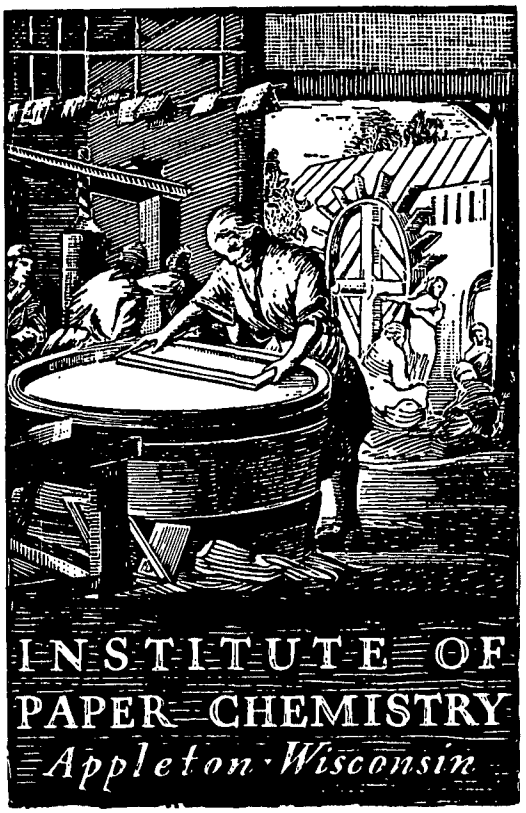

MASS PRODUCTION OF CONIFER HYBRIDS

Project 3223

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

March 15, 1987

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THE INSTITUTE OF PAPER CHEMISTRY

Appleton, Wisconsin

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THE INSTITUTE OF PAPER CHEMISTRY

Appleton, Wisconsin

MASS PRODUCTION OF CONIFER HYBRIDS

SUMMARY

Overall thrust of this project remains concentrated on developing procedures for obtaining somatic embryogenesis and recovering plantlets. Resources for tissue culture research have been increased, and heavy use has been made of the Norway spruce model system since its adoption last year. Biochemical work, though somewhat diminished, has continued but more in direct support of cell and tissue culture activities. Increased emphasis has been placed on integrating results from past work on nonconifer model systems and from research on natural embryo development and organogenesis.

Work with immature Norway spruce embryos advanced greatly in 1986. Combining our own approaches with those of others, we have refined the system such that embryogenic callus can be initiated and maintained routinely, numbers of somatic embryos can be quantified, and plantlets can be recovered. Capitalizing on results from 1985, we further refined the "optimum window" and are in a position to extend the findings to other species. By investigating and tracking several genotypes, we were also able to glimpse how different genetic backgrounds affect initiation and maintenance. Using explants of similar size (and presumably developmental stage), we found roughly equal initiation frequencies, but over fourfold differences in ease of maintenance. On the whole, initiation, growth, and maintenance of embryogenic callus from immature Norway spruce embryos is quite straightforward. Given this proficiency, we are pleased to offer samples of both embryogenic and nonembryogenic callus to Member Companies for experimentation in their own laboratories.

Access to embryogenic and nonembryogenic callus of Norway spruce expedited our search for biochemical markers. Although some are more useful than others, assays for ethylene, glutathione, total reductants, protein synthesis rates, and isozymes proved useful in distinguishing embryogenic from nonembryogenic callus. In addition, ultraviolet to infrared spectral analyses can be used to sort callus types. With the exception of ethylene, these markers also worked with larch and white spruce.

Increased work on new and refined protocols has resulted in our achieving somatic embryogenesis with explants from fully developed Norway spruce embyros. The newly adapted protocols are reliable and give consistent results. In addition, we can maintain embryogenic callus for considerable periods of time, and have recovered a number of plantlets. As in most systems, however, maturation and conversion frequencies remain low. Success with fully developed seed provides a stepping stone to more mature material and permits us to conduct experiments throughout the year. Results also affirm that reduced nitrogen, light, and/or their interaction are critical factors.

Considerable differences have been noted among Norway spruce callus lines in terms of somatic embryo yields. Our best lines produce 1000 or more embryos per gram of callus, but most lines produce far fewer. Possibilities for increasing yields and fostering maturation include more frequent subculturing (one vs. two or three weeks) and treatment with BSO. Results from earlier work on the biochemistry of nonconifer model systems are proving useful in these regards. Taken together, the several approaches are yielding more plantlets for transfer to and evaluation in the greenhouse. Alternative culture systems also hold promise. As an example, culture on agarose beads facilitated maintenance, caused an eightfold increase in growth over agar culture, and yielded much

larger numbers of mature somatic embryos. Although this system would be cumbersome for routine use, such findings underscore the merits of investigating alternative systems. Since these several steps are important barriers to efficient mass propagation, work in the aforementioned areas is being increased and focused on the most promising approaches. In addition, the agarose bead system will be useful for exploratory work on gene transfer.

Similar progress has been made with white spruce, a commercially important species. As for other species, stage of explant development was related to frequency and ease of initiation. Developing embryos that had cotyledons and were roughly 1.5 mm long gave the highest initiation frequencies (39%). In addition, embryogenic callus from larger explants grew better and was easier to maintain. If desired, this species could replace Norway spruce as the model system without loss of momentum. In addition, large quantities of genetically improved seed can be obtained from a variety of genetic backgrounds, and the species is utilized by some Member Companies.

Somatic embryogenesis has also been induced in white pine, another species of commercial importance and of potential interest to Member Companies. Progress has been marked, but development and maturation have not yet been achieved. Embryogenic callus type is similar to that of spruces, but origin and factors promoting formation appear different. Given these findings and the close botanical relationship, further work on white pine should hasten progress with loblolly pine.

We have repeated our earlier success with Douglas-fir, but are not yet able to initiate embryogenic callus on a reproducible basis. Frequencies of initiation remain low, and the few responsive cultures have proven difficult to

grow and maintain. That each such culture was obtained with the same protocol nevertheless provides us with a positive direction for future work.

Results from summer work on the "juvenility window" in loblolly, pond, and pitch pines and Douglas-fir verified that windows of morphogenic potential exist for each of the several explant types. Although unable to initiate embryogenic callus, we were able to discern the developmental stage and explant type best-suited for future work. Callus could be initiated from proembryos only with difficulty. Initiation frequencies improved with stage of development, but material at stages optimal for initiation in spruce were inappropriate for pine. Explants taken between fertilization and formation of cotyledons seem most appropriate.

Using the Smith Protocol, we recently observed white, mucilaginous callus typical of spruce in a small number of cultures derived from loblolly, pond, and pitch pines. However, neither clearly discernible somatic embryos nor other attributes typical of spruce have been noted. Biochemical and histological tests, including total reductants and chloroplast assays are being applied to the few available cultures to discern whether or not they truly are embryogenic. In addition, these several results suggest a clear path toward obtaining somatic embryogenesis in our target species: precotyledonary embryos, culture medium with low or no ammonium but supplemented with an organic form of reduced nitrogen, and culture in the dark.

Work on the "juvenility window" for loblolly pine paid yet other dividends. Tracking anatomical and morphological characteristics of cones, ovules, and embryos over time improved our understanding of how developmental stage *in vivo* affects response of explants *in vitro*. As an example, best performance in

culture for most protocols occurred with embryos excised when cones first reached maximum length. Also, cone length was correlated with gametophyte size during early summer, and can be used as an index of when to collect for culture with the Smith protocol. Not unexpectedly, results also demonstrated considerable differences in rates of cone development among parent trees. Taken together, the several findings should help make the timing of cone collections more precise, thereby reducing workloads and increasing efficiency in both field and laboratory.

Efforts to apply the biochemical marker, ethylene, to explants obtained in the work on the "juvenility window" were not helpful. The objective involved using ethylene evolution from explants to predict which would yield embryogenic calli. As noted above, however, we were unable to obtain embryogenic callus in the target species. In addition, the ethylene assay did not prove reliable for distinguishing embryogenic from nonembryogenic callus in white spruce. On the other hand, the total reductants test worked well with callus from several species. Whether this more reliable test will work with explants as well is currently being investigated.

Other biochemistry work centered on inhibiting key enzymatic reactions in efforts to promote embryogenesis. Specific inhibitors of GSH and phenolic acid synthesis reduced embryogenesis if applied during the embryo proliferation stage. However, embryogenesis was enhanced if the same inhibitors were added during the development stage. Such results suggest that some of our biochemical markers are associated not with deterministic events, but rather with those biochemical events that foster embryo development. Indeed, perturbation of certain biochemical pathways was shown to smooth the transition between embryo proliferation and development. In addition, the efficiency of embryo maturation

was increased. Further work along these lines should help remove a major barrier to use of somatic embryogenesis for mass propagation.

Histological assays have shown that spruce embryogenic callus originates from the outermost cell layers of the hypocotyl region. New work with explants from more mature materials will therefore be focused on tissues similar to hypocotyls.

The abnormal chloroplasts observed earlier in embryogenic spruce and larch calli as well as carrot somatic embryos apparently are "proplastids." Literature reports indicate occurrence in early zygotic embryos and meristemic tissues of several plant species. In our recent assays, they have also been found in zygotic embryos of Norway spruce and carrot. On the other hand, normal chloroplasts occur in more developed spruce somatic embryos. Such findings suggest that progression of plastid development in our somatic embryos corresponds to that in maturing zygotic embryos. We are therefore extending examinations to suspected embryogenic callus from other species, and to zygotic and somatic embryos at varying stages of development.

INTRODUCTION

Advances in plant cell and tissue culture have been rapid and dramatic in recent years, and developments at the Institute are no exception. Norway spruce was adopted as a model system in early 1985, and our work with it since that time has disclosed much about somatic embryogenesis in conifers.

Combining our own approaches with those of others, we have made Norway spruce a controlled and reproducible system in which somatic embryo numbers can be quantified and one from which plantlets can be recovered. Initiation of embryogenic callus from immature spruce embryos has become a straightforward process. Much the same holds for white spruce. Constant refinement of protocols has also resulted in similar success with mature embryos of Norway spruce, thereby permitting year-round research and setting the stage for work on explants from older material.

Significant progress has likewise been made with our target species, loblolly pine and Douglas-fir. We have produced callus from these species much like that routinely observed in spruces. In accordance with preliminary reports from elsewhere, we are confident that such cultures can be made to express their embryogenic potential.

Effects of explant age, maturation of somatic embryos, recovery of plantlets, and a rapid, inexpensive system for mass propagation nevertheless remain formidable challenges. For these reasons, we have added new talent on the tissue culture front, and have continued shifting resources away from biochemical research. Efforts in the biochemistry area are concentrated in support of our main tissue culture thrusts, particularly on improving our abilities to develop, mature, and recover plantlets.

Our focus has thus been tightened to work on those barriers that stand in the way of making somatic embryogenesis a usable technology. We are also enthusiastic about applying newer biochemical and molecular techniques to the aforementioned issues as well as to verifying fidelity of recovered plantlets and transferring genetic material.

This report summarizes the many accomplishments made during a year of major transition, and is intended to serve as a stepping stone to accelerated work on our main objectives and exploratory forays in new areas.

SOMATIC EMBRYOGENESIS RESEARCH

INITIATION FROM IMMATURE EMBRYOS

Initiation and Maintenance of Embryogenic Callus Derived from Immature Embryos of Norway and White Spruce

Introduction

In the summer of 1985 the IPC tissue culture team initiated embryogenic callus lines of Norway spruce. The protocol used was that reported by Hakman et al.,[1] with immature embryos as explants. In an effort to further define the optimum "window" for initiation of embryogenic callus from immature embryos of Norway spruce, new embryogenic callus lines were initiated in the summer of 1986. Particular attention was devoted to the following three areas. First, the frequency of initiation of embryogenic callus as a function of the development stage of the immature embryo. In numerous plants, for example in maize, there is an optimum immature embryo explant size for efficient initiation of embryogenic callus.[2] Second, determining the effect of cold storage on initiation of embryogenic callus from immature embryos of Norway spruce. Hakman and von Arnold[3] reported callus initiation frequency was increased by a cold storage treatment of cones. Thus, an effort was made to verify this. Third, embryos were collected from immature cones of several trees in an attempt to determine if differences in initiation frequencies were observed between trees.

Due to poor cone production of Norway spruce trees, an effort was also made to initiate embryogenic callus from white spruce, a species in which cones were abundantly available. During Dr. Inger Hakman's visit to IPC in February 1986, she informed us that she had initiated embryogenic callus in white and black spruce. Since that time an abstract of her work has been published.[4] In white spruce the frequency of initiation of embryogenic callus was also determined

in relation to the developmental stage of the immature embryo explant, and the results are presented here.

Methods

Norway spruce embryogenic callus lines used in experiments reported on here were derived from immature embryos of cones from trees at three locations: 1) The Municipal Golf Course, Appleton, WI, 2) Greenville, WI, and 3) Fayetteville, Ark. Embryogenic callus lines derived from the Appleton tree were initiated in 1985 and a description of collection dates and callus initiation frequencies is contained in IPC Project 3223 Progress Report Thirteen and has also been published.[5] The 1986 collections were from individual trees located at Greenville, WI, approximately 10 miles from IPC, and Fayetteville, Arkansas. The latter tree was located on the Univ. of Arkansas campus, and immature cones were provided by Brad Murphy of the Dept. of Horticulture and Forestry. The white spruce immature embryos were derived from two trees at the IPC Greenville Nursery (here after denoted as Trees A and B). Cultures from both species were initiated from immature embryos as described by Hakman.[1] The basal medium used was a modification of that reported by von Arnold and Eriksson[6] (refer to Appendix).

For initiation and maintenance of callus the basal medium was supplemented with (in mg/L) 2,4-D (2) and BAP (1). Thirty to fifty embryo explants were cultured from each tree at each collection date. Five explants were cultured per each 55 mm plastic Petri plate which contained approximately 6 mL medium. Explants were transferred and calli subcultured every two weeks and maintained at 23°C with 16 h irradiance (15-50 $\mu E\ m^{-2}s^{-1}$ at culture level) from cool-white fluorescent and incandescent lights.

At the time of culture initiation the following parameters were measured using a dissecting microscope with a micrometer eye-piece: embryo length (embryonal mass only, excluding suspensor) and the presence or absence of developing cotyledonary primordia. The initiation frequency of embryogenic callus was determined after 6 weeks in culture as the percentage of explants which formed white to translucent mucilaginous callus with somatic embryos visible by dissecting microscope observation.

For determination of the gravimetric composition of Norway spruce embryogenic callus the following methods were used. Pieces of embryogenic spruce callus (~ 100-200 mg wet weight) were aseptically weighed to the nearest milligram and dispensed (3 calli/9 cm petri dish) on basal medium containing 2 mg/L 2,4-D and 1 mg/L BA. At the end of 1, 2, and 3 weeks, three callus pieces were weighed to determine the wet weight. These callus pieces were transferred to tared Whatman GA/A glass microfiber filter pads, and rinsed with distilled water (under aspiration) for 30-60 s. The pads were reweighed (to calculate fresh weight) and were placed in an oven overnight at 60°C. A final weighing was then made for dry weight determination.

Results and Discussion

The frequency of initiation of embryogenic callus from immature embryos of Norway spruce collected from the Greenville, WI tree is presented in Table 1. Cones were collected from June 23 through August 4 and cultures were initiated within 24 hours of collection time. The development stage of immature embryo explants was determined at the time of culture initiation. Viewing explants with a dissecting microscope enabled accurate measurement of the length of the embryonal mass. Immature embryos derived from the June 23 collection were small (mean length less than 0.2 mm), precotyledonary, and initiated embryogenic callus at low frequencies.

Mean size of embryo explants collected between June 30 and July 21 ranged from 0.5 to 3.4 mm. During this four week period from 44 to 62% of the immature embryos initiated embryogenic callus and the levels of initiation were significantly higher than both earlier (June 23) and later (August 4) collection times. Therefore, a rather broad (four week) "window" was found during which high frequencies of initiation occurred using the Hakman protocol. In reference to findings that will be presented later for white spruce, it is interesting to note that embryogenic callus was initiated at high frequencies from both precotyledonary (June 30) and cotyledonary (July 7 to 21) embryo explants (Table 1). This result suggests that in Norway spruce the initiation of cotyledonary primordia did not serve as a marker for the beginning of the responsive stage of explant development.

Table 1. Frequency of initiation of Norway spruce embryogenic callus from immature embryos collected in 1986 from Greenville, WI tree.

Collection Time, month/day	Number of Explants	Explant Length, mm	Explants With Cotyledons, %	Embryogenic Callus Initiation, % explants*
6/23	31	0.16 ± 0.04	0	6[a]
6/30	33	0.50 ± 0.11	0	59[b]
7/7	28	0.93 ± 0.44	64	44[b]
7/14	36	2.26 ± 0.44	83	62[b]
7/21	11	3.38 ± 0.17	100	55[b]
8/4	11	ND	100	0[a]

*Means followed by a common superscript are not significantly different as determined by ANOVA with Duncan's New Multiple Range Test ($p < 0.05$).

In summary, the 1986 results presented in Table 1 both reinforce and extend the findings reported for the 1985 Norway spruce collection in the Appleton, WI region. In both years immature embryos cultured by the Hakman procedure lost the ability to produce embryogenic callus in early August. Our 1986

results provided information on the beginning of the window of morphogenic potential which was not studied in 1985. A sharply defined increase in embryogenic potential occurred near July 1, when embryos had reached 0.5 mm in length.

The results of our study on the effect of a cold pretreatment of immature Norway spruce cones showed that cold storage did not significantly increase embryogenic callus initiation (Table 2). The only statistically significant effect within collection dates was a decrease in frequency of embryogenic callus from embryos derived from cold stored cones relative to embryos from fresh (control) cones on July 7. It should be noted that our cold pretreatment period was for 14 days. Hakman and von Arnold[3] reported that a cold pretreatment of cones for two months increased embryogenic callus initiation. Perhaps the 14 day cold pretreatment period we tested was not long enough to significantly increase embryogenic callus initiation frequency. Regardless, we know from this study and the 1985 studies that we can cold store immature cones and effectively initiate embryogenic callus. This is of considerable utility because it enables us to spread the isolation work over a longer period of time.

Table 2. Effect of cold (4°C) pretreatment of explants on initiation of Norway spruce embryogenic callus.

Collection Time, month/day	Embryogenic Callus Initiation* % explants	
	Control	Cold Pretreatment
6/23	6[a]	22[a]
6/30	59[a]	34[a]
7/7	44[a]	6[b]
7/14	62[a]	72[a]
7/21	55[a]	65[a]
8/4	0	---

*Means followed by a common superscript are not significantly different as determined by ANOVA with Duncan's New Multiple Range Test ($p < 0.05$).

As indicated previously, embryogenic callus was initiated primarily from immature embryos of two Norway spruce trees in 1986. Most of the developing cones were obtained locally from the Greenville, WI tree (Table 1), but one collection was obtained from a tree in Fayetteville, AR. A comparison of initiation and maintenance frequencies of embryogenic callus derived from these two trees is presented in Table 3. The mean length of the Arkansas embryos was 3.1 mm (100% cotyledonary primordia). For comparative purposes the Greenville, WI collection that was closest in size is presented in the second row of Table 3. This was the July 21 Greenville collection with a mean length of 3.4 mm (also 100% cotyledonary primordia). Even though this was the most equivalent comparison possible in terms of embryo development stage, the Wisconsin embryos were significantly larger than the Arkansas embryos. It is important to note that although the initiation frequencies were similar between the explant source trees (67 vs. 65%), the percentage of embryogenic callus lines that could be maintained from the Arkansas tree was much higher (70%) than the percentage maintained from embryos of similar developmental stage from the Greenville tree (15%). Also listed in the third and fourth column of Table 3 are initiation and maintenance data from the "best" collection of the Greenville tree, i.e., June 30 when the immature embryos were at a much earlier developmental stage. The initiation frequency from this earlier Greenville collection was similar to the Arkansas tree, and a high percentage (89%) of the lines initiated were maintained.

Quantitative data on growth of the embryogenic callus derived from the Arkansas and Wisconsin trees are also presented in Table 3. After 15 weeks in culture the mean weight per individual line of embryogenic callus from the Arkansas tree was considerably higher than either Wisconsin collection. Because of the large variation in growth among lines these differences were not statistically

significant. Regardless of the lack of statistical differences in growth, the total weight of callus accumulated emphasizes the increased vigor of the Arkansas lines (Table 3).

Table 3. Initiation and maintenance of embryogenic callus derived from immature embryos of two Norway spruce trees: in Greenville, WI and Fayetteville, AR.***

Tree Location	1986* Collection Date, month/day	Embryo Explant, mm	Embryogenic Callus Lines Initiated %	Embryogenic Callus Lines Maintained** %	Callus Weight at 15 Weeks Mean/ Line, g	Callus Weight at 15 Weeks Total, g
Arkansas	6/23	3.1[a]	37/55 (67)[a]	26/37 (70)	0.61[a]	15.91
Wisconsin	7/21	3.4[b]	26/40 (65)[a]	4/26 (15)	0.27[a]	1.09
Wisconsin	6/30	0.5[c]	19/32 (59)[a]	17/19 (89)	0.37[a]	6.27

*Both the June 23, Arkansas and July 21, Wisconsin cone collections were cold stored 2 weeks. The data from the June 30 Wisconsin collection are from fresh cones (no cold pretreatment) and for comparative purposes, are presented as the "best" data from the Wisconsin tree.

**Percentage of initiated callus lines that were maintained after 15 weeks in culture.

***Means followed by a common superscript are not significantly different as determined by ANOVA with Duncan's New Multiple Range Test ($p \leq 0.05$).

In summary, the results of the Arkansas vs. the Wisconsin tree comparison provided evidence that more vigorous embryogenic callus was initiated from immature embryos of the Arkansas tree. It is of ultimate interest to address the question of the degree of variation in the capacity for initiation, maintenance, and subsequent plant regeneration from embryogenic callus derived from genetically different trees. This is of considerable importance for potential applications of somatic embryogenesis to existing tree improvement programs where select families serve as primary reservoirs for propagation purposes. Genotype differences in the potential for somatic embryogenesis of cultured explants are manifested in different ways, especially in species that are highly heterogeneous as the result of open pollination.[7] Considerable genotypic

variation in the in vitro response of alfalfa has been observed (Brown and Atanassov[8] and references therein). This is not unexpected as alfalfa is an open pollinated polymorphic species with a great deal of inherent variation. Similarly, conifers are highly heterozygous (Bonga,[9] p. 387-412) and one might expect considerable variation in their in vitro response between genetically different trees. This has proven to be the case with regeneration from loblolly pine cotyledons via organogenesis. The North Carolina State Univ. work of R. Mott and H. Amerson found certain loblolly pine families, such as family 9-17, that regenerated from cotyledons more efficiently than other families.

Initiation of embryogenic callus, per se, by the IPC Tissue Culture team has been very successful using at least three separate trees at three distinct locations: the Appleton golf course (1985), Greenville, Wisconsin and Fayetteville, Arkansas. Thus, we have not found evidence for considerable variations in initia-tion of embryogenic callus among these trees. It is quite possible that variation will be found among genetically different trees in other aspects, e.g., the vigor of callus growth and maintenance, or efficiency of somatic embryo maturation.

Studies were also conducted in 1986 to determine composition and growth characteristics of Norway spruce embryogenic callus initiated from immature embryos on the Hakman protocol. Embryogenic Norway spruce callus maintained on HM 2/1 medium is composed of an extracellular mucilaginous matrix in which are embedded elongated suspensorlike cells, somatic embryos, and dense clusters or "growth centers" of callus. Results reported in the North Carolina State Tree Improvement Cooperative 1986 Annual Report[10] suggest that these clusters of callus are derived from embryonal heads of somatic embryos which proliferate into callus. Growth was characterized by three gravimetric parameters: wet

weight (the extracellular material and tissue), fresh weight (tissues washed free of extracellular material), and, dry weight (oven-dried material from the fresh weight determinations). The embryogenic callus had a doubling time (twofold increase in dry weight) of ~ 6-8 days (Fig. 1 top panel). The results also showed that embryogenic spruce callus undergoes significant changes in composition with respect to time on 2,4-D and BA supplemented medium (see Fig. 1 bottom panel). In particular, the extracellular component (mucilage) is initially large (~ 80% of the total mass) and decreases to around half the total culture mass after three weeks. This "drying out" of the callus is manifested as the cultures appear to densify from the inside to the outer edges, which in turn remain quite wet in appearance. The net effect is that some extracellular water in the mucilaginous material becomes incorporated intracellularly. Inasmuch as the gravimetric composition is dependent on the subculture interval, attention must be paid to the duration of this interval when mass determination is employed in an experiment. If the subculture interval is known, then the mass determination that is expedient for tissue culture purposes (wet weight) can readily be related to more relevant determinations (fresh and dry weight) that are used in biochemical experiments.

In white spruce the developmental stage of each embryo explant was also determined at the time of culture initiation. Viewing explants with a light dissecting microscope enabled accurate measurement of the length of the embryonal mass which appeared white and opaque compared to the suspensor cells which appeared translucent (Fig. 2A). Embryo explants derived from cones collected June 19 were 0.1 to 0.4 mm in length with no visible cotyledonary primordia development (Fig. 2B). Nearly all of the embryo explants collected after July 1 had initiated cotyledonary primordia development (Fig. 2C).

