed by the growth of a shoot from one end and a root from the other end.

Embryogenesis - Initiation of embryoids or embryos from cultured cells.

Embryoid - A cell group approximating an embryo, but having a more random cell arrangement.

Enzyme - A protein molecule that catalyzes a specific chemical reaction.

ER - Endoplasmic reticulum. A system of membranes (originating from the external membrane of the nuclear envelope) that permeates the cytoplasm and that may or may not be covered with ribosomes.

Erosion zone - Zone in the gametophytic tissue below the archegonium that is degraded by the developing embryo.

Eucaryotic cells - Cells with true nuclei bounded by nuclear membranes and which undergo meiosis.

Excise - To cut or isolate callus tissue from its parental explant or to remove adventitious shoots from callus tissue for rooting.

Explant - A plant part excised and prepared for aseptic culture by surface sterilization followed by the exposure of live cells to a nutrient medium.

Fertilization - The normal union of two gametes during sexual reproduction.

Flavonoids - A class of phenolic compounds usually consisting of two hydroxylated aromatic rings joined by a three-carbon chain.

Gametophytic tissue - Haploid tissue of the seed that surrounds the developing embryo during the latter stages of embryogenesis.

Gel electrophoresis - A method for separating molecules based on their size and/or electrical charge. Molecules are forced to run through a gel (e.g., agarose or polyacrylamide) by placing them in an electric field. The speed at which they move depends on their size and/or charge.
Gene - One of the units of inherited material carried on a chromosome; arranged in a linear fashion and indivisible.

Gene cloning - A way to use microorganisms to produce millions of identical copies of a specific region of DNA or gene.

Gene pool - Reservoir of genetic variability available for use in genetic improvement of tree species.

Genetic engineering - The formation of new combinations of heritable material by the insertion of nucleic acid molecules into a vector system so as to allow their stable incorporation into a host organism in which they do not naturally occur.

Genetic gains - Average improvement in progeny over the mean of the parents.

Genetic variability - The variation existing in a given population (species, for example) with respect to particular genes or arrangement of genes.

Genome - May refer to the full genetic complement in the haploid set of chromosomes of a species, but one may speak of nuclear, chloroplastid and mitochondrial genomes.

Genotype - The genetic makeup of an individual; carried in the chromosomes.

Grana - Association of thylakoids in a stack.

Groundplasm - Homogeneous plasma (matrix) remaining after cell organelles and particles have been excluded.

Haploid - Having the reduced chromosome number, i.e., having one set of chromosomes in the nucleus. This is normal in sex cells, which have only half the number of sets occurring in diploid vegetative cells.

Homologous - Describing regions of DNA molecules that have the same nucleotide sequence. Complementary base pairing can occur between homologous regions in two different DNA molecules.

Hormone - Any growth substance which is generally transported to the site of action and can stimulate growth or cell enlargement (auxins), cell division (cytokinins), stem elongation (gibberellins), or can retard growth as in the abscission of leaves (ethylene).

Hybrid vigor - The increase in vigor, size and fertility of a hybrid as compared with its parents, resulting from the union of genetically different gametes and assumed to be due to special recombinations of dominant and recessive genes (heterosis).

Hybridization - The production of offspring of genetically different parents.

Hypocotyl - The part of a seedling axis between the radicle and the cotyledon(s).
Induction - To cause initiation of a plant structure, organ or process.

Inoculation density - "ID" is the volume of cells per unit of medium, i.e., µL/mL.

Inoculum - A small piece of tissue cut from callus, or a small amount of cell material from a suspension culture placed in contact with fresh medium for continued growth of the culture. Inocula (plural).

Interspecific hybrid - The progeny from matings between species.

Intraspecific hybrid - The progeny from matings within species.

Intron - A noncoding section of a gene that is spliced out of mRNA before translation into proteins.

In vitro - Outside the living organism.

In vivo - Within the living organism.

Isozymes - Multiple forms of a single enzyme.

Kanamycin - Antibiotic that disrupts protein synthesis in some bacteria and plants.

Lambda - The name of a particular bacteriophage (virus) used extensively in gene cloning.

Launch - (Induction), to cause the initiation of a process that will result in the development of a plant structure (shoots, roots, or embryos); sometimes used to describe the log phase of the growth cycle.

Lipids - Any of a group of biochemicals which are variably soluble in organic solvents and barely soluble in water.

Maturation - Development of proembryo to cotyledonary embryo.

Milieu - The whole chemical and physical environment of a culture.

Meristem - A localized group of cells, actively dividing and undifferentiated but ultimately giving rise to permanent tissue such as shoots, roots, wood or bark.