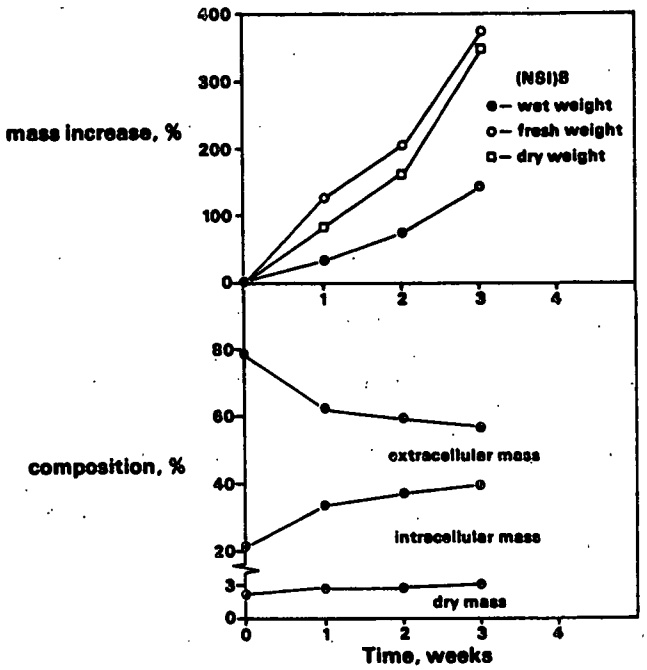

Figure 1. Growth and composition of embryogenic Norway spruce callus during a three week culture period on HM 2/1 medium. The top panel shows changes in gravimetric composition with time. The bottom panel shows changes in three fractions (extracellular, intracellular, and dry mass) with time.

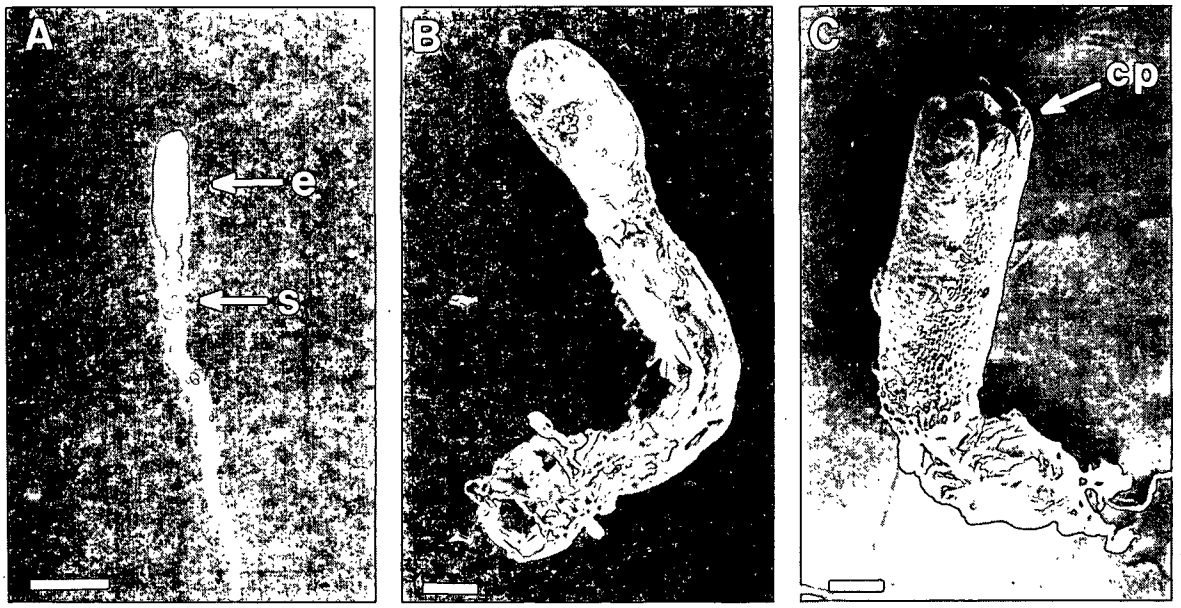

Figure 2. Immature zygotic embryo explants of white spruce. A. Precotyledonary embryo showing embryonal mass (e) and suspensor (s) regions. B. Scanning electron micrograph of precotyledonary embryo collected June 19. C. Scanning electron micrograph of embryo collected July 1 with development of cotyledonary primordia (cp). Scale bars: A = 1mm, B = 100 μ, and C = 100 μ.

A white to translucent embryogenic callus formed after 14 to 21 days in culture on some of the embryo explants. The embryogenic callus phenotype initiated and maintained in white spruce appeared very similar to Norway spruce, i.e., somatic embryos, elongated suspensorlike cells, and dense cell clusters growing in a mucilaginous matrix.[1,11,5] Figure 3 shows examples of white spruce somatic embryos. In both white and Norway spruce, investigations in our laboratory have shown that the origin of embryogenic callus is from the hypocotyl region of immature embryo explants.[12] Investigations by Henry Amerson of NC State University on the origin of embryogenic callus from white spruce immature embryos corroborate the hypocotyl region as the site of initiation.[10] We have not observed initiation of embryogenic callus from the radicle or suspensor cells of white spruce as reported by Gupta and Durzan in Norway spruce.[13]

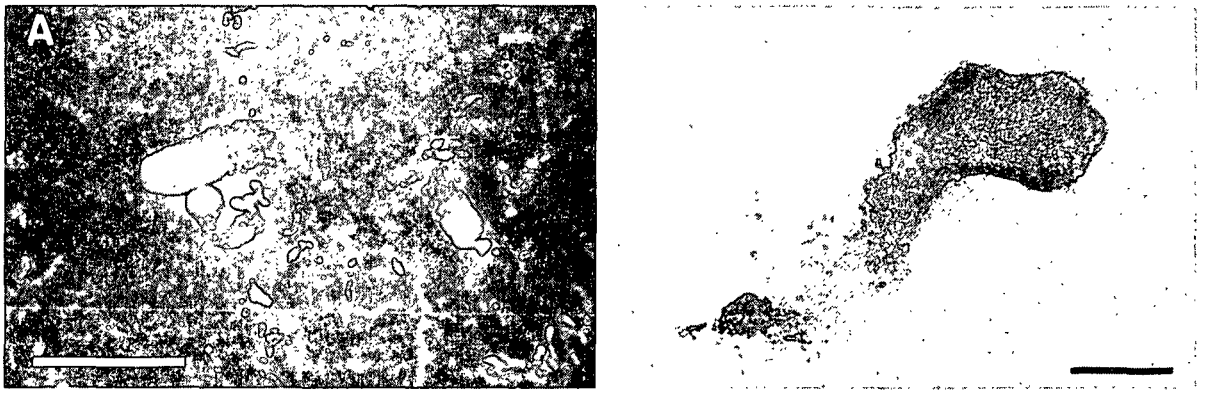

Figure 3. Somatic embryos of white spruce. A. Embryogenic callus maintained on Hakman medium with 2 mg/L 2,4-D and 1 mg/L BA was dispersed and plated in thin agarose layer. Note also numerous individual elongated suspensor cells. B. Whole mount of individual white spruce somatic embryo. Scale bars: A = 1 mm, B = 500 µ.

The mean embryo explant length, the percentage of explants with visible cotyledonary development, and the initiation frequency of embryogenic callus were plotted as a function of explant collection time in Fig. 4. There was no significant difference in the length of embryos collected from the two trees on

any collection date (Fig. 4A). The time course of cotyledonary development was also very similar between trees (Fig. 4B). By July 1 more than 97% of all embryos showed developing cotyledonary primordia.

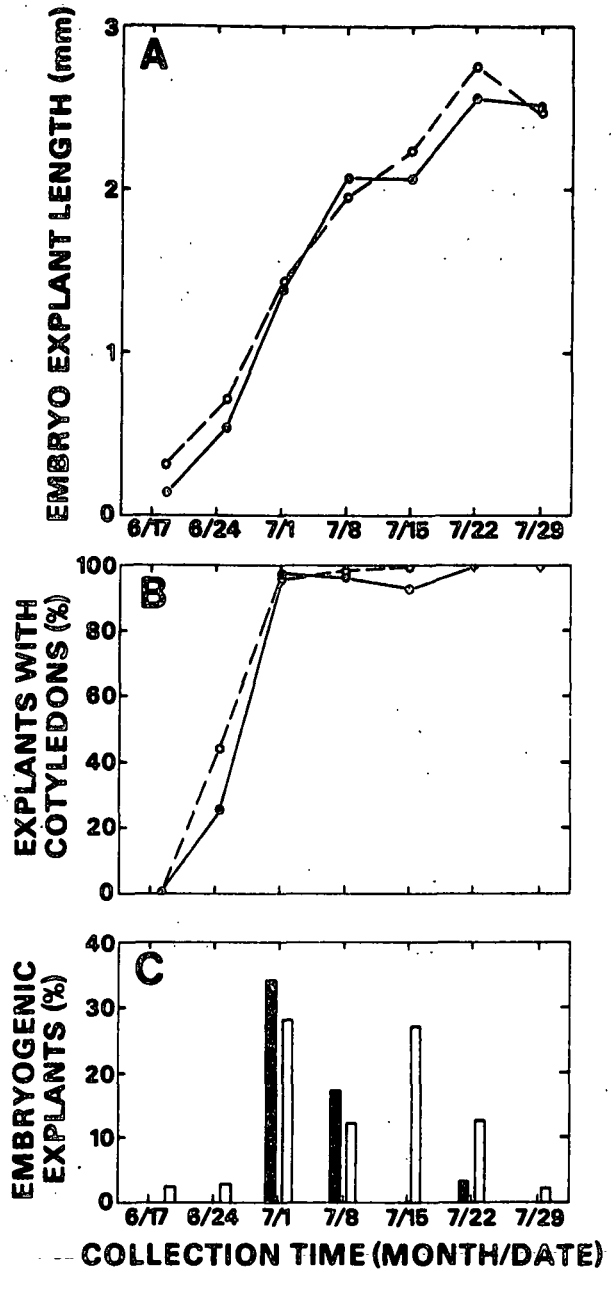

Figure 4. The relationship between the developmental stage of immature embryo explants of white spruce and the frequency of explants forming embryogenic callus. A. Mean embryo explant length. B. Percentage of embryo explants with visible cotyledonary primordia. C. Percentage of embryo explants initiating embryogenic callus. Closed symbols are data from tree A and open symbols from tree B.

Embryogenic callus was initiated at low frequencies (< 3%) from immature embryos of early collection dates, June 19 and 25 (Fig. 4C). These embryo explants ranged in mean size from 0.1 to 0.7 mm and a majority (84%) were precotyledonary. The highest frequency of initiation of embryogenic callus was from explants collected on July 1 when the mean embryo length was 1.4 mm and over 95% of the explants had visible cotyledonary development (Fig. 4C). Thus, in white spruce the transition from low to high initiation frequencies of embryogenic callus coincided with the development of visible cotyledons. There was no significant difference in embryogenic callus initiation frequencies between trees within collection dates with the exception of July 15 when no embryogenic callus was initiated from Tree A, whereas 26% was initiated from Tree B.

A total of 388 embryo explants derived from two white spruce trees were cultured between June 19 and July 29. A histogram of embryogenic callus initiation frequency further emphasizes the optimum explant size (Fig. 5). Only 3% (3/102) of embryo explants between 0.1 to 1.0 mm initiated embryogenic callus, whereas 21% (24/117) between 1.0 to 2.0 mm initiated embryogenic callus. The highest (39%) initiation frequency of embryogenic callus was from 1.4 to 1.6 mm explants. Fourteen percent (23/169) of the most mature explants, 2.0 to 3.0 mm, initiated embryogenic callus.

Numerous embryogenic callus lines could not be maintained for long-term culture due to poor growth rates. After 15 weeks in culture, 38% (19/50) of the embryogenic callus lines derived from embryo explants were maintained and grew vigorously. Similar to initiation frequency, the highest percentage of embryogenic callus lines maintained (13/24 or 54%) was derived from 1.0 to 2.0 mm explants. In comparison, only 33% (1/3) and 17% (4/23) of the embryogenic callus lines derived from smaller and larger explants, respectively, were maintained.

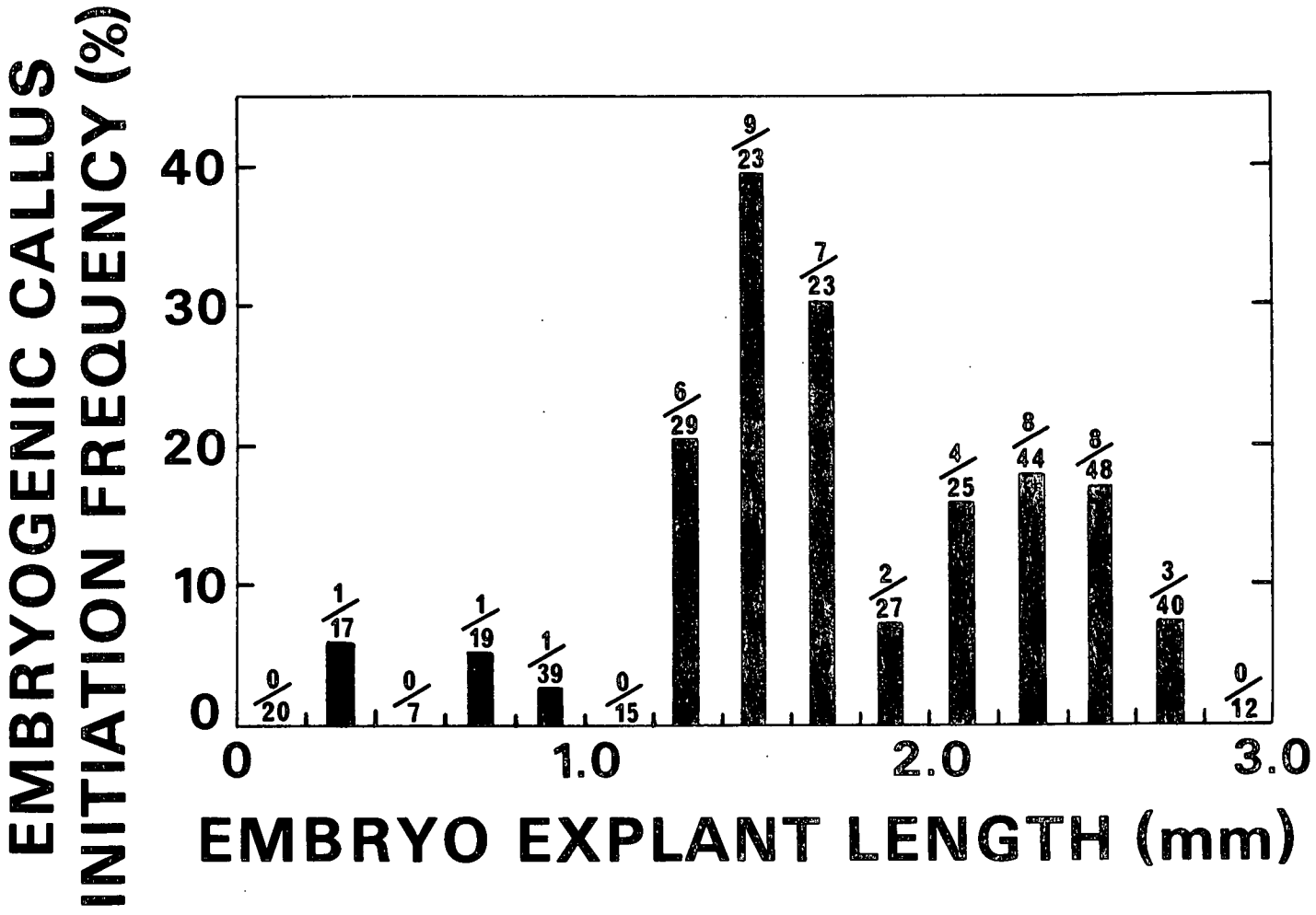

Figure 5. The frequency of initiation of embryogenic callus in white spruce as a function of immature embryo explant size. Data are pooled from Trees A and B.

In Picea simple polyembryony can occur due to fertilization of several archegonia of an ovule.[14] If multiple fertilization from genetically different pollen occurred, the resulting multiple embryos would be genotypically different. It is therefore possible, if embryogenic callus initiated from both dominant and accessory embryos, that resulting callus lines contained multiple genotypes. Multiple embryo explants from an individual seed were occasionally

observed from the June 19 and 26 collections, but these early embryos did not initiate embryogenic callus effectively. Furthermore, only one of the three callus lines could be maintained after 15 weeks. After July 1 when embryogenic callus was initiated at high frequencies, the larger size of embryo explants made it feasible to more conclusively track the origin of embryogenic callus.[12] Our observations have shown that embryogenic callus clearly initiated from the hypocotyl region of primary embryos. There was no evidence for initiation from accessory embryos. These observations provide evidence that the most vigorous embryogenic callus lines initiated in white spruce (e.g., those initiated after July 1) were of a single genotype in origin.

In summary, on the medium tested, the highest initiation frequency of embryogenic callus from immature embryos of white spruce was obtained from 1.4 to 1.6 mm embryos. Ninety-seven percent of the embryos in this size range had initiated cotyledonary development. Smaller embryos, which were predominantly precotyledonary, were much less effective on embryogenic explants. Embryo size and visible cotyledonary development are easily determined by microscopic observation and therefore serve as useful indices or markers of embryogenic potential of white spruce immature embryo explants.

Initiation Studies on Hard Pines, Douglas-fir and White Pine Using Explants Associated with Seed Development

Introduction

In 1985, the effort to obtain somatic embryogenesis in loblolly pine and Douglas-fir was modeled after the successful procedure used to induce somatic embryogenesis in Norway spruce. Some of the features critical to the successful initiation of embryogenic tissue in spruce that were incorporated into

1985 initiation studies were (1) a requisite developmental stage of the explant and (2) culture of explants onto a solidified medium containing a combination of auxin and cytokinin. In Norway spruce, the abovementioned features are embodied in (1) immature embryos at or near the stage of cotyledon development and (2) subsequent culture of these embryos onto an MS based medium containing a 25% reduction in the level of ammoniacal nitrogen and supplemented with 2 mg/L 2,4-D and 1 mg/L BA.

The incorporation of these two features was insufficient for the initiation of embryogenic tissue in the target species of loblolly pine and Douglas-fir. The failure of the Norway spruce protocol to initiate embryogenic tissue in the target species led to an alteration of procedure in several important areas. Although the developmental state of the explant was still identified as a concern of overriding importance, it was postulated that each explant type associated with in vivo embryology (nucellus, ovule, immature embryo) may have its own time period in which it will be receptive to in vitro environment and express its morphogenic potential. Incorporation of this postulate formed the backbone of the "conceptual summer plan" and resulted in the extensive examination of explant material associated with seed development.

The association of developmental windows with nucellar and ovular explants is not without precedent in woody species. Embryogenic tissue has been derived in nucellar and ovular tissue of temperate and tropical fruit trees.[15] In gymnosperms, fertilized ovules gave rise to embryogenic tissue in European larch,[16] and in the cycad, Zamia pumila (R. Litz, personal communication, 1985).

In regard to the culture medium, several limited studies from 1985 suggested that MS-levels of ammonium ion were inhibitory to the initiation of at

least nonembryogenic callus from immature embryos. For this reason, a medium devoid of ammonium ion (MSG, see Appendix) was used. This medium was very similar in composition to a medium developed for the micropropagation of loblolly pine.[17] The effect of the level and form of nitrogen was tested by a limited comparison of MS with MSG during the summer of 1986.

Two ancillary issues that were highlighted from the 1985 studies were the influence of genotype and growth regulator levels on the initiation of embryogenic tissue. In 1986, the decision was made to enlarge the number of clones tested in loblolly pine from 4 to 10 (11 clones counting the limited study with NC State) and to maintain clonal identity of the cultures. The influence of genotype on morphogenic potential has been well documented in most plant genera except conifers. Initiation studies in 1986 were aimed in part at exploring genotypic influences on initiation.

Including more clones necessitated reducing numbers of culture treatments. However, results from 1985 indicated little utility in examining large numbers of treatments that differed slightly in growth regulator levels. For this reason only four treatments were chosen per developmental stage, but they differed substantially in terms of growth regulator supplements. Except for the Smith protocol, which lacked growth regulators, all treatments employed 2,4-D and BA exclusively.

In summary, the conceptual summer plan involved

1. Examining embryogenic potential of different explants (nucellus, ovule and immature embryo) over their respective developmental periods.

2. Testing effects of reduced nitrogen forms (ammoniacal or organic) on initiation of embryogenic tissue.

3. Examining influence of three growth regulator regimes (auxin only, cytokinin only, and auxin-cytokinin combinations) on initiation.

Aside from the conceptual summer plan, "model systems" approach was also taken. Areas of investigation can be broadly categorized as:

1. Attempts to initiate embryogenic tissue in coniferous species that have a natural propensity for vegetative propagation.

2. Attempts to extend procedures for the initiation of embryogenic tissue in sugar pine and radiata pine to the target species.

For Category 1, pond pine (Pinus rigida var. serotina) and pitch pine (Pinus rigida Mill.) were chosen. Explants from these species were cultured under the protocols that comprise the conceptual plan as well as protocols described under Category 2.

For Category 2, two recently described procedures for the initiation of embryogenic tissue in sugar pine[11] and radiata pine (D. Smith, personal communication, 1986) were tested on both target and model species. The procedure for sugar pine was tested extensively with white pine, the most readily accessible 5-needled species. This procedure has very recently been applied to loblolly pine and pond pine as well. The procedure for initiation in radiata pine was tested on loblolly, pond and pitch pine.

Methods

Plant Material. Developing female cones of loblolly pine were provided by Union Camp Corp. (Rincon, GA), Westvaco Corp. (Summerville, SC) and North Carolina State University (Raleigh, NC). Cones of pond and pitch pine were provided by Union Camp and Westvaco, respectively. Cones of Douglas-fir were provided by Weyerhaeuser (Federal Way, WA) and cones of white pine were provided by Ohio State University (Wooster, OH) and the IPC greenhouse staff (Freedom, WI). Explants were harvested from these cones within 24-48 h of arrival, and unused cones (except Douglas-fir) were stored in kraft bags for up to five months at 4°C.

For gametophyte and embryo explants, 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. For nucellar explants the entire cone was sterilized by the above procedure, and the nucellus was aseptically removed.

Culture Initiation. In the southern pines and Douglas-fir, cultures were initiated on agar solidified medium (Bacto; Difco, 0.8%) containing, either singly or in combination, 2,4-D and BA (see Table 4). Nucellar and embryo explants were plated intact while gametophytic explants were longitudinally bisected prior to culture. Embryos were also cultured on MSG lacking growth regulators to examine the frequency of embryo rescue. In the southern pines cultured under the Smith protocol, intact gametophytes (presumably containing embryos) were cultured after the nucellus had been removed. All cultures of the southern pines and Douglas-fir were initiated at 23°C under a 16 h irradiance (15-50 $\mu E\ m^{-2}\ s^{-1}$) from cool-white fluorescent and incandescent lights. In white pine, immature embryos were

cultured under the media and hormonal conditions given in Tables 11-13. In addition to the environmental conditions cited above, white pine embryos that had been subjected to cold storage for several weeks were also cultured in the dark.

Table 4. Summary of growth regulator regimes used with MSG medium with explants of southern pines and Douglas-fir.

Growth Regulators, mg/L		Explant Type		
2,4-D	BA	Nucellus	Gametophyte	Embryo
0	2	x	--	--
0	5	--	x	--
2	0	x	--	--
2	1	--	x	x
2	2	x[a]	--	--
2	5[b]	--	--	x
10	2	--	x[a]	x
0	0	--	--	x[c]

[a]MS medium also employed at this growth regulator combination.
[b]Macro- and microelements at half strength.
[c]Medium supplemented with 1% sucrose and 1% activated charcoal; used with hard pines only.

In all species, clonal identity was maintained for each explant type. All cultures were initiated in 5 cm plastic petri plates, containing five explants/plate. In the southern pines and Douglas-fir, five plates/treatment were used for a total of 25 explants per medium-growth regulator combination. Due to various biotic and abiotic vagrancies (contamination, incomplete shipments, aborted seed development, etc.) the desired total of 25 explants per treatment was usually but not always realized. Table 5 shows the total number of cultures initiated from all explant types in the southern pines and Douglas-fir.

Table 5. Census of culture initiation from nucellar, gametophytic and embryo explants, April-August, 1986.

Species	Collections, no.	Clones, no.	Total Cultures
LP	16	10	15,461 (16,000)[a]
PO	15	4	5,053 (6,000)
PP	13	2	2,412 (2,600)
DF	13	9	4,394 (6,500)
Totals	57	25	27,320

[a]Number in parentheses refers to the ideal total assuming four treatments/clone, 25 explants/treatment.

Culture Maintenance and Evaluation. After four weeks, all treatments were scored as a percentage of explants responding by callus formation, whether embryogenic or nonembryogenic. For the embryo rescue treatments, the percentage of embryos that followed a course of normal plant development was also noted.

All explants in all treatments producing callus were subcultured after 4-6 weeks by transfer to medium of the same composition. After an additional 4-6 weeks, callus cultures exhibiting a nonembryogenic phenotype were usually discarded, although a small percentage were maintained for comparative histological and biochemical analysis. Callus cultures exhibiting an embryogenic phenotype were maintained on their initiation medium for establishment of a callus line, except for callus derived from the Smith protocol. Lines were established from callus initiated by the Smith protocol by transfer of the callus to MSG medium lacking charcoal but containing 3% sucrose and no growth regulators.

Results and Discussion

The Conceptual Summer Plan. The callus initiation frequency as a function of seed development is shown for the southern pines and Douglas-fir in Fig.

6-9. The initiation frequencies plotted in Fig. 6-9 represent the combined frequency for (1) all clones and (2) all cultures initiated on media supplemented with growth regulators. The combined frequency (across clone and treatment) was used because (1) virtually all callus initiated (> 99.99%) exhibited a nonembryogenic phenotype irrespective of clone, explant, or treatment and (2) all growth regulator regimes (except cytokinin only) were similarly adept at the initiation of nonembryogenic callus.

The overall failure to initiate embryogenic tissue, coupled with the propensity of all growth regulator regimes to initiate nonembryogenic callus makes for little meaningful interpretation of the results. However, several trends (or tentative conclusions) can be identified, and, in two collections (P09 and 10) an embryogenic phenotype was produced in a small number of cultures. The coupling of these trends and observations to the results obtained in the 1985 initiation studies identifies some of the critical features that will shape future initiations.

Examination of Fig. 6-9 reveals that a principle tenet of the conceptual summer plan was verified. Namely, each explant type has a particular developmental stage during which it will be most responsive to the in vitro environment. In nucellar explants this was particularly true in pitch pine (Fig. 7) and pond pine (Fig. 8). For gametophytic explants (ovule), all species exhibited a fairly well defined time interval in which callus could be initiated. This is in sharp contrast to previous work on gametophytic explants in which little if any callus was initiated from the limited number of collections examined (see Annual Report Thirteen). Clearly, encompassing a wider range of explant development can reveal windows that previously would have gone unnoticed.

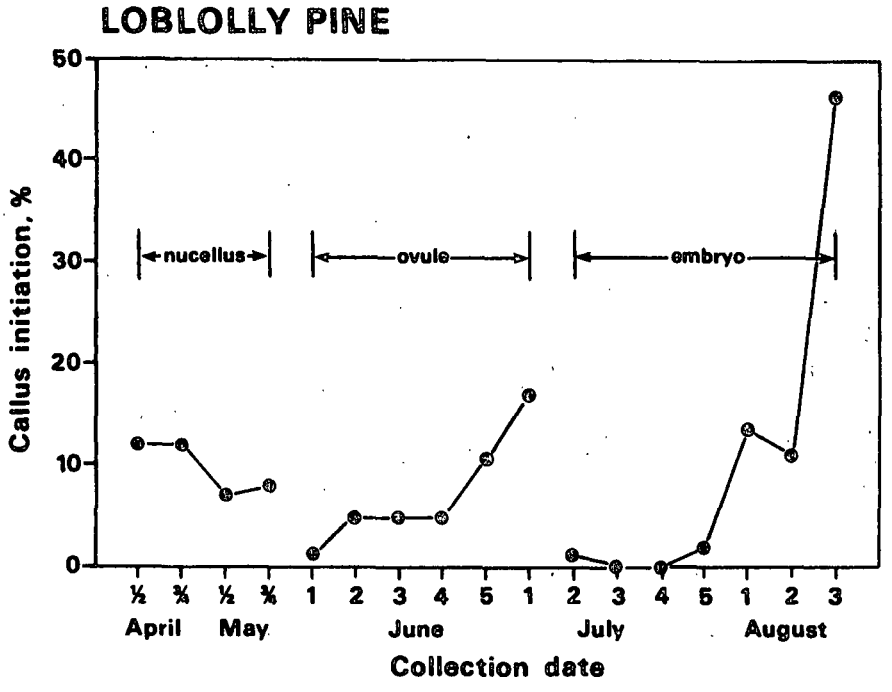

Figure 6. Loblolly pine initiation frequency as a function of collection date (1986).

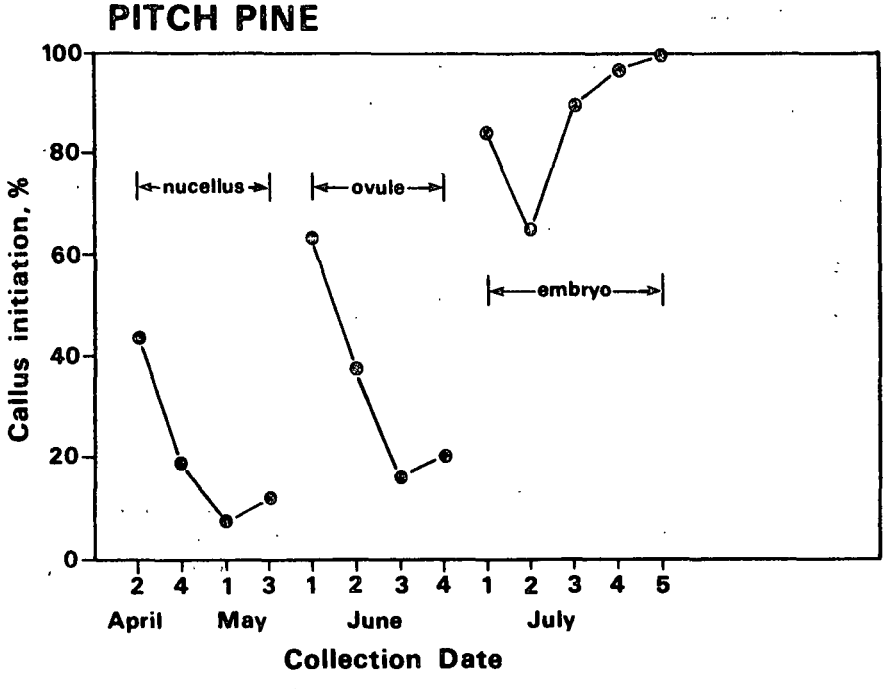

Figure 7. Pitch pine initiation frequency as a function of collection date (1986).

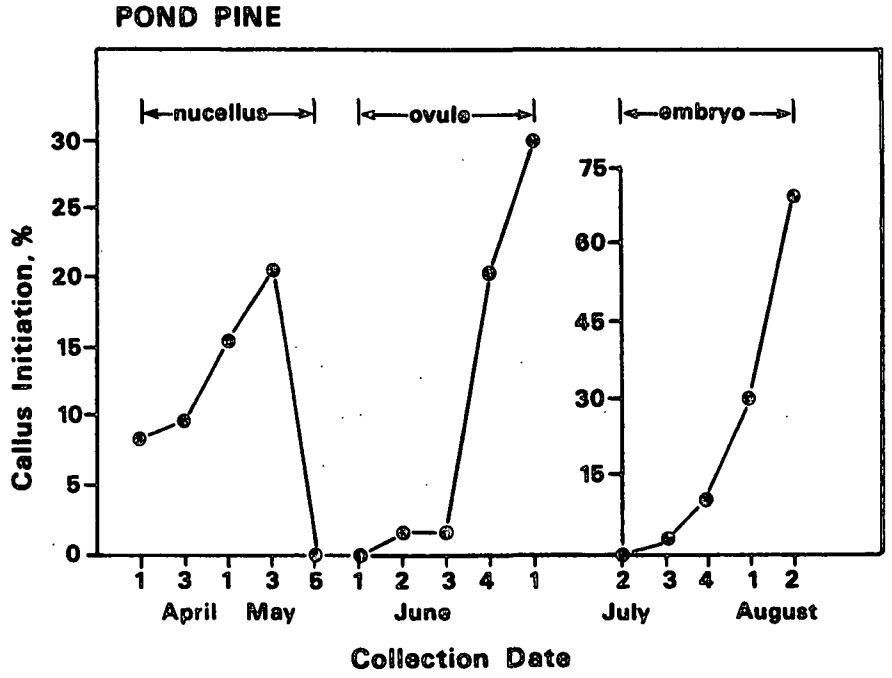

Figure 8. Pond pine initiation frequency as a function of collection date (1986).

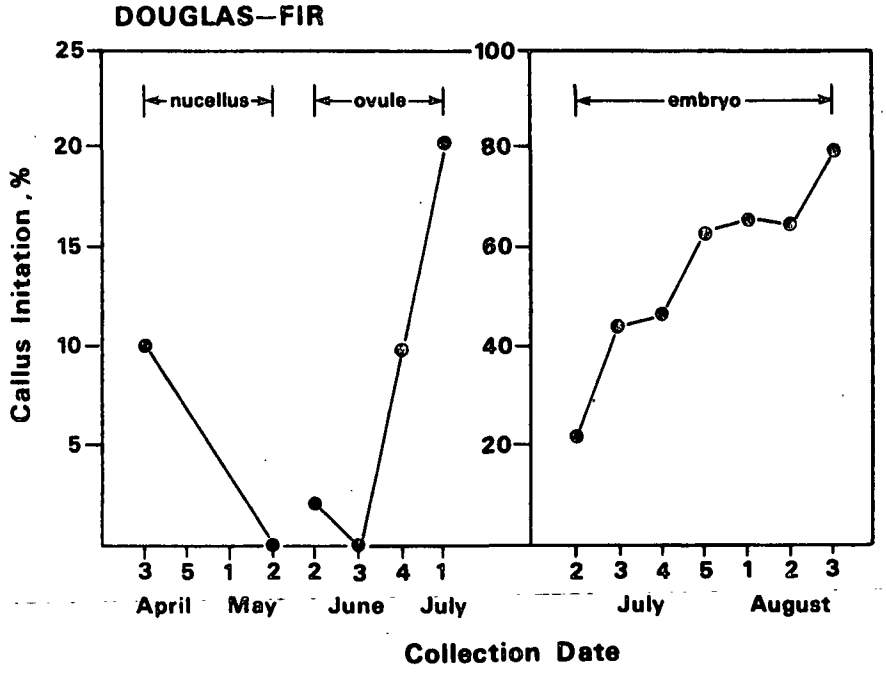

Figure 9. Douglas-fir initiation frequency as a function of collection date (1986).

The response of embryo explants over time is highly reminiscent of similar curves obtained in the past and follows quite closely the embryo rescue curves reported last year (see Annual Report Thirteen). Once again, the tacit correlation can be made that in loblolly pine, pond pine, and Douglas-fir the frequency of nonembryogenic callus initiation increases with the ability to grow immature embryos into plants. This correlation may be indicative of an increasing inadequacy of nutritional and environmental aspects of the culture conditions as embryo maturity decreases. However, the results with pitch pine (Fig. 7) do not bear this out.

In pitch pine (and to a slightly lesser extent, Douglas-fir), high initiation frequencies were obtained from embryos at all stages of development. Although most of these initiation events never proliferated to the extent that a callus line was established, the high initiation frequencies suggest that nutritional and hormonal components of the media were not necessarily deleterious to initiation. Aside from investigation of environmental conditions, the implication is that the medium is sufficient to support initiation of at least nonembryogenic callus. In pitch pine and Douglas-fir, failure to initiate embryogenic tissue may not be the result of "poisoning" the proembryo explants but may be due to what are at the present, unknown factors. Reports of embryogenesis in sugar pine indicate that culture in the dark may be one of these factors. Results from initiation studies in these two species support the notion that the stage of embryo development that is most efficient at the initiation of embryogenic callus in spruce is inappropriate for pines and Douglas-fir (Fig. 7 and 9). The caveat to this postulate is that the inappropriateness of the developmental stage of pine and Douglas-fir explants may not be permanent but rather a function of the nutritional, hormonal and environmental conditions. Nevertheless, it is

becoming increasingly apparent that features of embryogenic callus initiation in pines and Douglas-fir are going to be considerably different than in spruce with respect to the conditions discussed above.

Tables 6 and 7 depict the influence of nitrogen form and quantity on the initiation of nonembryogenic callus from nucellar and gametophytic tissue, respectively. The results show that when significant differences occurred, callus was always initiated more frequently on MSG medium. The large amount of scatter in the data was due only in part to the pooling of the data for all clones in a given collection in Tables 6 and 7. Invariably, in each collection one or more clones would be absolutely unresponsive to the treatments. However, from collection to collection, the particular clones that were unresponsive varied. Therefore, statistical variation was not minimized by elimination of a particularly unresponsive clone. In all likelihood, the sources of the variation were differences in the rate of explant development between clones (and between seeds within a clone) and did not necessarily reflect a genotype influence on the in vitro competence of the material toward callus formation. Bearing the above discussion in mind, it appears that high ammonium media formulations are not as conducive to callus initiation as formulations devoid of ammonium but supplemented with glutamine. Comparisons between MSG and MS were not made with embryo explants, in part due to the results obtained with nucellar and gametophyte explants but also due to the poor initiation frequencies from embryo explants observed with high ammonium formulations in 1985. In the collection P010 (1st wk, July), several gametophytic explants responded by formation of a white to translucent, mucilaginous callus similar in appearance to embryogenic spruce callus, except that somatic embryos have not been observed. Two of these initiation events (from MSG 2/1 and 10/2) have been proliferated to the extent that

callus lines (P010 Ag) 1 and 2 have been established. In these lines, embryogenic potential has been suggested by

1. Requisite visual and tactile markers
2. Low total reductants test (see p. 102)
3. Auxin-autotrophic growth

The latter observation has become so pervasive in embryogenic conifer systems that it can almost be considered as another marker. Embryogenic callus of larch (also derived from gametophytic tissue) exhibits sustained embryogenesis in the absence of exogenous auxin. Radiata pine (D. Smith, personal communication, 1985) and white pine (this report) likewise exhibit sustained embryogenesis without the addition of auxin. In (P010 Ag) 1 and 2 auxin-autotropic growth has been maintained for 14 weeks (to date) and the rate of growth is indistinguishable from that on media with growth regulator supplements. Currently, marker evaluation and attempts to induce embryo formation are under way with this material.

Table 6. Comparison of MSG vs. MS medium for callus initiation from nucellar explants.

Collection	Clones	Callus Initiation, % MSG 2/2	Callus Initiation, % MS 2/2
LP3	A-E	9 ± 7	1 ± 2
LP3[a]	F-J	13 ± 3	2 ± 4
P04	A-D	22 ± 16	17 ± 18
PP3	A-B	23 ± 18	4 ± 1
DF1	A-E	13 ± 25	3 ± 5

[a]Indicates significant differences ($p = 0.05$) between media by ANOVA.

Table 7. Comparison of MSG vs. MS medium for callus initiation from gametophytic explants.

Collection	Clones	Callus Initiation, % MSG 10/2	MS 10/2
LP9[a]	F-J	20 ± 16	1 ± 2
LP10	A-E	25 ± 18	3 ± 6
PO9	A-D	21 ± 25	2 ± 4
PO10[a]	A-D	46 ± 25	12 ± 10
PP5	A-B	75 ± 28	40 ± 33
PP6[a]	A-B	60 ± 6	14 ± 8
DF5	A-E	12 ± 12	6 ± 7
DF6	A-E	38 ± 46	9 ± 11

[a]Indicates significant differences ($p = 0.05$) between media by ANOVA.

Smith Protocol. Results from application of the Smith protocol to southern pines are shown in Tables 8-10. The embryogenic phenotype tabulated in Tables 8-10 appeared as a white to translucent mucilaginous callus that extruded from the archegoneal end of the gametophyte within 3-6 weeks of culture initiation (see Fig. 10). In loblolly and pitch pine rapid initial growth has not been maintained and no lines have been established. However, in pond pine rapid sustained callus growth from some of the initiation events has resulted in the establishment of four callus lines.

In agreement with the results in radiata pine communicated by Smith, the frequency at which this "extruded callus" is formed is dependent on the stage of embryo development. Collections from which the highest frequency of extruded callus was obtained were those in which proembryos were present within the gametophyte. The evidence for the presence of proembryos is poor seedling

Table 8. Callus initiation in loblolly pine by the method of Smith.

Collection	Date	Initiation Frequency, %	Germination, %[a]
LP11	7/10	8	0
LP12	7/17	8	0
LP13	7/24	12	16
LP14	7/31	6	28
LP15	8/7	6	53
LP16	8/14	0	69

[a]Efficiency at which excised embryos cultured on MSG 0/0 developed into seedlings.

Table 9. Callus initiation in pond pine by the method of Smith.

Collection	Date	Initiation Frequency, %	Germination, %[a]
PO11	7/11	12	0
PO12	7/18	2	8
PO13	7/25	5	68
PO14	8/1	23	96
PO15	8/8	1	--

[a]Efficiency at which excised embryos cultured on MSG 0/0 developed into seedlings.

Table 10. Callus initiation in pitch pine by the method of Smith.

Collection	Date	Initiation Frequency, %	Germination, %[a]
PP9	6/30	10	0
PP10	7/7	0	0
PP11	7/14	--	--
PP12	7/21	0	89
PP13	7/28	0	100

germination in the embryo rescue studies performed concomitantly (see Tables 8-10). As embryos matured within the seed (and embryo rescue was possible) the initiation frequency of extruded callus dropped in all species except pond pine.

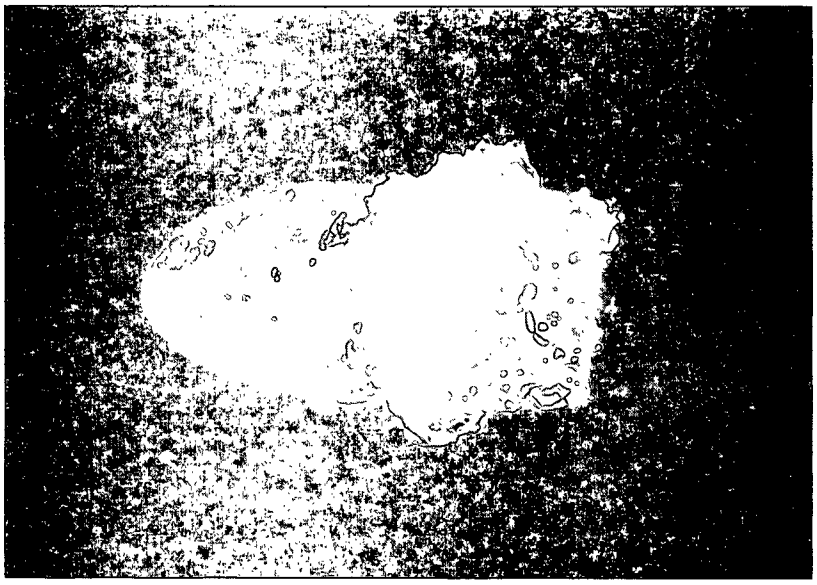

Figure 10. White mucilaginous callus extruded from loblolly pine gametophyte (LP12F; 15X).

The four callus lines obtained in pond pine represent a unique material in that the lines have never been exposed to exogenous growth regulators, and like pond pine gametophytic callus lines, they maintain vigorous growth. Initiation on growth regulator-free medium argues against any type of auxin habituation as being responsible for the autotrophic growth, which cannot be ruled out as the reason behind auxin-autotrophy in the gametophyte derived callus lines. Initiation of extruded callus was also fairly insensitive to genotype, as most clones produced extruded callus. For this reason, the results in Tables 8-10 represent the combined initiation frequency for all clones in a given collection.

To determine the origin of extruded callus, gametophytes were slit open at the time when the callus was isolated from the gametophyte for subculture. In all cases the primary embryo had either developed or atrophied to a significant degree, and callus proliferation was confined to near the suspensor region. In lieu of detailed study, it can only be tentatively stated that the extruded callus appears to arise from embryo suspensors or gametophytes, but not from tissues present in the embryo proper.

Histological examination of the extruded callus revealed a mixture of unaggregated suspensorlike cells and cytoplasmically filled cell clumps that resembled the globular stage of angiosperm embryogenesis (see Fig. 11).

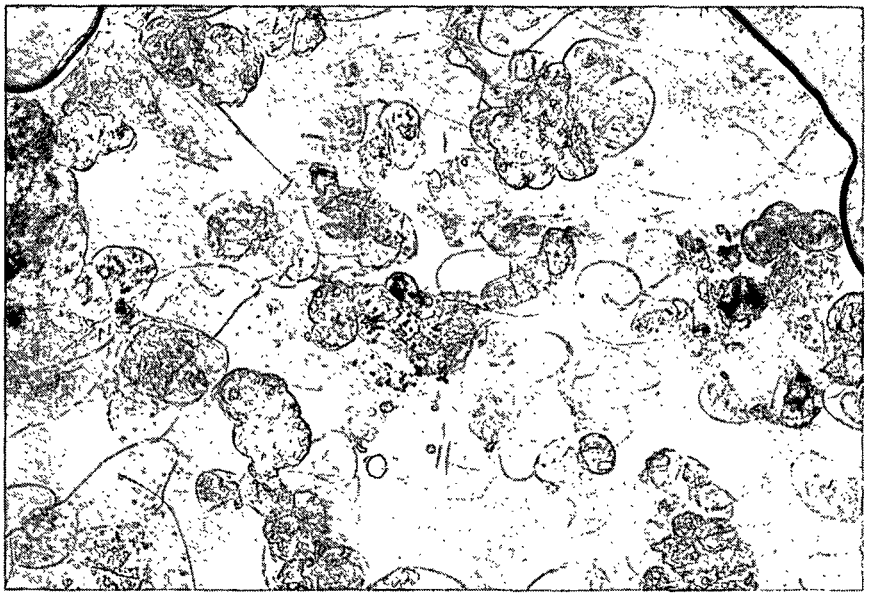

Figure 11. Globular cell masses in extruded callus of pond pine (P012 Ao)1 (200X).

The majority of the globular structures appeared similar to somatic embryos lacking suspensors. In a very small percentage of the callus, suspensor-like regions appeared to be developing (see Fig. 12 and 13). Efforts are underway to characterize this material biochemically and to induce embryo formation.

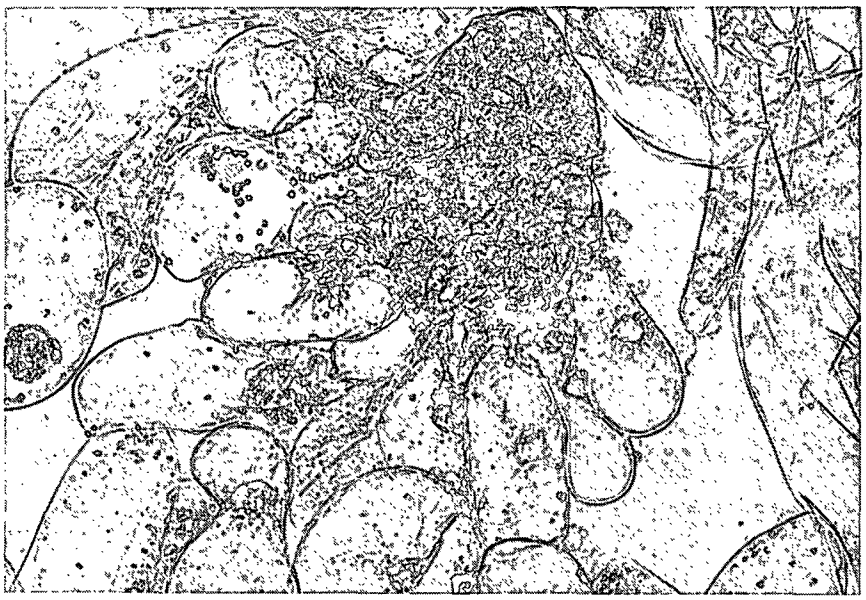

Figure 12. Embryolike structure in pond pine extruded callus (P012 Ao) (200X).

Figure 13. Embryolike structures in pond pine extruded callus (P012 Ao)(200X).

Somatic Embryogenesis in White Pine. Attempts to apply the protocol for somatic embryogenesis in sugar pine[11] to white pine were successful. Aside from verifying the published procedure for another five-needled pine, we examined additional factors affecting initiation of embryogenic tissue. These factors were developmental stage, culture medium, auxin concentration, and light vs. dark incubation conditions.

Experimental design precluded examining interactions among the above factors, but important results were obtained that could lead to a protocol for extending somatic embryogenesis to other pines. To highlight critical features in white pine, the above four factors will be considered consecutively.

Developmental Stage of the Explant. Table 11 shows quantitatively the course of embryo development in the two white pine clones (A and B) obtained near Wooster, OH. Although the size data and presence of cotyledons were not recorded in the white pine clones collected near Freedom, WI (clones I, II, III), the embryos were nominally of the same stage of development as the Ohio clones (and are thus grouped together with the Ohio clones in Tables 12 and 13). In white pine, as in other conifers, soon after the cotyledonary stage has been reached near the end of July (WP5), there is very little increase in embryo length. By the middle of July (WP3) nearly all immature embryos of all clones had cotyledons. If white pine embryogenesis were analogous to spruce, the period at or near the time of completed cotyledonary development for all explants (equivalent to WP3 and 4 in Table 11) would be optimum for initiation. However, in Tables 12 and 13 the earlier, precotyledonary embryos (WP1 and 2) were the best explants for initiation. Especially noteworthy was initiation of embryogenic tissue from proembryos in WP1 where cotyledon development was completely lacking (Table 12).

Table 11. Effect of medium and stage of embryo development on initiation of embryogenic tissue in white pine (cultured under 16/8 photoperiod).