Meristemoid - A localized group of cells in callus tissue, characterized by an accumulation of starch, RNA and protein, and giving rise to adventitious shoots or roots.

Mitochondria - Small bodies in spaces of the cytoplasm. They are spheres or rods, and are the sites of many important aerobic enzymatic processes. The inner layer of the wall is infolded into fingerlike processes.

Morphogenesis - Initiation of organized tissue in callus or suspension cultures.

mRNA (messenger RNA) - RNA that is used by the ribosome to synthesize proteins.
Nick translation - A procedure for radiolabelling DNA *in vitro*. Used to make a radioactive probe.

Nuclease - A general term for an enzyme that cuts DNA or RNA.

Nucleic acid - DNA or RNA.

Nucleotide - One of the building blocks of nucleic acids. A nucleotide consists of three parts: a base, a sugar and a phosphate.

Nutrient medium - A solid or liquid combination of major and minor salts, an energy source (sucrose), vitamins, hormones, and occasionally other defined or undefined supplements. Usually made up from previously prepared stock solution, then sterilized by autoclaving or filtering through a micropore filter. Media (plural).

Organized tissue - Tissue composed of regularly differentiated cells.

Organelle - A complex cytoplasmic structure of characteristic morphology and function, such as a mitochondrion or plastid.

Organogenesis - Initiation of roots or shoots from callus meristemoids.

Packed cell volume - "pcv" is the volume of cells determined by centrifugation.

Parasexual hybridization - Hybridization resulting from asexual fusion of cells, either diploid or haploid.

Passage - The duration of growth of callus or cell material from one subculture to another.

Photoperiod - Length of daily light cycle.

Plasmalemma - The semipermeable unit membrane surrounding and containing the cell cytoplasm. In plant cells, it is pressed up against the inner surface of the cell wall.

Plasmid - A small circular DNA molecule found inside bacterial cells. Plasmids reproduce every time the bacterial cell reproduces. Once infected, the bacteria will always contain a plasmid. Some plasmids continue to replicate in a bacterial cell so that a single cell may contain 200 plasmids. Plasmids are thus used to clone a gene.

Polyploidy - Having three or more times the haploid number of chromosomes.

Procaryotic cells - Single-celled organisms and reproducing entities that lack a membrane-bound nucleus; they do not undergo meiosis; these include the viruses, bacteria, and blue-green algae.

Probe - A radioactive DNA or RNA molecule used to detect the presence of its complementary strand on an electrophoretic "gel" by hybridization and autoradiography.
Proembryo - Used here to mean the embryo in very early precotyledonary stages of development.

Prolamellar body - Semicrystalline structure from which thylakoid membranes arise during chloroplast development in dark grown seedlings.

Promotor - A short nucleotide sequence on DNA recognized by RNA polymerase to initiate transcription (synthesis of mRNA).

Proplastids - A group of plastids which are progenitors of chloroplasts.

Protoplast - Spherical cell protoplasm (cytoplasm + nucleus) bounded by a membrane but no cell wall.

Protoplast fusion - Union of two protoplasts into one cell.

Recombinant DNA (rDNA) - Chimeric DNA molecule formed by cutting and splicing of DNA (genes).

Restriction endonucleases - (Restriction enzymes) enzymes that cut DNA at specific nucleotide sequences yielding fragments of various sizes. These enzymes are isolated from a variety of bacteria, and are identified by a three letter abbreviation consisting of the first letter of the genus and the first two letters of the bacterial species name, followed by the strain number (e.g., a particular enzyme isolated from an E. coli strain is designated Eco RI).

RFLPs (restriction fragment length polymorphisms) - DNA molecules from the same gene in two different individuals may differ slightly, and fragments of different length are formed when the gene is digested with a restriction enzyme. Since unequal-sized fragments travel at different speeds in an electrophoresis gel, the two fragments visualized by a radioactively-labeled homologous probe would appear as different bands on the gel. This is a RFLP.

Reverse transcriptase - An enzyme purified from tumor viruses that synthesizes DNA complementary to an RNA template.

Ribosomes - Organelles containing protein and RNA. They are seen as dense particles in electron micrographs. They are found in all types of cells in which protein is being synthesized.

RNA - Ribonucleic acid. RNA is usually single stranded.

RNA polymerase - The enzyme responsible for making RNA complementary to a DNA template. RNA polymerase binds at specific nucleotide sequences (promoters) in front of genes in DNA. It then moves through a gene and makes an RNA molecule that contains the information contained in the gene.

SEM - Scanning electron microscope.

Sequence - The order of the nucleotides in the DNA or RNA chain.
Somatic - Diploid body cells of an organism; those cells other than germ cells.