Collection, Date	Clone	Embryo Length, mm	Embryos with Cotyledons, %	Initiation Frequency, %[a]	
				MSG 2/1	HM 2/1
WP1	A	0.6 ± 0.2	0	0	0
(7/2)	B	0.4 ± 0.2	0	0	0
WP2	A	1.1 ± 0.6	35	0	0
(7/9)	B	0.7 ± 0.2	11	0	0
WP3	A	3.2 ± 1.2	95	0	0
(7/16)	B	1.9 ± 0.7	95	0	2
WP4	A	5.3 ± 0.6	100	0	0
(7/23)	B	4.8 ± 0.7	100	0	0
WP5	A	5.8 ± 0.4	100	0	0
(7/30)	B	5.6 ± 0.3	100	0	0

[a]40-50 explants/treatment.

Table 12. Effect of medium and stage of embryo development on initiation of embryogenic tissue in white pine (cultured in the dark).

Collection	Clone	Initiation Frequency, %[a]		
		MSG 2/1	DCR 3/0.5	DCR 3/0
WP1	I	--	0	--
	II	--	8	--
	III	--	0	--
WP2	A	0	0	0
	B	3	0	0
	I	0	0	0
	II	3	6	0
	III	3	5	0
WP3	A	0	0	0
	B	0	0	0
	I	0	0	0
	II	0	0	0
	III	0	0	0

[a]15-30 explants/treatment.

Table 13. Effect of medium and auxin level on the initiation of embryogenic tissue in white pine (WP2; culture in the dark).

	Initiation Frequency, %					
	MSG		DCR			
Clone	2/1	10/1	3/0	0.1/0.5	3/0.5	10/0.5
B	3	0	0	--	0	0
I	0	--	0	0	0	0
II	3	--	0	0	6	0
III	3	--	0	0	5	0

Effect of Culture Medium. Three media were employed for initiation studies in white pine. Although considerable variation exists in both the macro and microelements of these media, the major changes were in nitrogen composition. In particular, HM is a high ammonium ion medium (1200 mg/L), DCR is a low ammonium medium (400 mg/L) and MSG is devoid of ammonium ion. To replace the ammonium ion, MSG is supplemented with an organic form of reduced nitrogen (glutamine, 1400 mg/L), whereas DCR contains both glutamine (500 mg/L) and casein hydrolyzate (500 mg/L). The results indicate that in the dark the low ammonium medium (DCR) and the medium devoid of ammonium (MSG) were about equally adept at initiating embryogenic callus. The high ammonium medium (HM), which was only used in the light, initiated embryogenic tissue in only 1 embryo out of the 47 cultured in WP3 (Table 11). In spruce, it may be recalled, the culture of immature embryos from the equivalent of WP3 on HM 2/1 in the light was precisely the condition required for high frequency initiation of embryogenic tissue. Therefore, with regard to three critical factors influencing the initiation of embryogenic tissue, (developmental stage, medium composition, and light) spruce and pine appear to have substantially different requirements.

Effect of Auxin Concentration. Table 13 shows the effect of varying the 2,4-D concentration within the MSG and DCR media on initiation of embryogenic

tissue in white pine. In a single experiment with clone B, MSG 2/1 would support the induction of embryogenic callus, whereas MSG 10/1 would not (Table 13). On DCR medium, there appears to exist an optimum 2,4-D concentration that is greater than 0.1 mg/L but less than 10 mg/L, as no embryogenic tissue was initiated at either extreme (Table 13). It should also be noted that a cytokinin supplement (0.5 mg/L) is also required inasmuch as no embryogenic callus was initiated under cytokinin-free conditions (DCR 3/0). Curiously, it was DCR 3/0 that was reported by Gupta and Durzan as the hormonal composition initiating embryogenesis in sugar pine.[11] However, DCR 3/0.5 was the growth regulator regime reported in their poster abstract at the IAPTC meeting, Minneapolis, MN, 1986. In regard to growth regulator regimes, white pine appears similar to spruce in that 2,4-D and BA in combination initiate embyrogenesis.

Effect of Light. Embryogenic tissue was initiated quite readily, albeit at a low frequency in the dark. Although a replicated study on the effect of light was not performed, reasonable comparison can be made between Clone B on MSG 2/1 in the dark (3%, Tables 12 and 13) and no initiation in the light on MSG 2/1 (Table 11). It should also be noted that all the initiation studies performed in the light were done on fresh material, whereas all dark initiations were performed on cold stored material. Although cold storage exerted no significant effect on initiation frequencies in spruce (Table 2), this has not yet been verified in pine.

The above discussion has served to highlight some similarities and differences between spruce and pine somatic embryogenesis. Considering that spruce and pine depart from each other in many features of the initiation process, it is not unexpected that differences in the maintenance and embryo development stages exist as well.

Embryogenic white pine callus is similar to embryogenic spruce callus in that it exhibits the white to translucent, mucilaginous phenotype. However, in one important aspect the appearance of embryogenic pine callus is different than that of spruce. When grown under proliferative conditions, somatic embryos in spruce callus undergo a measure of development that allows clear discernment of the embryogenic nature of the callus. In white pine this is not the case. While whole mount preparations show easily recognizable somatic embryos (see Fig. 14 and 15), these embryos undergo very little development on any of the media used to proliferate the callus. The extent of development is so small that embryogenic pine callus appears to be dominated by small dense cell clusters with an appearance similar to globular embryos in angiosperms (see Fig. 16). Cell clusters such as these might be embryos in which suspensor development is lacking.

Figure 14. White pine proembryo (79X).

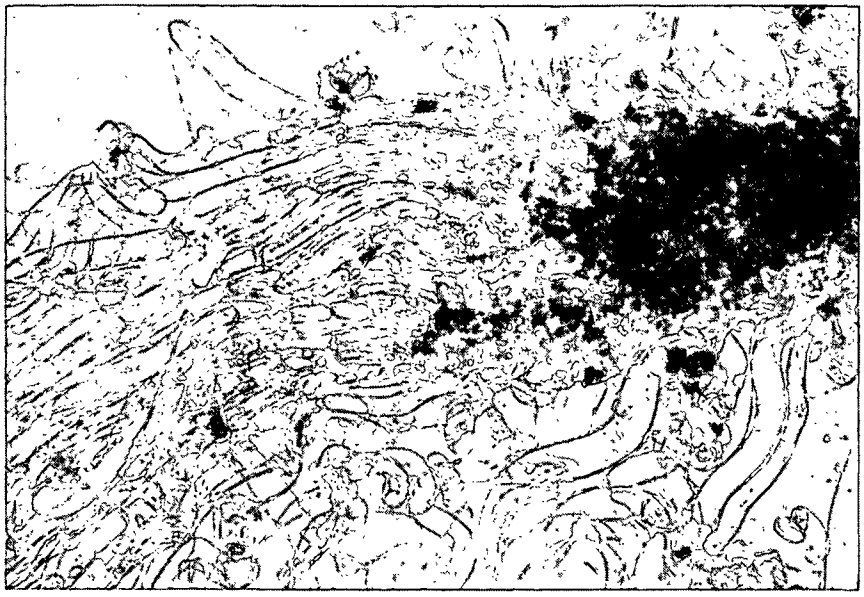

Figure 15. White pine proembryo (31X).

As noted above, very little development has occurred on any of the media to date. However, callus can be easily maintained in an embryogenic state. It appears that while a medium supplemented with 2,4-D and BA is required for initiation, this growth regulator regime may not be the best for maintenance. In particular, embryogenic white pine callus appears to proliferate best in the dark under auxin-free conditions supplemented with BA (DCR 0/0.2). Should auxin-free proliferation continue (as it appears it will), this would make the third embryogenic conifer system (along with larch and radiata pine) that continually undergoes repetitive embryogenesis in the absence of auxin.

Conclusions

For the first time, embryogenesis in a member of the genus Pinus has been achieved in this laboratory. The embryogenic callus phenotype was easily

recognized in white pine due to the past experience in spruce. Aside from the similarity in appearance and texture of the callus in these two genera, factors influencing both the initiation and maintenance of an embryogenic state were markedly dissimilar. When results with white pine are coupled with those from the Smith protocol and the conceptual summer plan, a well defined pathway emerges toward obtaining embryogenesis in loblolly pine and Douglas-fir.

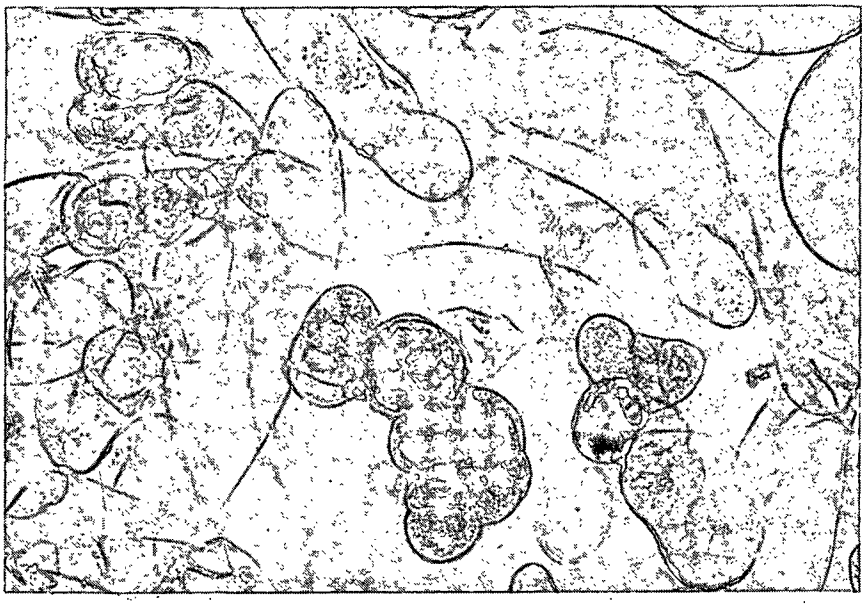

Figure 16. Proembryonic cell clusters of white pine (200X).

The results from the conceptual summer plan verified that windows of morphogenic potential are present for each of the major explants (nucellus, gametophyte, embryo) examined. Later stage, presumably fertilized gametophytes represent an explant that may still hold promise for the initiation of embryogenic callus. Proembryos were again the most difficult explants to initiate callus, but initiation frequencies (of nonembryogenic callus) improved with maturity. Embryo maturation stages equivalent to the optimum for embryogenic callus initiation in spruce proved inappropriate for pine.

Results from the Smith protocol and white pine suggest a reason for the inappropriateness of immature embryo explants in pine by pointing to the suspensor region of the embryo as the critical explant. Suspensor tissue appears to be most responsive to the *in vitro* environment (i.e., window) during the period of time from fertilization to a time coincident with the appearence of cotyledons on the embryos. In light of the above, the Smith protocol can be understood to provide the requisite nutritional and hormonal environment to the proembryo (via the gametophyte) for proliferation of embryogenic callus from the suspensor region. In white pine, the same result can be achieved with proembryos (sans gametophyte) with a combination of darkness, balanced nitrogen nutrition, and exogenous plant growth regulators.

Taken together, the results from all of the initiation studies in 1986 provide a "recipe" for extending somatic embryogenesis to all pines. However, embryo development and establishment of free-living plants will likely remain as the greatest stumbling block in the utilization of this technology, as has been the case in all reports of somatic embryogenesis in woody species.[15] Toward further application of this technology, the extension of the technique to mature explants would greatly increase the practicality of somatic embryogenesis. In spruce, where embryogenic tissue arises from hypocotyl epidermal or subepidermal cells,[12] it does not seem altogether unlikely that a similar explant type could be found in a mature tree. However, in pines the challenge to extend embryogenesis to mature tissues may be greater still due to the unique degree of specialization in suspensor cells compared to hypocotyl epidermis. Fortunately, it appears that the embryogenic state in conifers will be similar regardless of the explant type. The ability to recognize this state and foster its growth and development may enhance the possibility of extending embryogenesis to mature tissue.

Characterizing in vivo Embryology in Loblolly Pine

Introduction

According to the terminology used by Dogra[18] embryology refers to both pre- and postfertilization stages of seed development. In 1986 all of our effort to initiate embryogenic callus in loblolly pine utilized explants derived from developing seeds. This included both prefertilization tissues (nucellus and early gametophytic tissue) and postfertilization tissues (gametophytes containing proembryos and subsequently immature embryos).

Of the explant tissues used in this study, nucellar tissue is the explant of choice for clonal forestry because it is of maternal cellular origin and diploid. It has proven to be an effective embryogenic explant in numerous woody plants. Gametophytic tissue is maternal in cellular origin and haploid. It has proven to be an effective explant for induction of embryogenic callus in European larch.[16] The developing embryo results from gametic fusion and is therefore diploid, but is not genetically equivalent to the maternal tree. It has proven to be a very effective explant for initiation of embryogenic conifer callus.[1,11,12]

The purpose of this study was to characterize in vivo development of the seed tissues used as explants so as to develop indices or practical markers of explant development, and to correlate specific developmental stages to the in vitro response of explants. The results presented here track the time course of three potential markers of development: cone length, female gametophyte length, and embryo length in five loblolly pine families. During embryo development the time of cotyledonary primordia initiation was also determined. In addition, micrographs were prepared, mainly from loblolly pine family 11-10, to characterize anatomical development of the explant tissues in relation to measured parameters.

Methods

The female cones used in this study were collected from the Westvaco Corp. seed orchard in Summerville, SC between April 7 and August 18, 1986. Cones were received at IPC within 24 h of collection. Cone length was measured on 7 to 10 cones of each family prior to seed extraction. Families studied were (IPC code, in parentheses) 7-34 (F), 7-56 (G), 11-9 (H), 11-10 (I), and 11-16 (J). The length of female gametophytes and immature embryos was measured at the time of culture initiation using a dissecting microscope with eye-piece micrometer. The presence or absence of cotyledonary primordia was also determined at culture initiation on immature embryos by viewing with a dissecting microscope.

Samples of tissues prepared for light microscopy were treated with 1) glutaraldehyde and 2) osmium tetroxide fixatives[19] followed by dehydration in a five step graded series using acetone. Dehydrated materials were impregnated with and mounted in Spurr resin[20] and sectioned on a Sorvall ultramicrotome at approximately 5 µm thickness. With some of the larger fragile tissues, the use of Farmer's fixative, tributyl alcohol dehydration and paraffin impregnation prior to sectioning at 7-8 µm on an American Optical rotary microtome gave better results. All are widely practiced sectioning techniques.[21] Sections were fixed on glass microscope slides and stained with toluidine blue[21] prior to examination.

Results and Discussion

A schematic diagram of the tissues of the developing seed structure of loblolly pine at the time of fertilization is shown in Fig. 17. All micrographs shown in this study are oriented as shown in Fig. 17, i.e., with the micropylar end of the developing seed tissue to the top and the chalazal end to the bottom. These directions correspond to the root radicle and cotyledonary ends of the developing embryo, respectively.

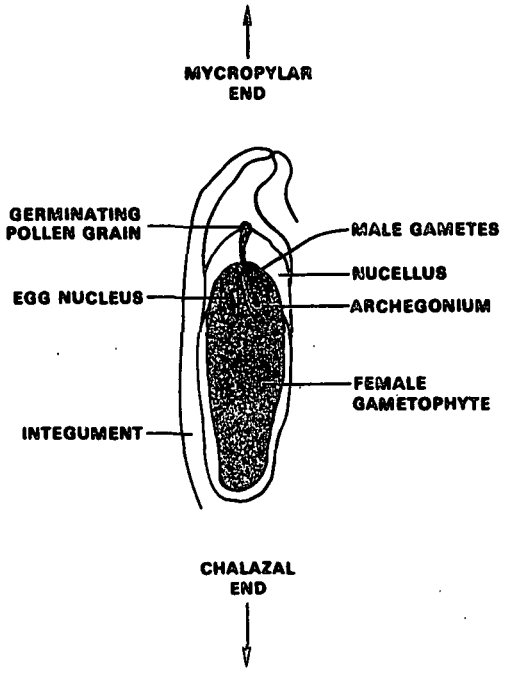

Figure 17. Schematic diagram of tissues of the developing seed of loblolly pine at the time of fertilization. The haploid tissues are shaded.

A series of micrographs on stages of in vivo development of loblolly pine seed tissues are shown in Fig. 18. In Pinus seed development extends over a two year period. Pollination occurs in early spring (March) of the first year. Figure 18a shows nucellar tissue surrounded by integument soon after the time of pollination. Note the pollen grain visible in the upper region of the nucellus. Nucellar explants as shown in Fig. 18a cultured from first year cones did not form embryogenic callus. All of the remaining tissues studied were derived from second year cones, and anatomical sections of these tissues are shown in Fig. 18b-i. Nucellar tissue derived from second year cones is shown in Fig. 18b. Note that this section contains evidence of a germinating pollen grain. An example of the female gametophyte tissue with several archegonia is shown in Fig. 18c. This sample was collected on June 9. The first microscopic section

to contain evidence of suspensor-embryo development is shown in Fig. 18d. Note the prominent erosion cavity of the gametophyte and the presence of dark stained suspensor tissue within the erosion cavity. The plane of this section did not contain all of the developing suspensor-embryo complex due to its coiled nature. The elongated and coiled nature of the developing suspensor tissue is more clearly shown in Fig. 18e. The earliest section of family 11-10 to show enlargement of the embryonal mass of the zygotic embryo is shown in Fig. 18f. Figure 18g shows a precotyledonary embryo of family 11-10 collected on Aug. 18. Considerable differences in the timing of embryo development between families is evident by comparing the family 11-9 embryo collected July 18 (Fig. 18h) and the family 11-10 embryo also collected July 18 (Fig. 18f). Note that the initial development of cotyledonary primordia are visible in the 11-9 embryo (Fig. 18h), whereas embryos of family 11-10 (Fig. 18f) were much smaller and precotyledonary on the same collection date. The most mature explants showed cotyledons, evidence of internal vascular development, and root and shoot meristems (Fig. 18i).

The length of cones from five loblolly pine families was determined from April 7 to August 25, 1986 (Fig. 19). Cone length as a function of time followed

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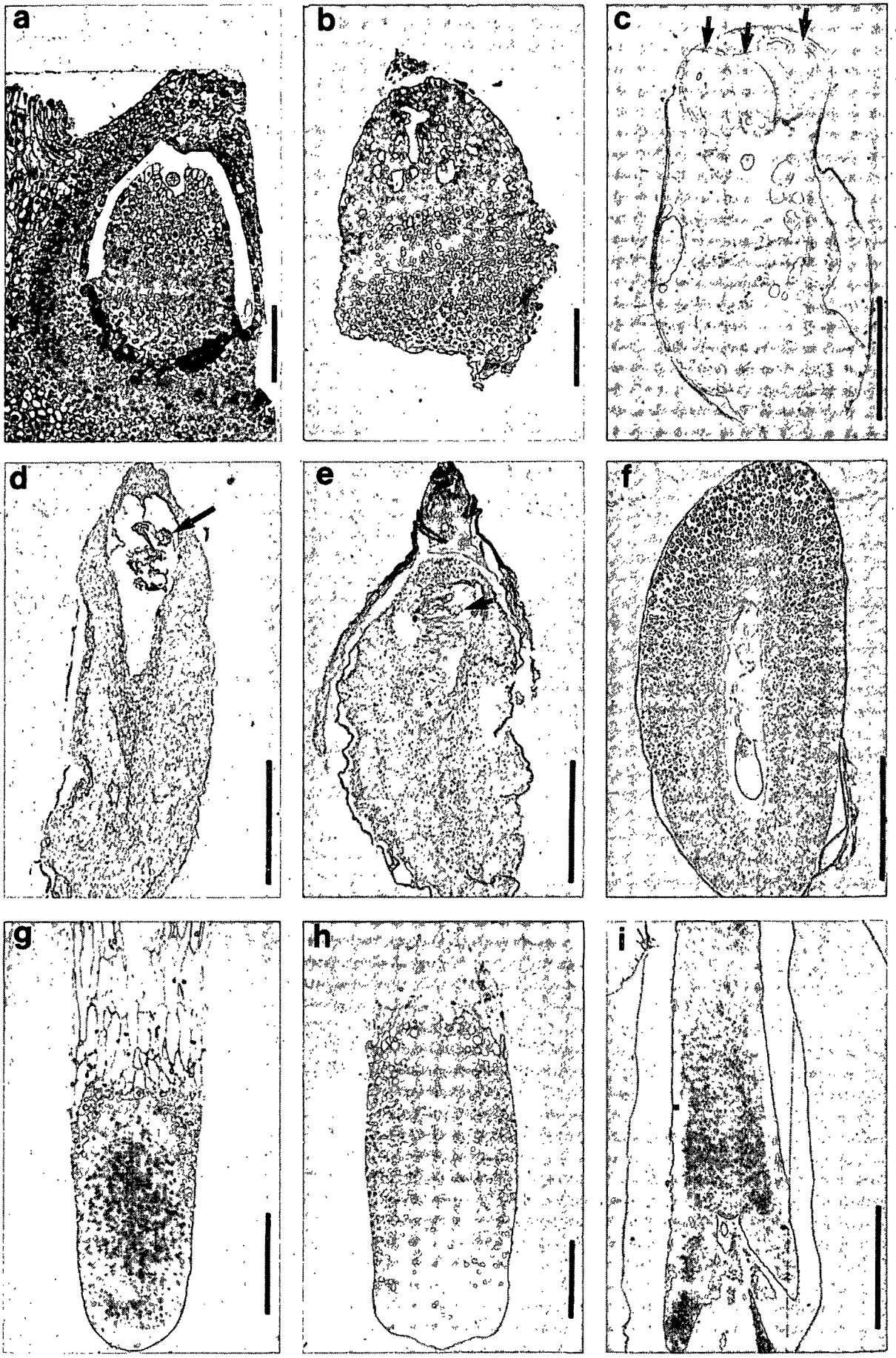
Figure 18. Embryology in loblolly pine. All micrographs are of family 11-10 except h which is of family 11-9. All micrographs are of tissues from second year cones, except a which is from first year cone. Collection dates of samples are in brackets. a. Nucellus tissue enclosed within integument [May 5, 1986]. b. Isolated nucellar tissue [April 21, 1986]. c. Female gametophyte with several archegonia (arrows) [June 9, 1986]. d. Female gametophyte with embryo suspensor tissue (arrow) within erosion cavity [July 7, 1986]. e. Suspensor tissue within erosion cavity of female gametophyte. Note deteriorating nucellus tissue attached [July 14, 1986]. f. Embryo within female gametophyte [July 28, 1986]. g. Precotyledonary embryo [August 11, 1986]. h. Embryo with cotyledonary primordia, shoot apex, and vascular development [July 28, 1986]. i. Embryo with cotyledons, shoot and root apices, and vascular development [August 25, 1986]. Scale bars in c-f and i are 1 mm. Scale bars in a, b, g, and h are 250 µ.


a
b
c
d
e
f
g
h
i

a typical sigmoidal growth curve. The exponential increase in cone length occurred from late April through the end of May. During this period we cultured nucellar tissue (as Fig. 18b). During the period in which we cultured female gametophytes (June 2 to June 30) the rate of increase in cone length declined. During the period in which we cultured embryos as explants (July 7 to August 25) there was very little increase in cone length, with mean lengths ranging from about 9 to 11 cm among the families studied. It was not feasible to culture large numbers of isolated embryo explants prior to July 7. The embryo-suspensor complex was extremely small and delicate prior to this date. In retrospect one can deduce that embryo development, namely, increase in embryo size to a stage practical for isolated culture, occurred during the stationary phase of cone development.

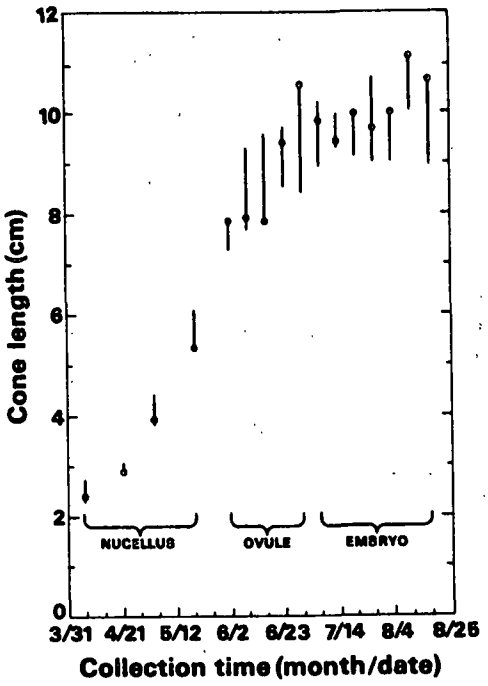


Figure 19. Cone length as a function of collection time for five loblolly pine families (7-34, 7-56, 11-9, 11-10, and 11-16). The solid circles are cone lengths for family 11-10 on which detailed anatomical studies were conducted. The bars indicate the range of cone lengths across all five families. The time period is also shown in which seed tissues (nucellus, ovule, and embryo) derived from cones were used as explants.

More time and effort would be needed to precisely determine the time of fertilization. Based on the micrographs of family 11-10 it occurred between June 9 (Fig. 18c) and July 7 (Fig. 18d). Thus, relative to cone length, fertilization occurred prior to the stationary phase of cone development. Although a review of literature on cone development in conifers relative to stages of embryology has not been completed, reference to the relationship of cone size and time of fertilization has been found.[22] Specifically, Buchholz stated that "fertilization usually occurs at about the time (or only shortly before) the cones have reached their maximum size." Our results are in complete agreement with this statement.

Mean lengths of female gametophytes was also determined (Fig. 20), and ranged from 2.5 mm on June 2 to 4.0 mm on June 30 (averaged across all five families). It was not feasible to culture earlier female gametophytes because they ruptured when removed from the nucellar tissue. There was a linear relationship (R = 0.80) between cone length and female gametophyte length in loblolly pine (Fig. 21). The results in Fig. 21 are based on a regression analysis of pooled data from all families. The results of regression analysis of cone length vs. female gametophyte length of individual families are presented in Table 14. The best linear relationship of cone to female gametophyte length was with family 11-16. These results, although preliminary, suggest the potential of correlating specific developmental stages, e.g., time of fertilization, to easily measured parameters such as cone length. Athough we did not measure specific gravity of cones during 1986, an effort will be made to do so in 1987. Possibly this may be an even more precise marker of cone development with less seasonal variation. Possibly this may be an even more precise marker of cone development with less seasonal variation.

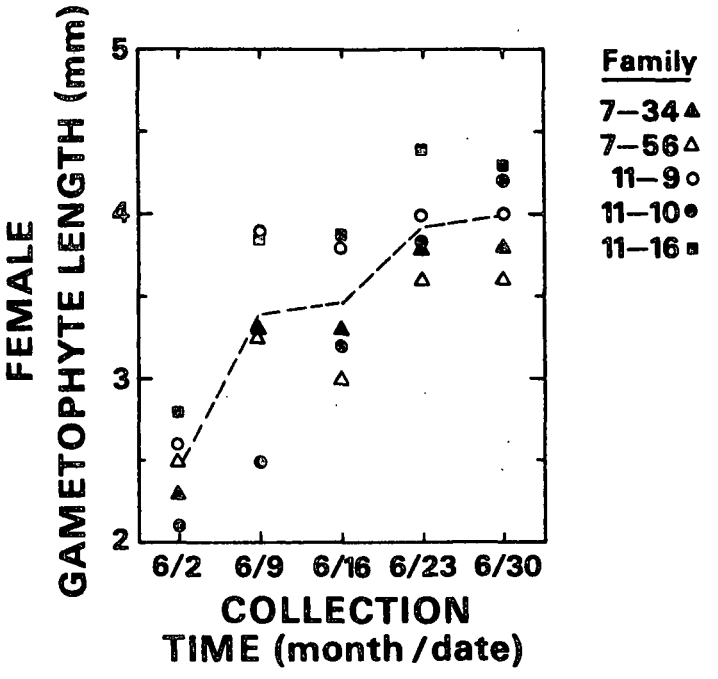


Figure 20. Female gametophyte length as a function of collection time for five loblolly pine families. The dashed line is the mean length across families.

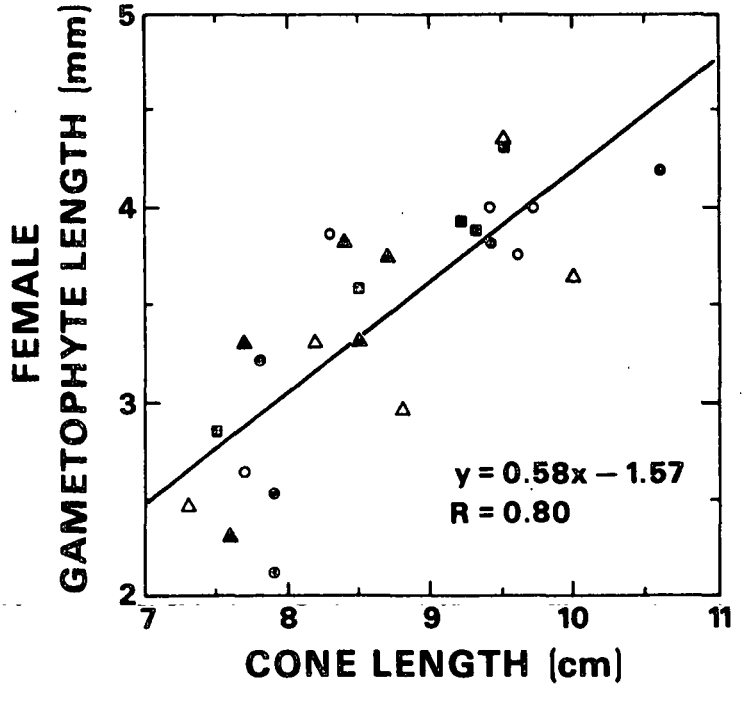


Figure 21. The relationship between cone length and female gametophyte length in loblolly pine. The data points are means across the five families collected between June 2 and 30, 1986. Refer to Fig. 20 for identification of family symbols.

Table 14. Relationship of cone length to female gametophyte length for five loblolly pine families.

Family (IPC Code)	Regression Line[a]	Correlation Coefficient
7-34 (F)	Y = 0.94X - 4.37	0.77
7-56 (G)	Y = 0.53X - 1.30	0.79
11-9 (H)	Y = 0.51X - 0.91	0.79
11-10 (I)	Y = 0.60X - 2.04	0.86
11-16 (J)	Y = 0.65X - 2.04	0.98

[a]Regression line where X is cone length and Y female gametophyte length.

The results of tracking development of loblolly pine embryos are shown in Fig. 22. The embryo explants cultured on July 7 and 14 were at a proembryonal stage, with numerous cleavage embryos attached to a massive suspensor system. The first measurable differences among families in embryo length occurred on July 21, 1986 (Fig. 22b). These differences persisted during the remainder of the period of the study. For instance, family 11-10 lagged behind other families in embryo development and had significantly smaller embryos than families 11-9 and 11-16. Similarly, development of cotyledonary primordia of family 11-10 lagged behind families 11-9 and 11-16 (Fig. 22a).

Comparing callus initiation results (refer to Fig. 6 and Table 8) to *in vivo* seed tissue development yields the following conclusions. First, the largest female gametophytes (ovules) collected after June 23 initiated callus (nonembryogenic) at least twice as frequently as earlier collections (Fig. 6). This increased *in vitro* response corresponds to female gametophyte lengths of 4

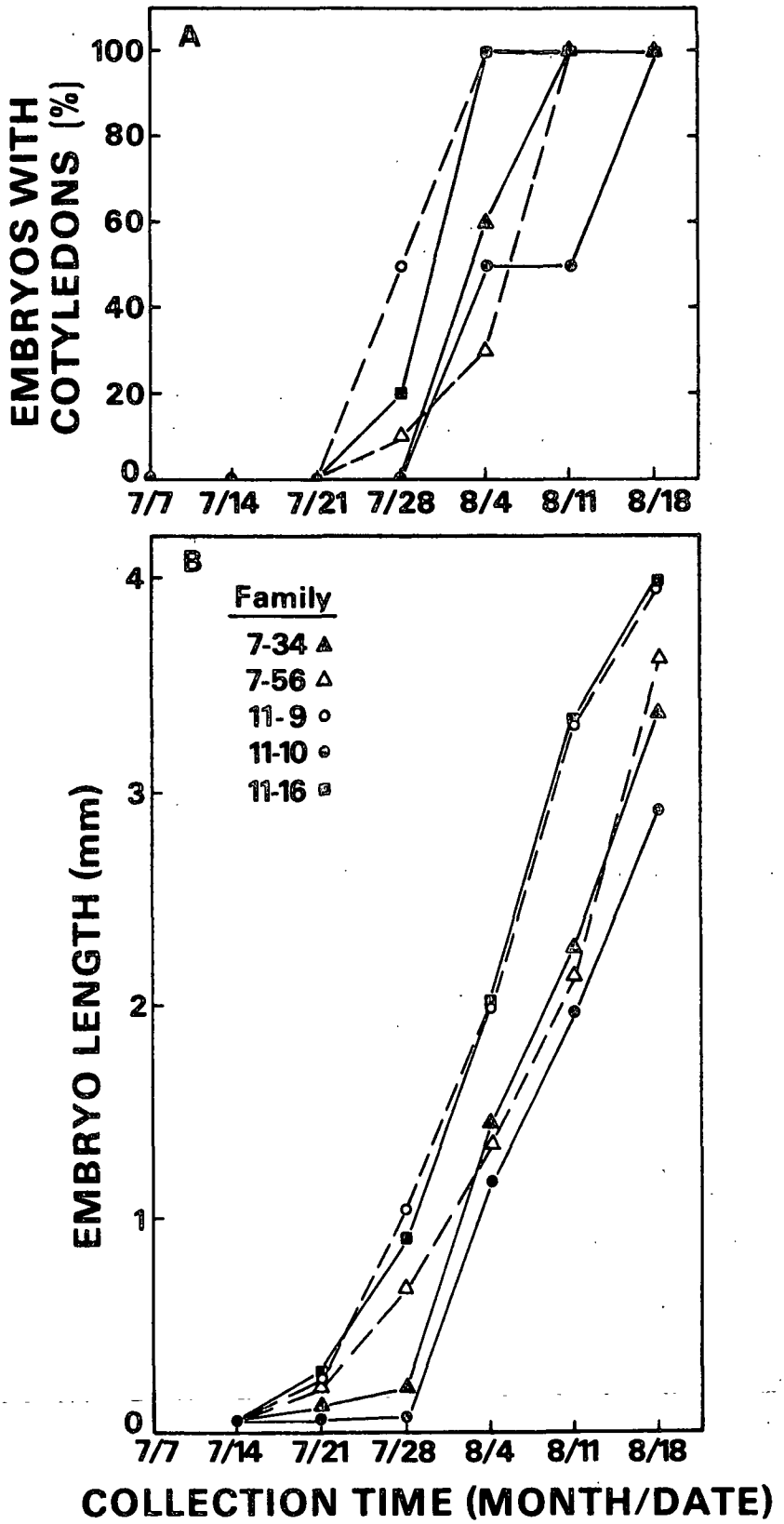


Figure 22. The stage of development of immature embryos as a function of collection time for five loblolly pine families. A: Percentage of embryos with cotyledonary primordia development. B: Embryo length.

mm (family mean) (Fig. 20), and mean cone lengths of 9 to 10 cm (Fig. 19). Second, embryo explants collected after July 28 initiated callus (although nonembryogenic) at higher frequencies than smaller embryos collected earlier (Fig. 6). On July 28 mean embryo lengths of all families were less than 1.0 mm (Fig. 22b) and a majority of the embryos were precotyledonary (Fig. 22a). After July 28 (August 4 to 18) mean embryo lengths ranged from 1 to 4 mm and a majority of the embryos had developing cotyledonary primordia. These two conclusions indicate the importance of developmental windows for initiation of in vitro response. A third and most significant conclusion can be made in relation to initiation of the white mucilaginous (embryogenic type) callus (Table 8). This callus type was initiated at highest frequencies prior to the July 31 and August 4 collections (LP14). This corresponds to when embryos were less than 1 mm in length and mostly precotyledonary (Fig. 22).

In conclusion, these results emphasize the importance and need of a thorough understanding of the stages and timing of explant development for successful initiation of specific types of callus from developing loblolly pine seeds. Perhaps the most potentially useful parameter was cone length and its relationship to gametophyte length. If the results presented here can be repeated with future collections, it may be possible to precisely define the optimum "window" for initiation based on easily measured parameters of cone development. The determination of embryo length was also useful in demonstrating that the most responsive embryos were precotyledonary and less than 1 mm in length. The ease and rapidity with which explant and cone parameters can be measured suggests that further studies are needed to determine their utility as predictive markers of embryogenic potential.

INITIATION FROM MATURE EMBRYOS

Cultures from Mature Embryos of Norway Spruce, Douglas-fir and Loblolly Pine

Introduction

Following reports of somatic embryogenesis from immature seed tissues,[1,3,16] our research has focused on the initiation of embryogenic lines from ovules and immature embryos of developing female cones. Studies from these tissues are necessarily restricted by time, being dependent on stages of embryo development and cycles of cone production. Induction of embryogenic callus from embryos excised from mature, fully developed seeds would provide a "timeless" and unlimited source of explant material. Toward this end, we have attempted to initiate embryogenic lines using mature embryo explants from Norway spruce, our model system, as well as from loblolly pine and Douglas-fir.

Repeating the protocol of Hakman et al.,[1] last year we initiated somatic embryogenesis from Norway spruce immature embryos. Following the same procedure with minor variations (Table 15), mature embryos produced very limited results. From one trial only, 3% of the Norway spruce mature embryos produced embryogenic callus, but embryo development was not achieved. This year we achieved somatic embryogenesis and plantlet regeneration from Norway spruce mature embryos. Although this was also reported by other researchers this year,[13,23] our method was arrived at independently, and differs both in media and culture conditions.

Methods and Materials

Loblolly pine, Douglas-fir and Norway spruce seeds were obtained from commercial seed companies and stored frozen or refrigerated (NS) until used. All seeds were surface sterilized and imbibed for approximately 12 hours prior to

excision. Table 16 summarizes the current attempts to initiate embryogenic tissue from excised embryos of mature seeds. The initiation of Norway spruce somatic embryos is described in greater detail.

Table 15. Culture method and conditions used in initial attempts to induce embryogenic callus from mature embryos of Norway spruce.

Culture Method/Condition	Medium Supplements, mg/L[a]	
Whole embryos		
1. Standard[b]	2	2,4-D
2. Dark	2	2,4-D
3. On embryogenic callus	2	2,4-D
4. On filter paper rafts placed on embryogenic callus	2	2,4-D
5. Standard	2	2,4-D; 100-1900 NO_3^-; 0-1000 NH_4^+; ± 1500 Glu
6. Standard	2	2,4-D - amino acids; ± 400 Glu
7. Standard	1	NAA
Sectioned embryos (cots, hypocots, radicle)		
1. Standard	2	2,4-D
2. Standard	2	2,4-D; 10-1900 NO_3^-; 0-100 NH_4^+; ± 1500 Glu

[a]Basal medium = HM + 1 mg/L BA
[b]Standard culture method refers to physical environment after Hakman et al.

Norway spruce seeds were obtained from Quality Tree Seed, Inc., Brewster, N.Y. in 1985 and stored at 4°C. Prior to excision of the embryos, seeds were surface sterilized in 30% H_2O_2 for 45 minutes and rinsed 3 times in sterile water. After imbibing overnight in final rinse water, embryos were aseptically excised to prepared nutrient agar plates. For all trials, 30-50 embryos were excised for each treatment.

Table 16. Summary of current attempts to induce embryogenic callus from mature embryos.

Research Plan	Date	Species	Medium + Supplements, mg/L		Environment
458	3/17	LP	HM-1% sucrose	2 2,4-D/1 BA	16/8 L/D
			1/2 BLG	2 2,4-D/1 BA	16/8 L/D
				2 NAA/1 BA	16/8 L/D
				2 NAA/5 BA	16/8 L/D
472	4/25	LP, DF, NS	1/2 BLG	2 NAA/1 BA	16/8 L/D
				2 NAA/5 BA	16/8 L/D
			1/2 HM	2 NAA/1 BA	16/8 L/D
477	4/30	DF, NS	HM-1% sucrose	2 2,4-D/1 BA	Dark
492	8/28	NS	1/2 BLG	2 NAA/1 BA	16/8 L/D
				2 NAA/5 BA	16/8 L/D
				5 NAA/1 BA	16/8 L/D
			BLG	2 NAA/1 BA	16/8 L/D
495	8/29	DF	1/2 BLG	2 NAA/1 BA	16/8:Dark
				2 NAA/5 BA	16/8:Dark
			BLG	2 NAA/1 BA	16/8:Dark
494	9/10	DF	1/2 MS to 1/2 MS	1.2 BA (3 wks)	D —> L —> D
				5.6 K/11 2,4-D/44 GLU	Dark
507	10/3	LP, DF, NS	1/2 BLG	2 NAA/5BA	Dark
			DCR	2 NAA/5BA	Dark
			DCR	3 2,4-D/0.5BA	Dark
512	10/27	DF	CBM (—) suc to CBM to HM	1 2,4-D liq. pulse	Dark
				2 2,4-D/0.2 BA	16/8 L/D
				1 2,4-D	16/8 L/D
516	11/11	DF, NS	1/2 BLG	NAA/BA matrix (0,1,2/0,0.5,1)	16/8 (NS) Dark (DF)
516a	12/21	LP	1/2 BLG + 400 mg/L N_4NO_3	NAA/BA matrix (0,2,5/0,1,5)	Dark

Norway spruce embryogenic callus was initiated and maintained on 1/2 BLG basal medium supplemented with 10.7 μM NAA and 4.5 to 22.5 μM BA. This is a half strength Brown and Lawrence modification of MS medium,[24] which omits NH_4NO_3,

decreases KNO_3 to 0.5 mM and replaces the NH_4NO_3 with 5 mM of L-glutamine (see Appendix). Cultures were incubated with a 16 hour photoperiod of cool-white fluorescent light. Embryogenic callus was subcultured at 21-28 day intervals.

Development of somatic embryos was induced following the protocol outlined by Becwar et al.[5] The HM medium used in their procedure, as well as the 1/2 BLG were tested as basal media (BM) with supplements: 1% activated charcoal followed by 1 µM each indole-3-butryic acid (IBA) and abscisic acid (ABA). Plantlet elongation was obtained on basal medium without growth regulators.

Results and Discussion

Norway Spruce Somatic Embryogensis. Two weeks after plating on 1/2 BLG medium, 40-50% of the embryos began to callus just below the green swelling cotyledons. At 3 weeks, isolated somatic embryos were often observed developing from the callus proliferating from the hypocotyl area (Fig 23a). These low-frequency proembryos continued to proliferate, producing fast growing embryogenic masses (Fig 23b). Most of the embryogenic callus, however, was first observed as areas of white, glassy tissue on or around the callusing explant (Fig 23c,d). By 4-6 weeks, the embryogenic callus had formed a gelatinous and filamentous mass of clear polarized structures. Isolated from the surrounding green or tanning callus, this tissue has been subcultured for over 9 months (Fig. 24).

The influence of basal media and BA concentrations is shown in Table 17. The number or percent of genotypes (embryo explants) producing the embryogenic callus phenotype decreased with time. This may be the result of inaccurate early evaluation of tissue potential, or to less than optimal conditions for continued embryogenic callus growth; i.e., basal media, growth regulators, subculture intervals, etc. The higher BA level, 5 mg/L, appeared to stimulate early induction

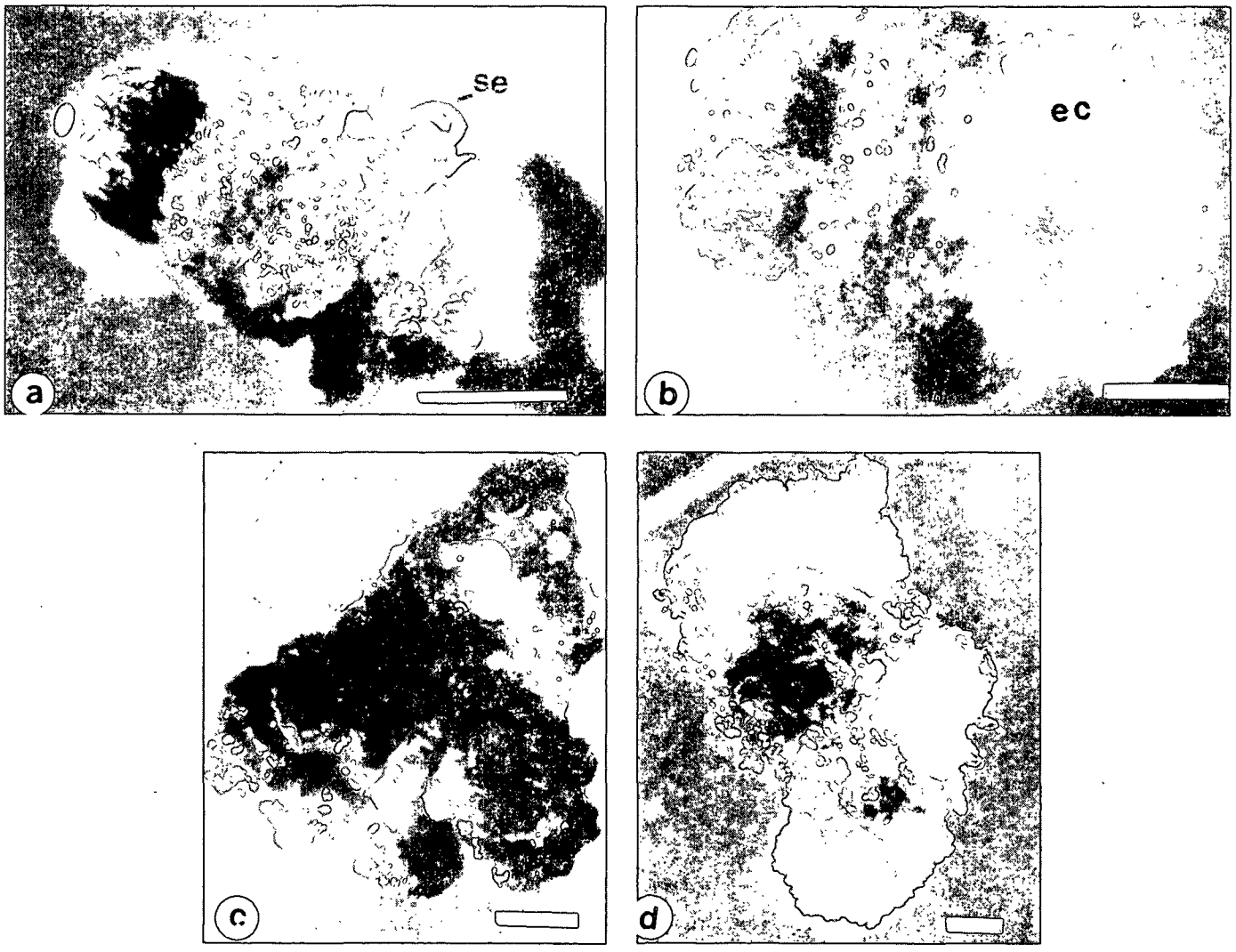


Figure 23. Initiation of embryogenic callus from mature Norway spruce embryos cultured in 16 h photoperiod on 1/2 BLG basal medium. a. Somatic embryos (se) arising from callusing hypocotyl area, 3 weeks after initiation. b. Same explant, 10 days later. Embryogenic callus (ec) is proliferating from the low-frequency somatic embryos, a. c-d. Typical induction of embryogenic callus 4-6 weeks after initiation.

of embryogenic-type callus; however, it was detrimental with time. The greatest number of maintained embryogenic lines was generated on 1/2 BLG 2/1 (2 mg/L NAA : 1 mg/L BA). With the same growth regulators, 1/2 HM medium failed to produce any embryogenic callus. Von Arnold and Hakman have reported embryogenic callus from mature Norway spruce embryos on full strength HM with a 1% sucrose level. However, it was necessary to initiate these lines in a dark environment.[23] The most recent report also states that "low levels of 2,4-D and darkness are essential for the initiation of somatic embryogenesis."[13] Our successful initiation of embryogenic callus under different conditions, a 16/8 light/dark cycle on 1/2 BLG medium, may be the result of our altered nitrogen regime. Further investigations of the relationship between light and inorganic/organic reduced nitrogen are underway.

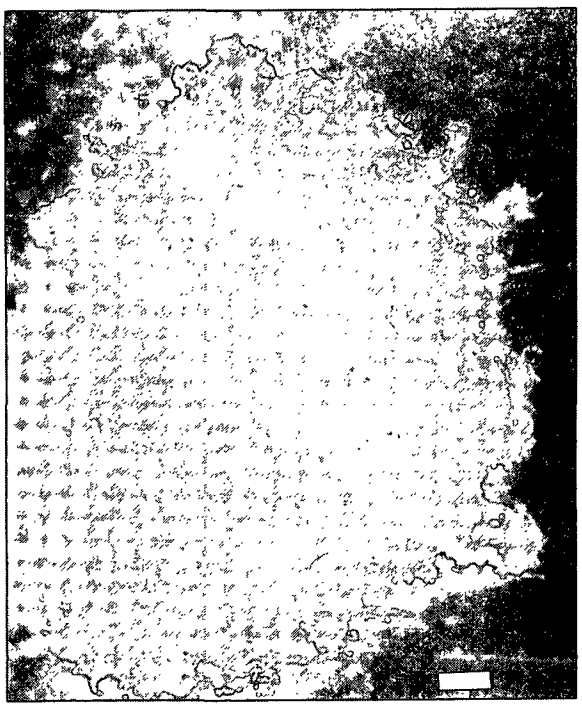


Figure 24. Embryogenic callus subcultured at 21-28 day intervals for 7 months.

Table 17. Percent embryogenic callus initiated and maintained from mature Norway spruce embryos on half (1/2) strength basal medium with 2 mg/L NAA and 1 or 5 mg/L BA.

Initiation Treatments		Percent Embryogenic Lines in Culture		
BM	NAA/BA	1 Month	2 Months	5 Months
1/2 BLG	2/1	14	21	17
1/2 BLG	2/5	37	7	7
1/2 HM	2/1	0	0	0

In a follow-up study with minor variations, full and half strength BLG media with the same growth regulators were compared. After 5 months, 14 additional embryogenic lines were in culture, or 28% of the genotypes plated to 1/2 BLG. In contrast, only proliferative, nonembryogenic callus was produced from full strength BLG.