Somatic cell hybrid - The plant resulting from fusion of protoplasts from somatic cells of genetically different sources.

Splicing - Removal of introns from the "immature" form of eukaryotic mRNA. Carried out in the nucleus of the cell.

Subculture - Dividing agar grown callus or liquid cell suspensions for transfer to fresh medium.

Suspension culture - Cells or cell aggregates dispersed and growing in moving liquid medium.

Suspensor - Elongated, vacuolated cells subtending the embryonal cells in a developing zygotic embryo.

Tannins - A class of complex phenolic compounds known for their astringency and ability to tan the proteins of animal skins. There are two major types of tannins, the hydrolyzable and the condensed tannins.

TEM - Transmission electron microscope.

Template - A pattern of nucleotide sequences in DNA or RNA used by polymerases to specify the sequence in a new polymer by complementarity.

Tetracycline - An antibiotic that kills bacteria by blocking protein synthesis.

Thylakoids - Complex system of flattened membranes within a chloroplast; are often found in stacks to form grana.

Ti plasmid - The plasmid carried by the bacterium Agrobacter tumefaciens which is used to carry foreign genes into a plant cell.

Tissue culture - General term for callus and cell cultures.

Totipotency - A cell characteristic in which the cell retains the potential of forming all the cell types of the adult organism.

Transcription - The process of converting information in DNA into information in RNA. The copying of a gene into RNA. RNA polymerase is the enzyme that executes this conversion of information.

Transformation - The process whereby a cell takes up free DNA such that the free DNA (gene) becomes a permanent part of the cell's genome.

Translation - The process of converting the information in mRNA into protein. Also called protein synthesis.

Transposon - A short section of DNA capable of "jumping" to another region of a chromosome or to a different chromosome.
Transposon tagging - Method of using a transposon to locate a gene. When a transposon inserts into a chromosome, it causes a knockout mutation leading to a distinct mutant phenotype. A radioactive probe made from this transposon can then be used to identify the DNA sequence (gene) into which it had been inserted. The gene can then be localized on a gel and perhaps on a particular chromosome from the mutant plant. In short, the mutated gene is tagged or made identifiable by the transposon.

Ultrastructural - Sublight microscopic, intracellular structure.

Vacuole - A fluid-filled space in a cell. A single vacuole, taking up most of the volume of the cell, present in many plant cells, and containing a cell sap which is isotonic with the protoplasm.

Vegetative cells - Nonreproductive cells such as haploid cells from female gametophytes of conifers or diploid somatic cells.

Vesicle - Small membrane-bound body in the cytoplasm.

Zygote - Fusion product of male and female sex cells or fusion product of protoplasts.
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: \( 20(\text{NS 384-1})2E \)

\( 20 = \text{subcultured 20 times} \)

\( \text{NS} = \text{Norway spruce} \)

\( 384 = \text{research plan (RP384)} \)

\( -1 = \text{time of initiation or treatment identification} \)

\( 2 = \text{line or genetic source, e.g., seedling No. 2} \)

\( E = \text{Immature embryo; explant type (only used if cell line derived from more than one explant within a research plan)}. \)

\( a \text{Each experiment initiated by any team member has an approved research plan with an identifying number. The tissue source origin (clone, seed lot, etc.) and initiation date is recorded under that number in the investigator's IPC research notebook and is available in the Tissue Culture Research Plan files.} \)
Cell lines less than one year old from immature cone collections:

Example: 5(LP6B)E - the RP No. is deleted and the letter within parentheses indicates cone source code.

Species Codes

- LP - loblolly pine
- DF - Douglas-fir
- PP - pitch pine
- PO - pond pine
- NS - Norway spruce
- WP - white pine
- WS - white spruce