Continued development or maturation of somatic embryos was very cell line dependent. Four genotypes, all determined to be highly embryogenic from tissue squashes prepared for the light microscope, were grown for 1 week on HM + 1% charcoal before transfer to HM + 1 μM ABA and IBA. After 3-4 weeks, multiple and dense embryonal heads were clearly visible (Fig 25a). Within 30-45 days from transfer to charcoal, only 2-4 of the approximately 50 potential embryos per callus mass (approximately 100 mg fresh weight) elongated and developed cotyledons (Fig 25b,c). From this stage, conversion to plantlets occurred readily on basal medium lacking growth regulators (Fig 25d). However, only 1 of the 4 lines developed to this stage. No maturation occurred from tissue transferred to the same sequence with 1/2 BLG as a basal medium.

Loblolly Pine and Douglas-fir Trials. To date, we have not induced embryogenic callus from loblolly pine or Douglas-fir mature embryos. Half

strength BLG basal medium was first tested with loblolly pine. Although embryogenesis was not induced, cultures were of a quality to warrant further testing of the medium and other species. Attempts to obtain somatic embryogenesis via the media and methods reported for mature Norway spruce[25] and sugar pine[11] were also unsuccessful. From general observations, however, dark-grown Douglas-fir and loblolly pine tissues appear to exhibit greater embryogenic potential than their light-grown counterparts. After 2-3 weeks, Douglas-fir cultures initiated in the dark appear similar to newly initiated spruce lines. However, the white glassy callus then reverts to the typical small-celled and opaque proliferative callus growth. The CBM treatments that generate the sporadic appearance of somatic embryos from immature Douglas-fir embryo callus are also being investigated.

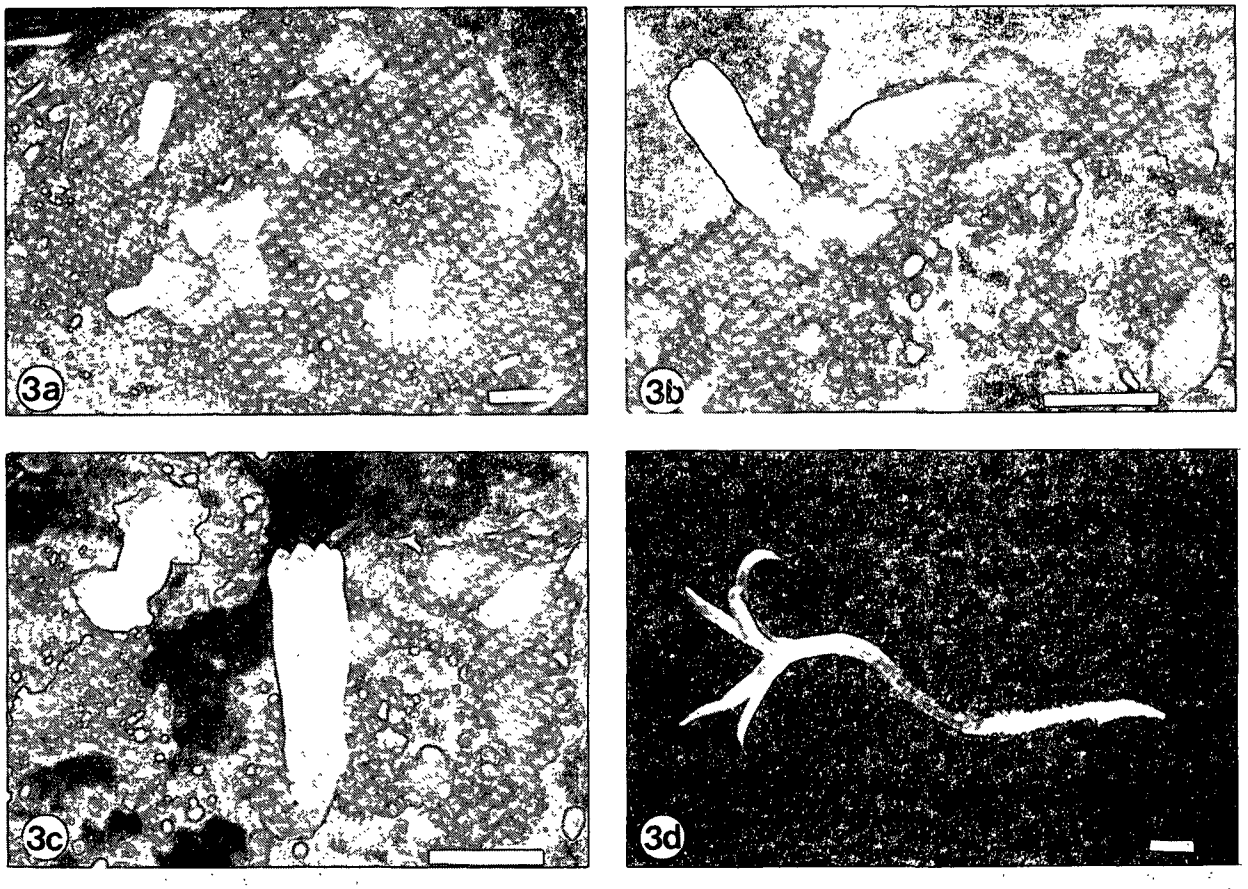


Figure 25. Plantlet development from subcultured light-grown embryogenic callus initiated from mature zygotic Norway spruce embryos. a. Smooth embryonal "heads" and early somatic embryos emerging after 21-28 days on HM medium with 1 μM IBA and ABA. b-c. Maturation of somatic embryos; elongating hypocotyls and development of cotyledonary nodes. d. Conversion of isolated somatic embryo to plantlet on basal medium without growth regulators, 1-2 months after transfer from HM I/A.

Conclusions

Induction of embryogenic callus from mature excised embryos has the obvious advantage of providing tissues for continuous studies of somatic embryogenesis, embryo maturation, and plantlet conversion. It may also provide a stepping stone to direct manipulations with tissues or cells from trees mature enough to have been proven genetically superior. We are now able to repeatedly generate embryogenic callus from embryos excised from mature Norway spruce seeds. In contrast to a recent report,[13] these somatic embryos emerge from the callusing hypocotyl area in a manner similar to that observed from immature zygotic embryos. To our knowledge, this regeneration in the light is unique to our laboratory. The interaction between light and reduced nitrogen components of our medium is now being investigated and may provide data critical to our understanding of somatic embryogenesis induction. Although Norway spruce plantlets have been regenerated from these somatic embryos, the maturation efficiency of the present protocol is low. Continued efforts are underway to increase the frequency of development. As genetic differences in embryogenic potential have been observed, both old and newly initiated lines will provide tissues for these studies. Somatic embryogenesis research using loblolly pine and Douglas-fir mature embryos will continue with emphasis on dark environment initiation (as suggested by earlier studies) as well as on alternate nitrogen and carbohydrate sources.

DEVELOPMENT OF NORWAY SPRUCE SOMATIC EMBRYOS AND CONVERSION TO PLANTS

Introduction

Although initiation of embryogenic callus is a prerequisite to developing an in vitro embryogenic system in conifers, embryogenic callus is of little practical use unless we can efficiently induce somatic embryos to develop and

grow into plants. This section covers our efforts in this regard and specifically, the protocol used to induce somatic embryo development. Also described are a somatic embryo dispersion and counting technique for quantifying levels of embryogenesis among callus lines and a method for quantifying growth and regeneration of embryogenic callus using an alternative culture system, "bead culture."[26] Using the counting technique, we have been able to quantify the effect of differing maintenance and development protocols on subsequent somatic embryo development. Preliminary information is presented on techniques to increase the frequency at which somatic embryos can be converted to plantlets. Lastly, our initial efforts to transfer somatic embryo plants to the greenhouse are discussed.

Methods

The protocol we used to induce development of Norway spruce somatic embryos is outlined in Fig. 26. Briefly, this involved the transfer of embryogenic callus from maintenance medium (basal medium with 2,4-D and BA) to medium with no growth regulators and 1% charcoal for one week and then to medium with low levels of IBA and ABA. On this latter medium, callus was transferred at 2 to 3 week intervals, and somatic embryos were developed directly on the callus clump.

As indicated in Fig. 26 a fraction of the embryogenic callus on the IBA/ABA development medium could also be dispersed for counting the somatic embryos. The somatic embryo dispersion and counting technique is described in Fig. 27 and has been published.[27] This method of plating dispersed callus and somatic embryos in a thin agarose layer was originally developed for protoplast culture.[26] Essential to the technique is plating in a medium containing a low gelling temperature agarose (e.g., Sigma Type VII, which can be maintained in a

liquid state at 38°C). To facilitate counting of somatic embryos on the dispersed layer the plate was placed on a background grid and observed through a dissecting scope at 15X. All cultures were grown in the light as previously described. Somatic embryo counts for a sample plate were means of three to four individual counts taken by different observers. The overall count mean presented is a mean of three to four replicate plates from each treatment.

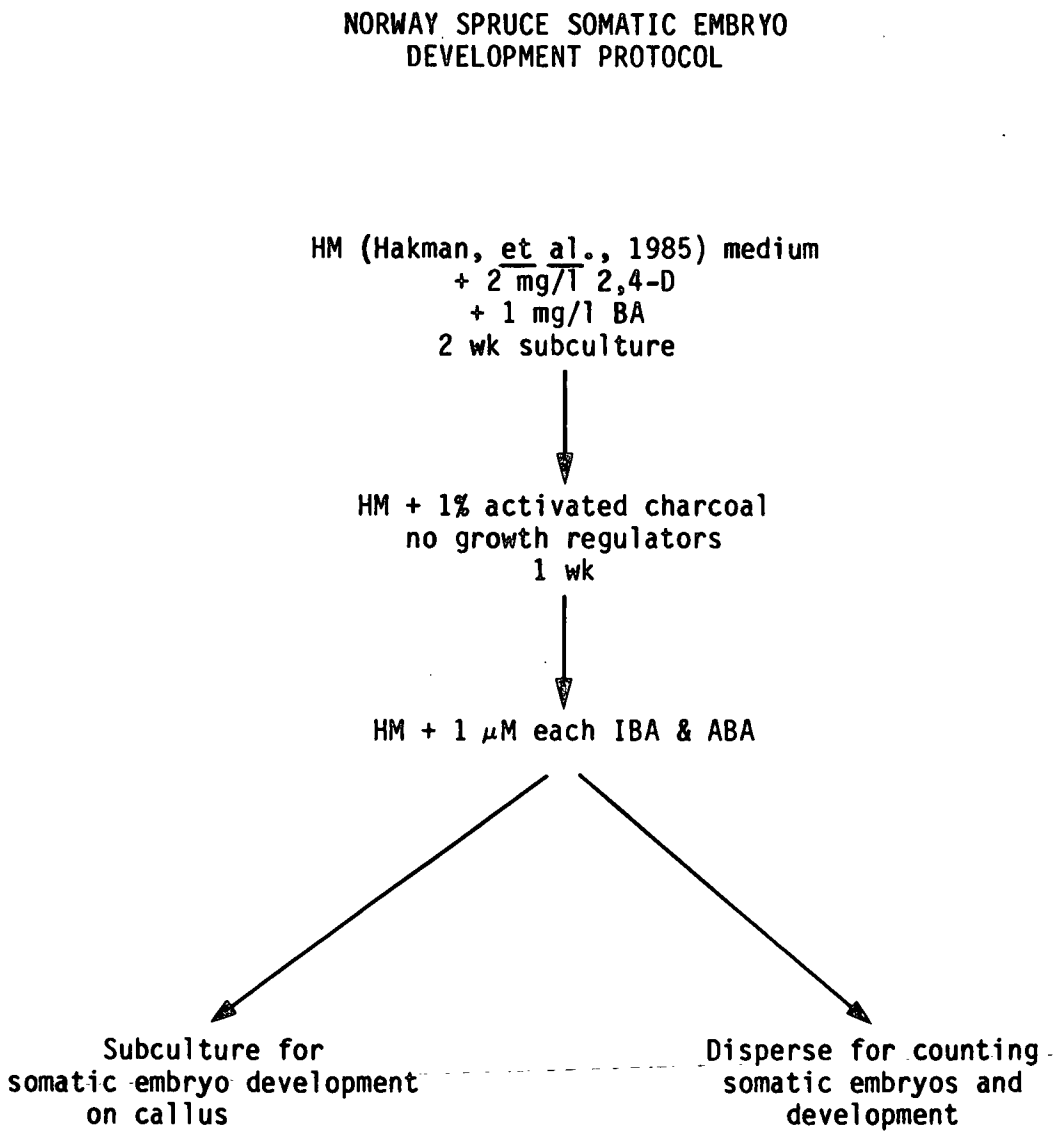


Figure 26. Norway spruce somatic embryo development protocol.

The effect of subculture interval on embryo development in embryogenic callus of Norway spruce was also studied as follows. Pieces of embryogenic callus (~ 100-200 mg wet weight) were kept on embryo proliferation medium (HM 2/1) for 0, 1, 2, and 3 weeks. At the end of each of these time intervals, six pieces were carried through the developmental protocol. After two weeks on HM I/A medium, the pieces of embryogenic callus were weighed, dispersed and the level of embryogenesis was determined as given in Fig. 27.

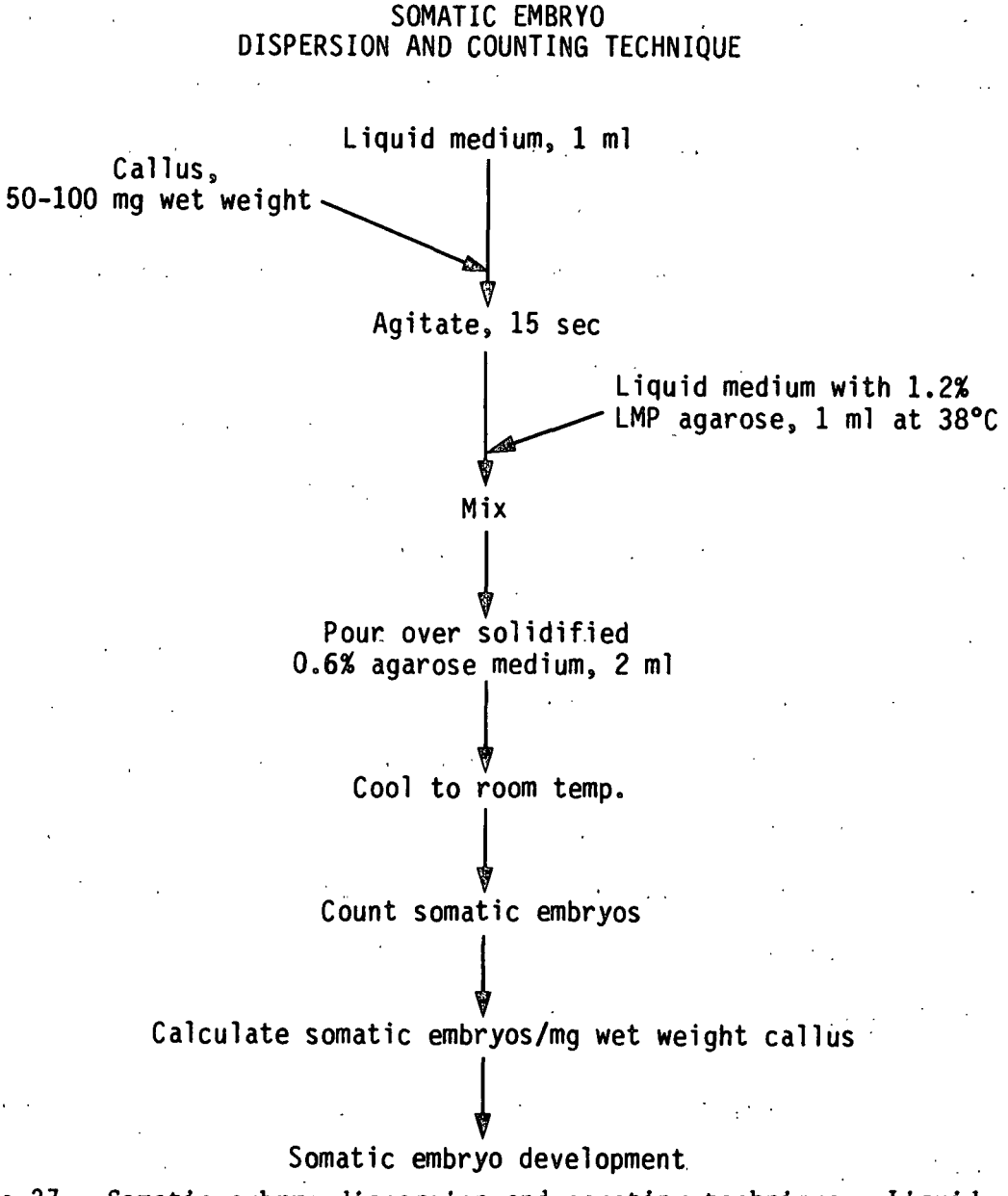


Figure 27. Somatic embryo dispersion and counting technique. Liquid medium is HM with 1 μM each IBA and ABA.

The agarose "bead culture" technique described by Shillito et al.[26] was used to study growth and regeneration. Briefly, the technique involves dispersing embryogenic callus [line (NS1)5] in liquid HM 2/1 medium, adding low gelling temperature agarose at final concentration of 0.6 to 1.0%, and plating the cell suspension in a thin layer. These agarose sheets or "beads" can be cultured in liquid HM 2/1 medium. The liquid medium can be removed and replaced with fresh liquid medium weekly. The culture was grown in 16 h light and slowly shaken at 50 rpm. For growth measurements the dry weight increase of embryogenic callus grown in the agarose bead culture was compared to the dry weight increase of embryogenic callus grown on agar-solidified HM 2/1 medium. For determination of somatic embryo development capacity, pieces of embryogenic callus were removed from the agarose bead and placed on our development protocol, as previously described.

Somatic embryos were removed individually from the callus or the agarose layer when cotyledons appeared distinct and green and a white hypocotyl was also visible. They were placed root end into quarter strength basal medium lacking growth regulators for further development.

When primary root length reached approximately 1 cm, somatic embryo plantlets were transferred from in vitro to soil (5:1, Jiffy soil mix:Perlite) in either Magneta GA7-3 plastic vessels or Spencer-Lemaire root trainers ("book planters"). Plantlets in the GA7-3 vessels were grown in the laboratory under 16 h incandescent and fluorescent light for approximately two months before transfer to the greenhouse. Plantlets transferred to root trainers went directly from in vitro to greenhouse conditions.

High relative humidity was maintained by controlling the tightness of the GA7-3 vessel lids or by enclosing the root training trays in large plastic

bags for six weeks. In the greenhouse plants were fertilized with osmocote (14-14-14) and grown under ambient light supplemented with fluorescent light (16 h, 100 $\mu E\ m^{-2}\ s^{-1}$). Temperature was maintained at 20-25°C. After six weeks in the greenhouse, the lids of GA7-3 were opened, root trays were removed from plastic bags, and all plants were periodically misted. After this two week acclimation period, plants were transferred to lower temperatures (5°C low and 15°C high) and ambient light for 7 weeks. Subsequently, plants have been maintained at 0 to 15°C with occasional lows to approximately -5°C.

Results and Discussion

The development of somatic embryos from embryogenic callus of numerous plant species is induced by either complete removal of auxin or greatly reduced auxin levels.[28] Although the synthetic auxin, 2,4-D, has proven very effective at initiation and maintenance of embryogenesis in plant tissue cultures, its potency and persistent effects are often suggested as causal agents in limiting subsequent expression of morphogenic potential.[29]

We have included a one week culture period on basal medium with no growth regulators and 1% activated charcoal as the initial step in our development protocol (Fig. 26). Activated charcoal is known to preferentially adsorb aromatic molecules (Bonga[9] p. 4-35 and references cited therein) and therefore may act to reduce 2,4-D levels in the embryogenic callus. The second stage of the developmental protocol is transfer to basal medium with low levels of IBA and ABA. IBA is a much less potent auxin than 2,4-D and has been effectively used at low levels to obtain soybean somatic embryo development.[30] ABA has been shown to suppress abnormal somatic embryo development in carrot[31] and was included in the developmental protocol. We have not determined the optimum levels of IBA and ABA for somatic embryo development of Norway spruce.

Callus lines derived from 23 different immature embryo explants (from the 1985 Norway spruce collections) and maintained under identical cultural conditions for seven months were evaluated for embryogenic capacity using the procedure outlined in Fig. 26 and 27. The callus lines are ranked according to somatic embryo density in Table 18. There were significant differences in the level of somatic embryogenesis among callus lines. Some lines such as (NS1)11 were highly embryogenic, whereas lines such as (NS1)2 had a very low somatic embryo density. It should be noted that both of these callus lines appeared similar visually, and both grew vigorously. Thus, our somatic embryo counting technique was useful for identifying callus lines with a high capacity for somatic embryo development among those with similar phenotypes. The most embryogenic line, (NS1)11, contained over 10^3 somatic embryos per gram of callus. The results in Table 18 also show that the callus lines initiated from the most immature embryos tested (NS1 lines) had higher levels of somatic embryogenesis than lines derived from immature embryos collected at later dates (NS2 and later lines). This suggests that developmental stage of explants affects both the initiation frequency and subsequent embryogenic capacity.

Eight green callus lines were surveyed for the presence of somatic embryos (Table 18). Somatic embryos were only found in one line, (NS5)17. This occurred at a very low frequency (two somatic embryos in one of four dispersed callus pieces) but has interesting implications. First, the green callus may not be nonembryogenic, per se. It may retain some limited capacity to form somatic embryos at a very low frequency. Second, the green callus phenotype may give rise to the embryogenic white at low frequencies.

The white embryogenic callus lines we tested all had a similar phenotype based on visual observation. Embedding dispersed embryogenic callus in a thin agarose layer provided a simple culture system for quantification of differences

in somatic embryo density and developmental capacity among callus lines. This technique should be applicable to other embryogenic callus systems that are easily dispersed in liquid by agitation. Embryogenic callus of European larch is very similar in morphology. It is also comprised of a heterogeneous mixture of somatic embryos, elongated suspensorlike cells, and cell clusters all embedded in a mucilaginous matrix.[16]

Table 18. Differences in somatic embryogenesis among Norway spruce callus lines.

Callus* Line	Callus** Phenotype	Somatic Embryos*** Counted per mg Wet Weight Callus
(NS1)11	W	1.5a
(NS1)12	W	0.8 b
(NS1)10	W	0.8 b
(NS1)8	W	0.7 bc
(NS1)5	W	0.7 bcd
(NS1)7	W	0.7 bcd
(NS1)13	W	0.6 bcd
(NS2)6	W	0.5 bcd
(NS2)5	W	0.5 cde
(NS1)9	W	0.5 cdef
(NS1)1	W	0.4 defg
(NS2)4	W	0.2 efgh
(NS1)6	W	0.2 fgh
(NS2)3	W	0.2 fgh
(NS8)1	W	0.2 gh
(NS1)2	W	0.2 gh
(NS4)4	W	0.1 h
(NS4)6	W	0.1 h
(NS5)17	G	0.003 h
(NS1)1	G	0 h
(NS1)4	G	0 h
(NS1)5	G	0 h
(NS1)8	G	0 h
(NS5)5	G	0 h
(NS1)15	G	0 h
(NS1)20	G	0 h

*Code in parentheses identifies explant collection. Number following parentheses identifies explant from which callus derived.

**W = white embryogenic callus. G = green callus.

***Mean value of four observers on each of four callus pieces per each line. Means followed by a common superscript are not significantly different as determined by ANOVA with Duncan's New Multiple Range Test ($p < 0.05$).

The utility of the method we have described for measuring embryogenic capacity in callus lines of Norway spruce is that it can do so in a quantitative manner, making statistical analysis of the results possible. The technique has already proven useful for evaluating the effectiveness of biochemical treatments aimed at enhancing the level of embryogenesis[32] (and refer to later section on biochemistry) and can be used to determine the importance of genetic, physiologic, or other components of embryogenesis.

The influence of the subculture interval on the level of embryogenesis (embryos per unit weight of callus) is shown in Table 19 for two "good" lines [(NS1) 1 and 8] and a "poor" line [(NS1)6]. Regardless of relative quality, all three lines had the highest embryo density one week after transfer to fresh medium. In (NS1)1 and 6 the embryo density after one week was significantly higher than at any other time. In the case of (NS1)6 the level of embryogenesis after one week was sufficiently high to move its classification from "poor" to "good" - a feature that would have gone unnoticed if the lines had been evaluated after the usual two week interval. After peaking at one week, the somatic embryo density declined. The decline can be accounted for by a subsequent callusing of newly differentiated embryos, which effectively prevents further development upon transfer to the developmental protocol. Thus, it appears that maintaining a high level of somatic embryogenesis requires weekly subculture.

The similar morphologies of somatic and zygotic embryos are shown in Fig. 28. The somatic embryo (Fig. 28a) is fully developed (at a "mature" stage) with a hypocotyl region and cotyledonary leaves, and can easily be removed from the callus.

Table 19. Effect of subculture interval on the level of embryogenesis in Norway spruce callus.

Time, wks	Somatic Embryo Density, embryos/g wet wt.* (NS1)1	(NS1)6	(NS1)8
0	728[b]	183[b]	503[c]
1	2148[a]	795[a]	1487[a]
2	975[b]	235[b]	1315[ab]
3	930[b]	130[b]	923[bc]

*Means followed by a common superscript are not significantly different as determined by ANOVA with Duncan's New Multiple Range Test ($p < 0.05$).