Explant Codes

- C = cotyledon
- H = hypocotyl
- B = bud
- E = immature embryo
- M = mature embryo
- N = nucellus
- G = gametophyte
<table>
<thead>
<tr>
<th>Species</th>
<th>Tissue Culture Code</th>
<th>Source</th>
<th>Industrial Codes</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Douglas-fir</strong></td>
<td>DF J</td>
<td>Weyerhaeuser, Federal Way, WA</td>
<td>WTC-357</td>
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<tr>
<td></td>
<td>DF K</td>
<td></td>
<td>WTC-358</td>
</tr>
<tr>
<td></td>
<td>DF L</td>
<td></td>
<td>WTC-359</td>
</tr>
<tr>
<td></td>
<td>DF M</td>
<td></td>
<td>WTC-360</td>
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<tr>
<td></td>
<td>DF N</td>
<td></td>
<td>WTC-361</td>
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<td><strong>Loblolly pine</strong></td>
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<td>10-1003 D-22 HQI</td>
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<tr>
<td></td>
<td>LP B</td>
<td></td>
<td>10-1007 F-21 HQI</td>
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<tr>
<td></td>
<td>LP C</td>
<td></td>
<td>10-1011 C-20 HQI</td>
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<td></td>
<td>LP D</td>
<td></td>
<td>10-1018 B-16 HQI</td>
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<td>LP E</td>
<td></td>
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<td>LP J</td>
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<td>LP L</td>
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<td>LP M</td>
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<td>LP P</td>
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<td>11-9a</td>
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<td></td>
<td>LP R</td>
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<td><strong>Norway spruce</strong></td>
<td>NS</td>
<td>Greenville, WI</td>
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<tr>
<td></td>
<td>NSA</td>
<td>U. Arkansas, Fayetteville, AR</td>
<td></td>
</tr>
<tr>
<td></td>
<td>SHNS</td>
<td>Shiocton, WI</td>
<td></td>
</tr>
<tr>
<td><strong>White spruce</strong></td>
<td>WS A</td>
<td>Greenville, WI</td>
<td>--</td>
</tr>
<tr>
<td></td>
<td>WS B</td>
<td></td>
<td>--</td>
</tr>
<tr>
<td></td>
<td>WS 65</td>
<td>Oconto River, Seed Orchard, WI</td>
<td></td>
</tr>
<tr>
<td><strong>Pitch/loblolly</strong></td>
<td>PL</td>
<td>Westvaco, Summerville, SC</td>
<td>65 x LP</td>
</tr>
<tr>
<td>Hybrid</td>
<td></td>
<td></td>
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</tbody>
</table>

*aCones obtained from progeny of the given clone.
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 ($P < 0.05$). The number of replications is indicated by $N$. 
### AMINO ACIDS ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Name</th>
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<tbody>
<tr>
<td>ala</td>
<td>alanine</td>
</tr>
<tr>
<td>arg</td>
<td>arginine</td>
</tr>
<tr>
<td>asn</td>
<td>asparagine</td>
</tr>
<tr>
<td>asp</td>
<td>aspartic acid</td>
</tr>
<tr>
<td>cit</td>
<td>citrulline</td>
</tr>
<tr>
<td>cys</td>
<td>cysteine</td>
</tr>
<tr>
<td>γ-aba</td>
<td>aminobutyric acid</td>
</tr>
<tr>
<td>gln</td>
<td>glutamine</td>
</tr>
<tr>
<td>glu</td>
<td>glutamic acid</td>
</tr>
<tr>
<td>gly</td>
<td>glycine</td>
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<tr>
<td>his</td>
<td>histidine</td>
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<tr>
<td>hyp</td>
<td>hydroxyproline</td>
</tr>
<tr>
<td>ile</td>
<td>isoleucine</td>
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<tr>
<td>leu</td>
<td>leucine</td>
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<tr>
<td>lys</td>
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<tr>
<td>met</td>
<td>methionine</td>
</tr>
<tr>
<td>orn</td>
<td>ornithine</td>
</tr>
<tr>
<td>phe</td>
<td>phenylalanine</td>
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<tr>
<td>pro</td>
<td>proline</td>
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<tr>
<td>ser</td>
<td>serine</td>
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<tr>
<td>thr</td>
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<td>trp</td>
<td>tryptophan</td>
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<tr>
<td>tyr</td>
<td>tyrosine</td>
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<tr>
<td>val</td>
<td>valine</td>
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**CUMULATIVE LIST OF ABBREVIATIONS**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>AA</td>
<td>Ascorbic acid</td>
</tr>
<tr>
<td>2,4-D</td>
<td>2,4-Dichlorophenoxyacetic acid</td>
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<tr>
<td>ABA</td>
<td>Abscisic acid</td>
</tr>
<tr>
<td>ACC</td>
<td>1-Aminocyclopropane-1-carboxylic acid</td>
</tr>
<tr>
<td>ADC</td>
<td>Arginine decarboxylase</td>
</tr>
<tr>
<td>ADP</td>
<td>5'-Adenosine diphosphate</td>
</tr>
<tr>
<td>AMP</td>
<td>5'-Adenosine monophosphate</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>AOA</td>
<td>Aminoxyacetic acid</td>
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<tr>
<td>AOAA</td>
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<tr>
<td>AOPP</td>
<td>α-Aminooxy-β-phenylpropionic acid</td>
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<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
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<tr>
<td>AVG</td>
<td>Aminoethoxyvinylglycine</td>
</tr>
<tr>
<td>BA</td>
<td>Benzylaminopurine = benzyl adenine</td>
</tr>
<tr>
<td>BAP</td>
<td>Benzylaminopurine = benzyl adenine</td>
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<tr>
<td>BLG</td>
<td>Brown and Lawrence medium + gln</td>
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<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
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<tr>
<td>BSO</td>
<td>Buthionine sulfoximine</td>
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<tr>
<td>cAMP</td>
<td>3',5'-Cyclic adenosine monophosphate</td>
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<tr>
<td>CBM</td>
<td>Bornman medium</td>
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<tr>
<td>C/N</td>
<td>Carbon/nitrogen</td>
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<tr>
<td>D</td>
<td>Dark</td>
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<tr>
<td>DCR</td>
<td>Durzan sugar pine medium</td>
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<tr>
<td>DF</td>
<td>Douglas-fir</td>
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<tr>
<td>DFMA</td>
<td>α-difluoromethylarginine</td>
</tr>
<tr>
<td>DFMO</td>
<td>α-difluoromethylornithine</td>
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<td>DCHA</td>
<td>Dicyclohexylammonium sulfate</td>
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<td>DW</td>
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<td>E</td>
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<tr>
<td>EC or ec</td>
<td>Embryogenic callus</td>
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<tr>
<td>EDTA</td>
<td>Ethylenediaminotetraacetic acid</td>
</tr>
<tr>
<td>Ed</td>
<td>Embryonal initial</td>
</tr>
<tr>
<td>FAA</td>
<td>Free amino acid(s)</td>
</tr>
<tr>
<td>FTIR</td>
<td>Fourier transform infrared</td>
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<tr>
<td>FW or fr.wt.</td>
<td>Fresh weight</td>
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<tr>
<td>G-1-P</td>
<td>Glucose-1-phosphate</td>
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<tr>
<td>GA</td>
<td>Gibberellic acid (gibberellin)</td>
</tr>
<tr>
<td>GC</td>
<td>Gas chromatography</td>
</tr>
<tr>
<td>GC/MS</td>
<td>Gas chromatography/mass spectrometry</td>
</tr>
<tr>
<td>GD</td>
<td>Gresshof and Doy medium</td>
</tr>
<tr>
<td>GSH</td>
<td>Glutathione (reduced)</td>
</tr>
<tr>
<td>GSSG</td>
<td>Glutathione (oxidized)</td>
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<td>HEPES</td>
<td>N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid</td>
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<tr>
<td>HFBI</td>
<td>Heptafluorobutyrylimidazole</td>
</tr>
<tr>
<td>HFSE</td>
<td>High frequency somatic embryogenesis</td>
</tr>
<tr>
<td>HM</td>
<td>Hakman medium</td>
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<tr>
<td>HPLC</td>
<td>High performance liquid chromatography</td>
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<tr>
<td>IAA</td>
<td>Indoleacetic acid</td>
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<td>Abbreviation</td>
<td>Definition</td>
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<tr>
<td>IBA</td>
<td>Indolebutyric acid</td>
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<tr>
<td>IEF</td>
<td>Isoelectric focusing</td>
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<tr>
<td>IPA</td>
<td>Isopentenylaminopurine = 2iP</td>
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<tr>
<td>L</td>
<td>Larch, light or liter</td>
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<tr>
<td>LFSE</td>
<td>Low frequency somatic embryogenesis</td>
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<td>LM</td>
<td>Litvay medium</td>
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<td>LP</td>
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<td>lx</td>
<td>Lux</td>
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<td>MEOI</td>
<td>Methylenoxindole</td>
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<td>Morpholinoethane sulfonic acid</td>
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<td>Methyloxindole</td>
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<td>MOPS</td>
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<td>MGBG</td>
<td>Methylglyoxal bis-guanyl hydrazone</td>
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<td>NAD+</td>
<td>Nicotinamide adenine dinucleotide (oxidized)</td>
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<tr>
<td>NADP+</td>
<td>Nicotinamide adenine dinucleotide phosphate (oxidized)</td>
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<td>Nicotinamide adenine dinucleotide phosphate (reduced)</td>
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<td>Nonembryogenic</td>
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<td>Nitroblue tetrazolium</td>
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<td>NOAA</td>
<td>Naphthoxyacetic acid</td>
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<td>NS</td>
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## BASAL MEDIA FORMULATIONS - COMPARISON

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\(^a\text{MSGa} = \text{MSG} + 1 \text{g/L casein hydrolysate} + 500 \text{mg/L GLN.}  \\
\(^b\text{DCl} = \text{DCR with 50 mg/L glutamine.}\)