A comparison of somatic embryo development of Norway spruce on our protocol (Fig. 26) and simply transferring embryogenic callus to basal medium lacking growth hormones is presented in Table 20. For line (NS1)5 our developmental

protocol yielded significantly higher somatic embryo counts and numbers of somatic embryos which reached the mature stage. For line (NS1)13 only the number of somatic embryos reaching the mature stage was significantly higher on our protocol. Thus, for both lines the maturation frequency, that is, the efficiency with which somatic embryos reached the mature stage, was higher using our protocol. Even so, a 3 to 15% maturation frequency is a low frequency in regard to potential utility of the process. These data can be considered base-line values from which we need to improve toward higher efficiencies. Results reported in the biochemical section of this report address potential ways to improve maturation frequencies.

bead culture *vs.* on agar medium is summarized in Table 21. Relative to growth on agar medium, growth in bead culture was increased nearly eightfold. The results are means of ten replications, and the experiment has been duplicated.

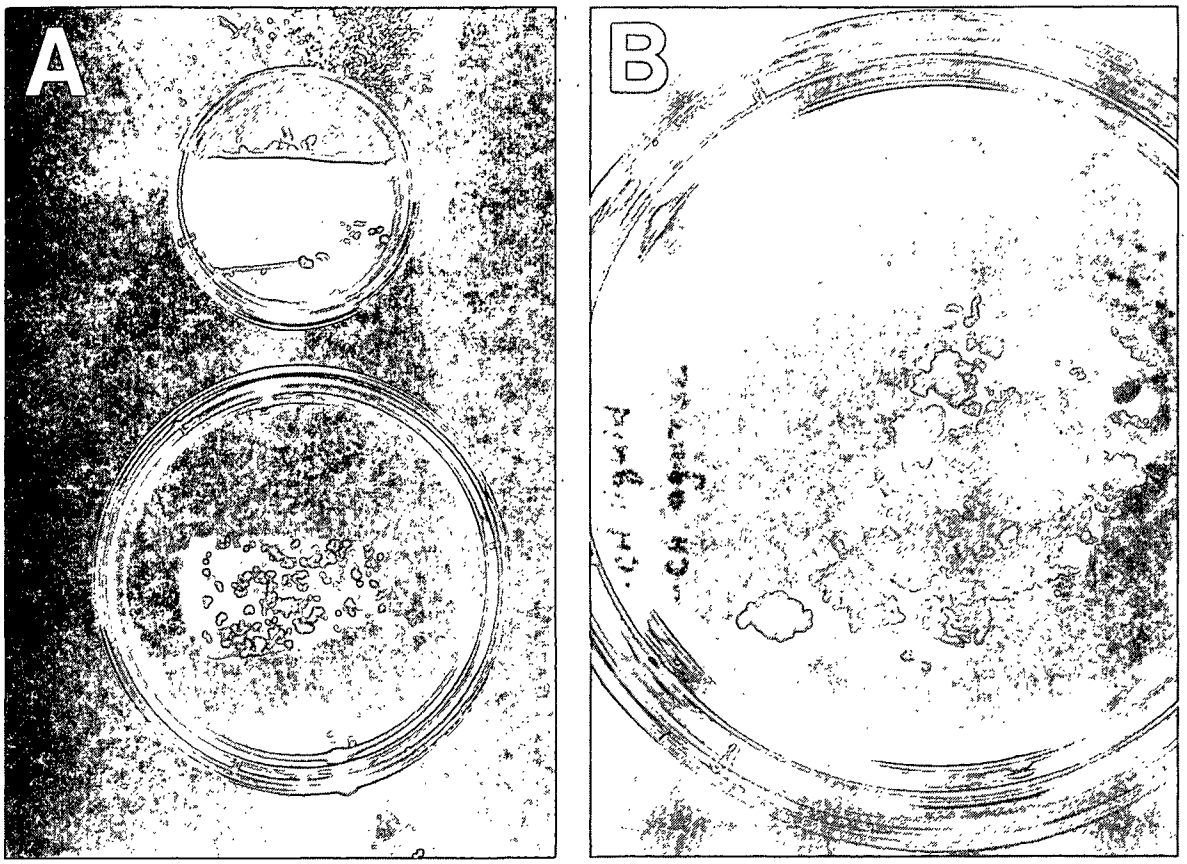


Figure 29. Growth of Norway spruce embryogenic callus in agarose bead culture. A: Dispersed embryogenic callus plated in thin agarose layer (small top plate). After agarose gells, a section is cut out and placed in larger plate (bottom). Liquid medium (HM 2/1) is added to surround agarose bead and is exchanged weekly. B: Typical growth of embryogenic spruce callus after 4 weeks. Scale: The large plates in A and B are 9 cm in diameter.

We have also verified that somatic embryos derived from embryogenic callus grown in bead culture develop to the mature embryo stage. In fact the number of somatic embryos developed from embryogenic callus grown in bead culture was considerably higher than from our routine protocol on agar (Table 21).

The significance of the results is the demonstration that an alternative maintenance system, in this case agarose bead culture, provides increased embryogenic callus growth rates and somatic embryo maturation. This may be a very important point, because maturation currently is the limiting step in obtaining

plants. Thus, inducing rapid growth of embryogenic callus may in itself improve later limiting steps in somatic embryo development. Although results with the bead culture technique are encouraging, the technique itself may not have direct application to large scale maintenance of embryogenic conifer callus. A conventional liquid suspension culture system is more amenable to large scale maintenance of embryogenic callus, and is also conducive to promoting rapid growth.

Table 21. Effect of embryogenic callus maintenance system on growth and somatic embryo development of Norway spruce callus [line (NS1)5].

Callus Maintenance Protocol	Dry Weight Increase*	Mature Somatic Embryos, number/gram callus
Agar	2.6[a]	17
Bead culture	20.0[b]	76

*Dry weight increase, $(DW_o-DW_f)/DW_o$, during 28 day growth period. Means followed by a common superscript are not significantly different as determined by ANOVA with Duncan's New Multiple Range Test ($p \leq 0.05$).

Perhaps the greatest utility of agarose bead culture will be in genetic transformation experiments. Protoplasts or cells can be transformed with exogenous DNA containing a marker gene, such as kanamycin resistance, and grown in agarose bead culture. The liquid medium surrounding the agarose bead would contain the selection antibiotic kanamycin. Thus, only transformed cells in the agarose bead survive. This technique has worked well with herbaceous plant species.[33] Experiments utilizing the agarose bead culture technique for transformation purposes with embryogenic spruce cells are in progress.

Preliminary results on further development of mature somatic embryos of Norway spruce are presented in Table 22. It is important to note that here we have defined somatic embryo conversion as initiation of primary root development.

This is analogous to germination. In two trials the conversion frequency ranged from 5 to 33% with an average of 11%. Again, there is room for considerable improvement in the efficiency of this phase of development of somatic embryos. In some *in vitro* plant systems, e.g., alfalfa and celery, somatic embryo conversion frequencies as high as 65% have been reported.[34]

Table 22. Conversion frequency of Norway spruce somatic embryos to plantlets.

Experiment	Number of Somatic Embryos		Conversion Frequency, %
	Mature Stage	Primary Root Development	
1	94	5	5
2	24	8	33
Totals	118	13	11

The results in Table 23 suggest that the positioning of somatic embryo radicles may affect the conversion frequency. These results were obtained from an experiment where differences in conversion frequency were noted between somatic embryos with their radicle placed in agar *vs.* somatic embryos which were positioned flat on the agar surface. Further experiments have been initiated to test the hypothesis that somatic embryo conversion frequencies are increased by avoiding immersion of the radicle in agar.

Table 23. Effect of position of somatic embryo radicle on conversion frequency.

Somatic Embryo Root Radicle Position	Number of Somatic Embryos		Conversion Frequency, %
	Mature Stage	Primary root Development	
In agar	88	6	7
On agar surface	30	7	23

A total of 31 somatic embryo plants have been transferred to the greenhouse, and seven survived as of December, 1986. One is shown in Fig. 30. The plants are phenotypically normal relative to seedlings at a similar stage of development. Continued efforts in 1987 will be made to establish an efficient *in vitro* to greenhouse transfer system and to establish larger numbers of somatic embryo plants in the greenhouse for eventual field planting and evaluation.

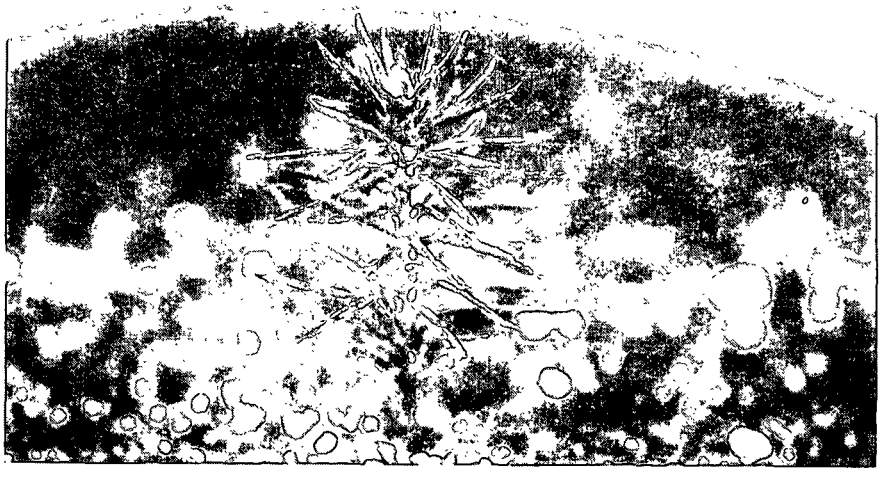


Figure 30. Norway spruce somatic embryo derived plant growing in soil in greenhouse. Plant height is 4 cm.

BIOCHEMICAL INVESTIGATIONS

CHARACTERIZATION OF EXPLANTS AND EMBRYOGENIC VS. NONEMBRYOGENIC CALLUS

Significant quantities of embryogenic and nonembryogenic callus from the new Norway spruce model system became available during 1986. This provided impetus for the further development, evaluation and utilization of biochemical markers to distinguish embryogenic from nonembryogenic tissue. Although initially all Norway spruce callus was readily identified by its white mucilaginous appearance, from the outset there was need for assurance that these visual/tactile markers were dependable and not just coincidental. Furthermore, there was no guarantee that appearance would be a sufficient criterion to pick out competent tissue of other conifers including the target species, Douglas-fir and loblolly pine. In this situation, biochemical characterization of these two callus types was undertaken to back up the obvious visual/tactile signs that had been encountered.

It was judged wise to seek these biochemical markers as insurance even though they might not be required to help sort tissues in many cases. Additionally, knowledge of biochemical parameters associated with embryogenic tissue would be valuable in our efforts to understand the molecular basis of embryogenesis and thereby enhance the reliability of the process. Consequently, 1986 witnessed the assessment of several marker possibilities and attempts to employ a couple of them to predict embryogenic potential.

Development and Evaluation of Biochemical Markers

Introduction

As is evident from Progress Report Thirteen, biochemical marker development was already underway prior to our use of Norway spruce. Based upon studies

of the old wild carrot model and observations on incompetent loblolly pine suspensions, ethylene production rate had been advanced as a primary marker candidate. Likewise, glutathione and polyamines had reached the status of secondary markers, and the potential of several other parameters, including most of those mentioned below, was already recognized and even explored in some cases.

Materials and Methods

Ethylene. Callus pieces of about 100 mg fresh weight were transferred to 19 x 48 mm glass vials containing 5 mL of solidified culture medium. After allowing the tissue to acclimate for 3 to 5 days, the vials were sealed with a rubber stopper containing a cylindrical half-hole rubber septum. After 24 hours, 1 mL of headgas was removed with a syringe and injected into a Packard Model 428 gas chromatograph equipped with a flame ionization detector and a 6 ft Porapak Super Q packed column. With an isothermal oven temperature of 45°C, an injector temperature of 200°C, and detector temperature of 200°C, the retention time for ethylene was normally between 1.5 and 1.6 minutes as determined daily with a purchased standard (Scott Specialty Gases).

Glutathione and Total Reductants. Callus pieces ranging from 30 to 110 mg fresh weight were extracted with 10 mL or less of Type I water in a Ten Broeck homogenizer for GSH analysis. Actual weights per volume to use may need to be adjusted for a given sample type. Immediately after centrifugation (39,000 x g for 20 min), 50 µL aliquots of supernatant were mixed with 950 µL of 0.01N HCl. From this point the assay was that adapted from Brehe and Burch[35] as recently described.[36]

For assay of total reductants (of sufficiently negative potential to reduce ferric iron), callus pieces 10 to 30 mg fresh weight were employed, but

the assay could be performed on a few mg of callus if necessary. The tissue was extracted with water as above using 1 mL of water per 1-2 mg of callus and centrifuged at low speed. Reductants were assayed in the supernatant by the method of Singh et al.,[37] i.e., equal volumes of extract and freshly prepared ferric chloride - potassium ferricyanide reagent were mixed, incubated 1 hour at 30°C, and read against a reagent blank at 760 nm. Given the heterogeneity of the responding compounds, the results are expressed simply as A760/g fresh weight. Where protein was determined on the aqueous extracts, the Bradford reagent[38] was used.

Protein Synthesis. Callus pieces of about 100 mg fresh weight were placed in wells of multiwell plates (24 wells per plate; 2 mL well volume) to which were added 300 µL liquid basal medium containing the growth regulators used for culture maintenance. To each well were added 5 µCi of ^{3}H-leucine [L-(3,4,5-^{3}HN)-leucine; 147 Ci/mmole]. After incubation under the usual light regime for 12 hours, the tissue was collected, rinsed with 10 mM (unlabeled) leucine, and homogenized in 500 µL buffer (50 mM HEPES, pH 7.6). After centrifugation in an Eppendorf microcentrifuge, the supernatant was treated with 3 mL cold 10% TCA, mixed, and stored at 0°C for 30 minutes. A portion of this supernatant was also used for protein determination by the method of Bradford.[38] Upon standing at 0°C, precipitates were collected on Whatman GF/A glass filter pads, dried, and measured for radioactivity in a scintillation counter.

Isozymes. The isozyme analyses reported here were conducted on crude extracts prepared according to the freeze-thaw technique in a plastic pipet tip described by Berger et al.[39] Both peroxidase and acid phosphatase isozymes were resolved by isoelectric focusing on agarose gel (pH 3.5 to 9.5). Esterase resolution was better on LKB PAGplates. The esterase detection method was that of

Everett et al.,[40] whereas peroxidase and acid phosphatase stains were from Hamaker and Snyder,[41] modified to use guaiacol in the case of peroxidase (gel stained 20 to 30 minutes in a solution containing 50 mL 0.1M acetate buffer, pH 5.0, plus 0.25 mL guaiacol and 1.0 mL of 0.5% hydrogen peroxide). Agarose gels could be preserved by rinsing with water and air drying after staining.

Spectra. The UV-visible spectra were taken of 95% ethanol extracts of cells relative to a solvent reference. For the infrared difference spectrum shown, callus was freeze-dried and analyzed in the diffuse reflectance (direct) mode by FTIR spectroscopy.

Results and Discussion

Ethylene. Earlier research in other laboratories had produced conflicting results regarding the effects of ethylene on plant morphogenesis (Report Thirteen, p. 93, 106). From studies conducted in this laboratory on embryogenically competent wild carrot and incompetent pine cell suspensions (Report Thirteen, p. 93-6, 106-117), it appeared that low ethylene evolution rates might be characteristic of competent cells. Therefore, as embryogenic and nonembryogenic Norway spruce callus became available, each was subjected to ethylene analysis. Some of the first findings (Table 24) indicated that indeed, relative to nonembryogenic callus, this conifer embryogenic callus evolved ethylene at a much lower rate.

The extension of this analysis to other dualistic cell lines comprised of both embryogenic (white) and nonembryogenic (green) callus confirmed the initial findings (Table 25, top). In fact, eventually this difference in ethylene evolution rate was also noted for callus derived from mature zygotic embryos of Norway spruce (Table 25, bottom). These data seemed to justify the prior assignment of ethylene evolution rate to primary marker status. However, as for any marker, the utility of ethylene in this role would depend upon how early in the

culture process reliable readings could be taken. This aspect of marker utility is addressed later in this report.

Table 24. Ethylene evolution by Norway spruce callus.

Cell Line	Ethylene Evolution, nL/mg fr. wt./day* Embryogenic Callus	Nonembryogenic Callus	N
4(NS1)8	n.a.	2.85[a]	3
4(NS1)2	0.32[c]	n.a.	3
4(NS1)3	n.a.	1.27[b]	3
4(NS1)9	0.18[c]	n.a.	3
7(NS7)X	n.a.	1.25[b]	5
5(NS1)12	0.17[c]**	n.a.	5
4(NS1)12	0.36[c]	n.a.	3

*n.a.= not available; means followed by a common superscript are not significantly different as determined by ANOVA with Duncan's New Multiple Range Test ($p < 0.05$).
**some green nonembryogenic callus present also.

Table 25. Ethylene evolution rate in embryogenic and nonembryogenic Norway spruce callus.

Callus Line	Ethylene Evolution, nL/mg fr. wt./day* Embryogenic Callus	Nonembryogenic Callus
15(NS1)1	0.086[c]	1.63[a]
15(NS1)5	0.013[c]	1.13[b]
5(NS1)8	0.150[c]	1.75[a]
5(MNS472-2)9	0.010(0.012)	n.a.
5(MNS472-2)8	0.012	n.a.
4(MNS472-3)4	0.014	0.216

*n.a.= not available; means followed by a common superscript are not significantly different as determined by ANOVA with Duncan's New Multiple Range Test ($p < 0.05$). For the mature embryo callus lines (MNS), no replication except for one duplicate in ( ).

Glutathione. Student research here at IPC on the role of glutathione (GSH) in wild carrot development had shown that the concentration of this as well as that of another antioxidant, ascorbic acid, declines to a low level as somatic embryo development proceeds to completion.[36] Analysis of GSH distributions between Norway spruce embryogenic and nonembryogenic callus revealed that a similar situation existed in this conifer (Table 26). Whether expressed on a fresh weight or a protein basis, the GSH content was higher in nonembryogenic callus than in the white callus containing developing embryos. This finding has been exploited to enhance the maturation stage of Norway spruce somatic embryo development (see Applications section below).

Table 26. Glutathione content of Norway spruce callus.

	GSH, nmoles/g wet wt.		GSH, nmoles/µg protein	
Callus Line	Embryogenic	Nonembryogenic	Embryogenic	Nonembryogenic
10(NS1)6	134^{c}	434^{a}	0.35^{b}	1.87^{a}
10(NS1)7	141^{c}	473^{a}	0.31^{b}	2.22^{a}
10(NS1)8	120^{c}	325^{b}	0.27^{b}	1.65^{a}

N = 3; water extracts; means followed by a common superscript are not significantly different as determined by ANOVA with Duncan's New Multiple Range Test run separately for the wet weight basis and the protein basis ($p < 0.05$).

Total Reductants. Because GSH is a quantitatively significant antioxidant in these plant cells which also contain several other reductants such as vitamin C and phenolic compounds, it was of interest to learn whether this GSH result (Table 26) was peculiar to GSH alone or whether reductants in general were more plentiful in nonembryogenic than embryogenic cultures as implied earlier.[42] To test this hypothesis in the Norway spruce system required a reagent to detect reductants in general. Although no such reagent seems to be in common use in this context, the ferric chloride/potassium ferricyanide reagent sometimes used

to measure total phenolics[37] was found to be suitable. As shown in Table 27, not only phenolics but other reducing agents respond, and in theory anything capable of reducing ferric to ferrous iron under the test conditions should react. When this colorimetric test was applied to simple water extracts of embryogenic vs. nonembryogenic Norway spruce callus, the differences seen were quite impressive. The nonembryogenic callus was loaded with reductants relative to the embryogenic callus (Table 28). Both types of callus were light-grown, so the presence of phenolic compounds might have been expected in both. Despite the probability that phenolic compounds are partly responsible for the color development, neither the nature nor the number of compounds responding in the test are known at this time. It is for this reason that the results must be expressed simply as A_{760}/g fr. wt. rather than, e.g., moles/g fr. wt.

Table 27. Total reductants test specificity.

Compound	A_{760}/mmole
Catechin	17.6
Ferulic acid	13.5
Caffeic acid	11.9
Ascorbic acid	5.5
Glutathione	3.2
p-Coumaric acid	1.5
Glucose	0.3

The simplicity of the total reductants test has made its use very attractive, and it has been applied to embryogenic versus nonembryogenic calli of larch and white spruce as well with the same results (Table 29). Likewise, mature zygotic embryo callus from Norway spruce behaves this way (Table 30). The only "exception" observed in model system callus extracts was a case where embryogenic larch callus contained highly developed embryos that were already exhibiting red anthocyanin in

their root systems. This was not really an exception but rather application of the test to a sample that falls outside of the realm of the test's utility. It is possible to state without reservation that to date nonembryogenic callus always contained more reductants than embryogenic callus, usually many fold more.

Table 28. Total reductants in Norway spruce callus.

Callus Line	Reductants, A_{760}/g fr. wt.	
	Embryogenic Callus	Nonembryogenic Callus
10(NS1)6	30[d]	518[b]
10(NS1)7	22[d]	438[c]
10(NS1)8	32[d]	535[b]
5(NS4)6	56[d]	1250[a]

N = 3 except for NS4 N = 4; means followed by common superscripts are not significantly different as determined by ANOVA with Duncan's New Multiple Range Test ($p < 0.05$).

Table 29. Total reductants in larch and white spruce callus.

Callus Line	Reductants, A_{760}/g fr. wt. ± S.D.	
	Embryogenic Callus	Nonembryogenic Callus
Larch		
12(L1-18)5	85 ± 37	n.a.
24(L253-2)12	n.a.	848 ± 206
White Spruce		
5(WS3A)2	56 ± 7	n.a.
5(WS3A)20	86 ± 37	n.a.
5(WS3A)31	n.a.	371 ± 20
5(WS3A)37	n.a.	512 ± 117

n.a. = not available; N = 3

It was found that the nonembryogenic tissue need not be green to be loaded with reductants. For example, 17(NS384-1)1 callus, which had been green

but turned white but not embryogenic when cultured in the dark, yielded an A_{760}/g fr. wt. value of 1405 ± 206 in the total reductants test. Of course, the reductants may have built up while the callus was still green. Moderate tanning of white embryogenic larch callus which remained mucilaginous did not cause significant changes in its A_{760} value nor in embryo yield. Whereas neither white nor mucilaginous are foolproof characters alone, in combination they seem to be and are then always associated with low A_{760} values in our experience to date.

Table 30. Total reductants in Norway spruce callus from mature seeds.

	Reductants, A_{760}/g fr. wt.	
Callus Line	Embryogenic Callus	Nonembryogenic Callus
5(MNS472-2)9	8.7[b]	n.a.
5(MNS472-2)8	3.3[b]	n.a.
5(MNS472-3)4	10.6[b]	308[a]

n.a. = not available; N = 3; means followed by common superscripts are not significantly different as determined by ANOVA with Duncan's New Multiple Range Test ($p < 0.05$).

Net Protein Synthesis. Given that a complex morphogenic process like somatic embryogenesis presumably involves highly regulated sequential gene expression,[43] i.e., specific protein production and degradation, it was considered likely that protein turnover might be quite different in an embryogenic callus relative to a nonembryogenic callus. The literature in this area fosters this expectation.[44]

Using as a yardstick the appearance of tritium label in precipitable protein following incubation of the two callus types from single cell lines of Norway spruce with ^{3}H-leucine, the amount of radioactivity found in the proteins of embryogenic callus was far greater than that found in nonembryogenic callus (Table 31). Nevertheless, the Bradford analysis indicated that the total extracted

protein differed little in the two callus types. While the particular protocol employed in this type of analysis readily distinguished the two callus types from each other in work to date, it must be kept in mind that the data gathered so far reflect the net result of protein synthesis and degradation following an overnight incubation. We have some indications ([45] and unpublished) that catabolism as well as anabolism figures significantly in results like this, and further investigations are planned along these lines.

Table 31. Incorporation of ^{3}H-leucine into protein by embryogenic and nonembryogenic callus.

	Radioactivity, cpm/µg protein	
Callus Line	Embryogenic Callus	Nonembryogenic Callus
Norway spruce		
8(NS1)1	688[ab]	32[c]
8(NS1)5	764[a]	60[bc]
21(NS1)8	5061[a]	157[c]
Larch		
14(L1-6)2	1859[b]	n.a.
25(LD12-253)2	n.a.	53[c]

n.a. = not available; means followed by a common superscript are not signifcantly different as determined by ANOVA with Duncan's New Multiple Range Test ($p < 0.05$) run separately for two sets of data above and below the -------- line.

Other Markers. Two other potential markers of callus competence were explored with the Norway spruce model. One of these, isozymes, appeared in last year's conceptual plan as a potential marker. The other, spectra of callus extracts, was so obvious that it was almost overlooked. Since nonembryogenic Norway spruce callus is green whereas embryogenic callus appears to lack pigments, one would at least expect to see differences in the chlorophyll region of visible spectra of ethanolic extracts. Indeed this is the case (Fig. 31a), but differences

are also seen in the accessory pigment region (Fig. 31b) and in the ultraviolet (Fig. 31c). The entities responsible for the latter absorbance are thought to be contributing to the total reductants assay response as well. Note that both larch and Norway spruce extracts were prepared, and the results for the two species are largely parallel. Other spectral analyses might also be expected to reveal differences between the two callus types if needed, and some exploratory probes have been made. For example, Fig. 32 shows a difference FTIR (Fourier transform infrared) spectrum for the two callus types from Norway spruce. Note that the major difference occurs in the vicinity of 1600 wavenumbers where functional groups like carbonyls are probably responsible; however, in the context of this project, interpretation at that level is, for the moment, less important than the overall spectral difference.

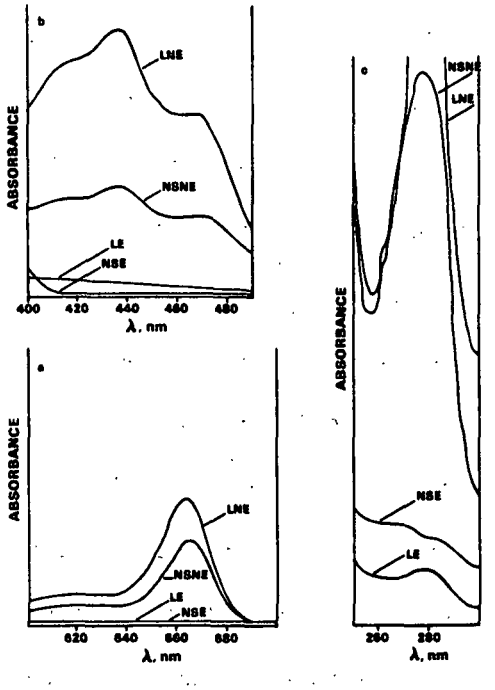


Figure 31. UV-visible spectra of ethanolic extracts of larch (L) and Norway spruce (NS) embryogenic (E) and nonembryogenic (NE) callus: (a) chlorophyll region, (b) accessory pigment region, (c) phenolic region.

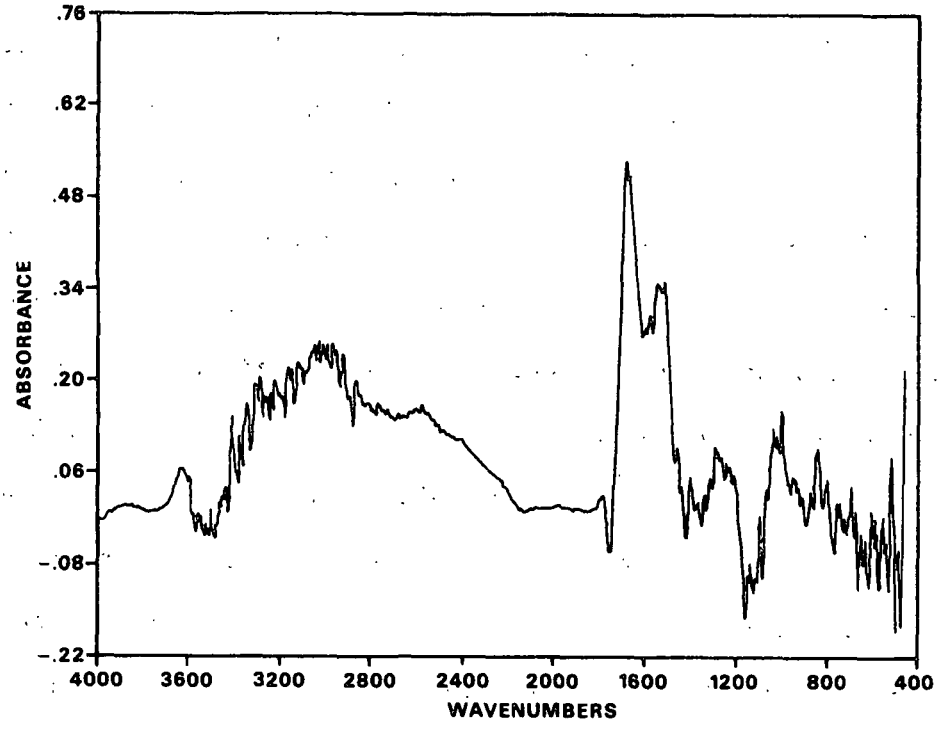


Figure 32. FTIR difference spectrum of nonembryogenic callus minus embryogenic callus of Norway spruce taken in the diffuse reflectance mode.

The isozyme patterns of a number of enzymes were examined for crude extracts of embryogenic vs. nonembryogenic Norway spruce callus. Both polyacrylamide and agarose gels were used in conjunction with electrophoresis or isoelectric focusing techniques. Most promising of the enzymes were acid phosphatase (Fig. 33) and peroxidase (Fig. 34), which both exhibited distinctive isozyme patterns for the two callus types across at least 3 cell lines. For some enzymes (17 stains were tried) activity could not be elicited from crude extracts; for others the patterns were too faint or indistinct, too variable, or not variable enough. However, these cursory results with crude extracts should not be taken to rule out the utility of other enzymes. Of four enzymes used recently to sort embryogenic callus of maize,[40] only esterase showed much promise in Norway spruce screening.

Conclusion

Several biochemical parameters were investigated and found capable of distinguishing embryogenic from nonembryogenic callus of the Norway spruce

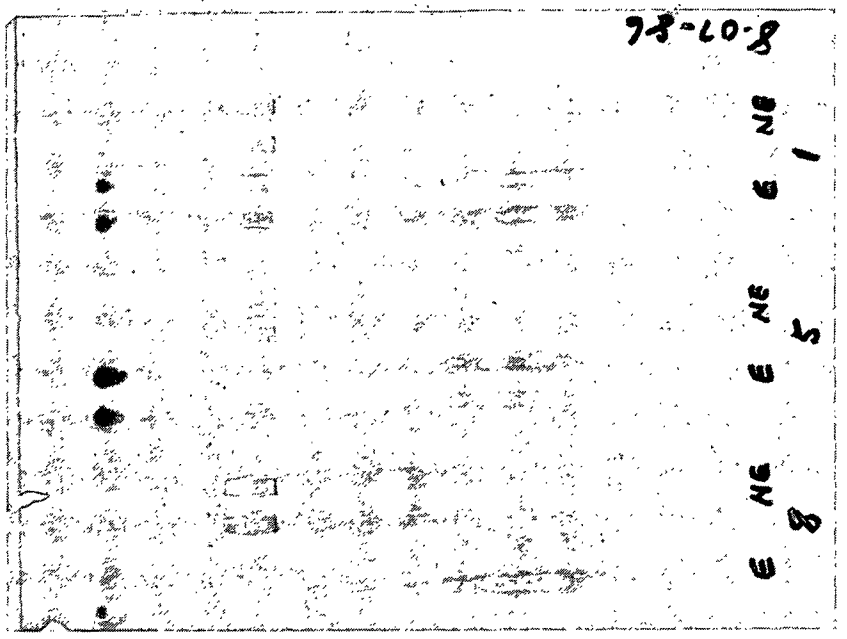


Figure 33. Acid phosphatase isozyme patterns of embryogenic (E) and nonembryogenic (NE) callus of Norway spruce cell lines 21(NS1)1, 21(NS1)5, and 21(NS1)8. N = 2.

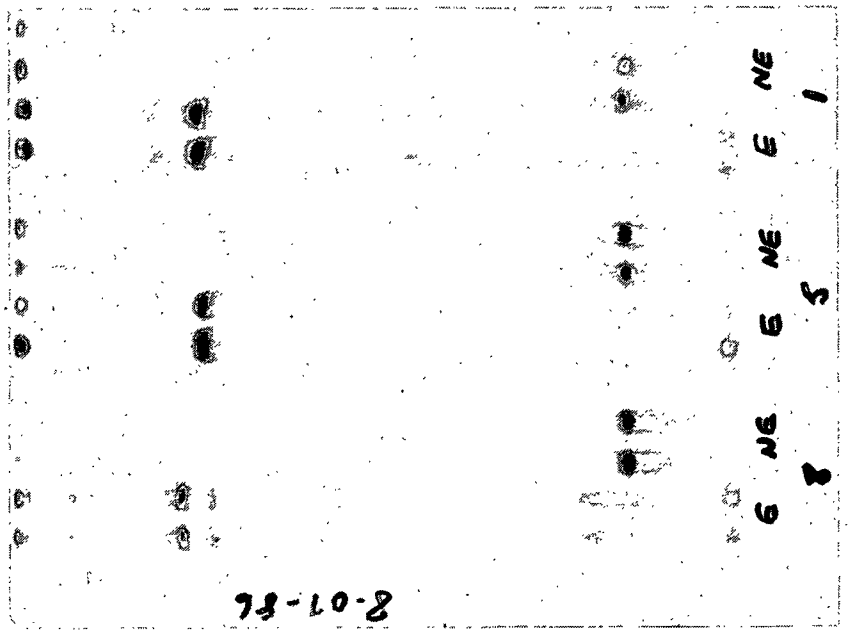


Figure 34. Peroxidase isozyme patterns of embryogenic (E) and nonembryogenic (NE) callus of Norway spruce cell lines 21(NS1)1, 21(NS1)5, and 21(NS1)8. N = 2.

model. Some, like the total reductants test and protein synthesis, also proved satisfactory for other conifer species such as larch and white spruce.

Deployment of Biochemical Markers in Support of New Embryogenic Callus Initiation Efforts

Introduction

Although it was established that many if not all of the biochemical markers discussed above could be marshalled to help sort embryogenic from nonembryogenic callus (at least in the case of Norway spruce), some of the tests were much easier to perform than others or held some other advantage such as being nondestructive. On the other hand, the greater value of markers rests with their predictive capacity, i.e., could they be used to sort the two types of callus well ahead of an investigator's ability to make an identification based solely upon visual/tactile markers? To this end, a fairly major but unsuccessful effort was mounted to use ethylene in this predictive manner. Ethylene evolution from embryogenic cultures had been consistently low in model systems. Furthermore, ethylene analysis did not require a large sample, it was nondestructive, and once set up, it was not terribly complex or time consuming to assay sizable batches of samples.

The total reductants test also has been moving in the predictive direction, being employed at early stages of callus formation with efforts currently under way to push it back still further if possible. These attempts to extract predictability from the markers are described below.

Materials and Methods

The analytical techniques for ethylene and total reductants were presented earlier in this report (p. 84-5). Likewise, the explanting of tissue from the 1986 summer cone collections for embryogenic callus production was described elsewhere (p. 10-11, 27-29).

Results and Discussion

The ultimate (or nearly so) in predictability was envisaged as being able to ascertain embryogenic competence at the explant stage. Accordingly, experiments were set up to see if there was any correlation between ethylene evolution rates from the original explants and subsequent embryogenic callus production. White spruce rather than Norway spruce was used in most of these highly replicated investigations because of its greater availability. Due to problems like contamination, reruns were necessary, and it took some time to bring this white spruce research to a conclusion. The results of the most recent trial are given in Table 32. In this case 3 of 20 replicates were lost to contamination, but beyond that, it is apparent that ethylene evolution rate provided no significant differential between embryogenic and nonembryogenic callus in white spruce, contrary to previous experience with Norway spruce. Furthermore, it does not appear hopeful from these data that one would be able to use ethylene evolution from the immature zygotic embryo to predict the subsequent appearance of embryogenic callus. However, subcultured white spruce callus free of explant has yet to be tested, and it may yet fall in line with the results on Norway spruce. What does fall out from the data is that explants showing very little ethylene production are not going to produce much callus of any type and could be discarded early in the case of white spruce.

Table 32. Ethylene evolution rates of zygotic embryo explants and resulting cultured tissue of white spruce.

	Ethylene Evolution, nL/explant or callus/day*		
Culture Growth	Embryo Explant	Cultured Tissue**	N
Little to none	0.048 ± 0.070	0.146 ± 0.176	4
Embryogenic	0.551 ± 0.145	0.965 ± 0.679	9
Nonembryogenic	0.444 ± 0.147	0.812 ± 0.521	4

*± S. D.
**Callus still attached to explant.

Meanwhile, the monitoring of ethylene from loblolly pine explants of nucellus, gametophyte, and immature zygotic embryo had to go forward as these tissues became available from the 1986 summer cone collections. Data for four collection dates of zygotic embryo explants of loblolly pine are presented in Table 33. Since none of these nor many other loblolly pine explants that had been examined for ethylene evolution resulted in embryogenic callus production, it was not possible to establish any positive correlations for loblolly pine explants. One might be tempted to conclude that there was perfect correlation between ethylene evolution and nonembryogenic callus production but for two caveats. First, unlike the white spruce case cited above, for loblolly pine no correlation was found between general transferable callus production and ethylene evolution rates. Secondly, the loblolly pine ethylene evolution rates such as found in Table 33 were not higher than those which accompanied embryogenic callus formation in white spruce. Although two species were compared here, the data weakens ethylene as a useful marker for the loblolly pine target species; however, it is still not known if it could sort embryogenic from nonembryogenic loblolly pine callus once produced, and some further checking on established white spruce callus is needed.

It should be noted that there were some aspects of experimental design that were investigated along the way. However, while these probes indicated that conditions may not have been optimal throughout these studies, their uniformity during the investigations reported here allays any doubt about relative values, i.e., the kinds of comparisons and interpretations made above should remain valid on this account. Among factors investigated were (1) the time lapse before measurement to allow any wound ethylene response to pass, (2) the need to subculture the explants during callusing after gas measurement, (3) media or stopper contributions to measured ethylene, (4) sample size versus gas

evolution rate, and (5) permeability of vial stoppers. The last mentioned was examined because of a report in the literature warning of the unsuitability of some types of stoppers.[46]

Table 33. Ethylene production by two sources of loblolly pine zygotic embryo explants from four cone collection dates.

Explant		Date of Analysis	Medium, auxin/cytokinin in mg/L Ethylene Evolution, nL/explant/day ± S. D. MSG2/1	MSG10/2	1/2MSG,2/5
LP12	G	7/30/86	0.5 ± 0.2	0.6 ± 0.2	0.6 ± 0.2
	I	7/30/86	0.6 ± 0.1	0.7 ± 0.2	0.5 ± 0.1
LP13	G	8/6/86	0.6 ± 0.1	0.6 ± 0.3	0.5 ± 0.2
	I	8/6/86	0.7 ± 0.2	0.6 ± 0.1	0.6 ± 0.2
LP14	G	8/13/86	0.8 ± 0.3	0.7 ± 0.2	0.8 ± 0.1
	I	8/13/86	0.5 ± 0.5	0.9 ± 0.4	0.6 ± 0.2
LP15	G	8/21/86	0.4 ± 0.2	0.5 ± 0.1	0.4 ± 0.2
	I	8/21/86	0.4 ± 0.4	0.2 ± 0.1	0.3 ± 0.1

For explant collection dates, see coding key. Correlation coefficients between ethylene production rates and transferable callus formed: for G only, r = -0.21; for I only, r = -0.29; for G and I, r = - 0.26. N = 4.

The total reductants test was also applied to several new calli arising from immature zygotic embryo explants of the target species set out in 1986. The range of values encountered for Douglas-fir and loblolly pine calli is exemplified by an array of 15 of these cell lines in Fig. 35. Based upon past experience with the model systems, it is unlikely that any of the depicted calli beyond the first 3 or 4 with the lowest A_{760} values would be embryogenic. Note that the latter are all Douglas-fir. As of this writing, none of the calli in this array have proven to be embryogenic; furthermore, several other cell lines tested but not shown with this group also failed to produce any embryogenic callus.

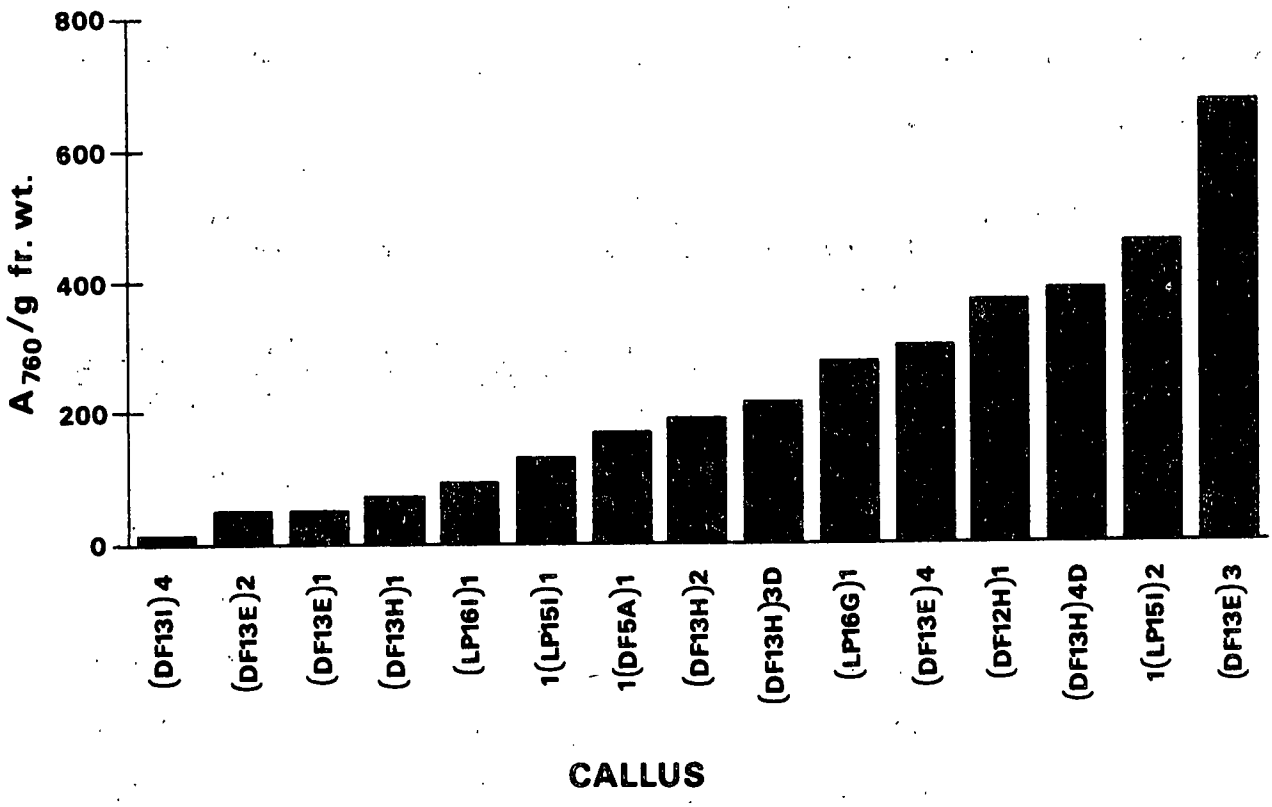


Figure 35. Total reductants in callus formed from some Douglas-fir and loblolly pine zygotic embryo explants (1986 summer collections).

Calli originating from explants of other pine species and from mature zygotic embryos of Douglas-fir were subjected to the total reductants test also. Test values for the other pines which include several promising cell lines are given in Table 34. Among these cell lines, there is at least one embryogenic callus with a low A_{760} value, namely, the white pine. Among cell lines not shown, no embryogenic callus was obtained from one of the mature seed Douglas-fir lines (MDF495-3)3D despite its low A_{760} value, but it failed to meet both of the visual/tactile criteria (it was white but not mucilaginous).

Conclusion

Contrary to expectations from earlier results with Norway spruce, the ethylene test has so far failed to sort callus types or predict the impending

appearance of embryogenic callus in white spruce. It did presage a lack of general callus production in white spruce, but even this correlation was not apparent for loblolly pine. While some improvements in technique are possible, the value of ethylene as a predictive marker at the explant stage for the target species has waned. Conceivably, it could still prove useful at a very early callus stage.

Table 34. Total reductants in callus from some zygotic embryo explants of various pine species (1986 summer cone collections).

Callus Line	Embryogenic	Reductants, A_{760}/g fr. wt. ± S. D.
Pitch Pine		
(PP6B)	no (white)	33 ± 6
1(PP6B)	no (white)	4 ± 7
(PP12B)1	no (white)	54 ± 11
White Pine		
5(WP3B)1	yes (white and mucilaginous)	28 ± 5
(WP3B)	no (white)	430 ± 26
(WP2A)2	no (green)	522 ± 150
3(WP5B)2	no (green)	298 ± 71
Pond Pine		
(P010 Ag)1	? (white)	7 ± 3
(P010 Ag)2	? (white)	5 ± 3
1(P015De)	no (green)	457 ± 136
2(P010 Ag)	no (green)	293 ± 64

N = 3

The total reductants test results to date indicate that a low A_{760} value is a necessary but perhaps not sufficient indicator of embryogenic callus in all cases. Whether it could be applied at the explant stage is not known but is currently under investigation.

APPLICATIONS OF BIOCHEMISTRY TO SOMATIC EMBRYO DEVELOPMENT IN NORWAY SPRUCE

Introduction

Since its inception, Project 3223 has been concerned with biochemical aspects of somatic embryogenesis. Attempts to identify metabolic pathways critical to or associated with somatic embryogenesis have historically centered around the process in wild carrot. In carrot, two distinct patterns of growth can be obtained by the simple manipulation of the exogenous auxin level (2,4-D). In the presence of 2,4-D small, nodular clusters of cells without any resemblance to a plant are continuously produced in liquid suspension cultures. Upon removal or dilution of the original 2,4-D level, signs of organization appear in as little as seven days, and readily distinguishable embryos are visible in 21 days. In the past, these two patterns of growth have been considered almost as unrelated processes - growth in the presence of 2,4-D had been considered as unorganized growth whereas only growth in the absence of 2,4-D was considered embryogenesis. However, as embryogenesis was observed in an increasingly diverse array of species, and, as the process was studied in much more detail in carrot, it was realized that embryogenesis was not simply triggered from an unorganized state by the removal of auxin. Several observations that have dispelled this notion are

1. The observation in many species that embryogenesis, and even limited embryo development can occur in the presence of 2,4-D. For our purposes, spruces are a noteworthy example in this regard, but many other examples exist as well.[15]
2. Embryogenesis can increase with increasing 2,4-D concentrations, as observed in celery.[47]

3. Addition of 2,4-D is required for embryogenic determination in domestic carrot.[48]
4. In carrot, chloroplasts specific to embryos are found in cultures grown under + 2,4-D conditions (Feirer, unpublished).

The four observations listed above all indicate that the removal of 2,4-D does not initiate embryogenesis but rather allows for the completion of embryo development. Thus, it can be stated that embryogenesis begins in the presence of 2,4-D, but embryo development is suppressed under these conditions.

The above discussion implies that previous biochemical studies of carrot embryogenesis were studies not so much of the biochemistry of somatic embryogenesis, but rather that portion relating to somatic embryo development. In order to examine the importance of various metabolic pathways in embryogenesis we must look not only at events associated with embryo development, but at embryo determination and initiation as well. The following section describes attempts to elucidate whether certain biochemical features that appear to be associated with an embryogenic condition (i.e., markers) are involved with embryo proliferation (+ 2,4-D) or embryo development (- 2,4-D).

The biochemical features examined in this section pertain to the two redox markers (GSH levels and nonspecific reducing agents) that have successfully identified an embryogenic condition. Inasmuch as low GSH levels and a lessened reducing capacity as measured by ferric ion reduction were associated with an embryogenic condition, inhibiting the biosynthesis of GSH and phenolic acids (i.e., the proposed nonspecific reductants) may promote or enhance some aspect of the embryogenic condition. For GSH and phenolic acids, two specific inhibitors of their synthesis are available in BSO (buthionine sulfoximine) and AOPP

(aminooxypropionic acid), respectively. The synthesis of GSH is blocked by BSO through inhibition of glutamyl cysteine synthase, and AOPP blocks phenolic acid synthesis through inactivation of phenylalanine ammonia lyase (PAL).

Methods

To test the effects of the specific enzyme inhibitors, buthionine sulfoximine (BSO) and aminooxypropionic acid (AOPP) in Norway spruce, the compounds were filter sterilized and added to either the embryo proliferation (containing 2,4-D and BA) or embryo development (containing IBA and ABA) medium. In experiments in which the inhibitors were added to the embryo proliferation medium (3 callus pieces/plate; 3 plates/treatment), calli were grown for 2 culture intervals of two weeks duration prior to transfer to an inhibitor-free developmental protocol. Three weeks into the developmental protocol (1 week on basal medium plus charcoal; 2 weeks on embryo development medium) callus pieces were weighed and dispersed, and the level of somatic embryogenesis was determined as described.[27] In experiments in which the inhibitors were added to the embryo development medium, callus lines were transferred from embryo proliferation medium to charcoal-containing medium for 1 week. After this time, callus pieces were kept for two weeks on embryo development medium containing the inhibitors, after which the level of somatic embryogenesis was determined as above.

In experiments pertaining to the effect of BSO on embryo maturation, callus pieces were transferred to renewed medium containing the inhibitor for an additional two weeks. At the end of this time, the number of embryos that had turned green and had begun to develop cotyledons was scored.

Results and Discussion

The results show that the addition of BSO and AOPP to embryo development media can result in an increase in somatic embryogenesis in some cell lines, but

addition of the inhibitors to embryo proliferation medium always resulted in a decrease in embryogenesis (Table 35 and 36). Therefore, it seems that both GSH levels and PAL activity (i.e., phenolics) are associated with an embryogenic condition, but probably in the developmental aspects of somatic embryogenesis. However, the role of GSH and phenolic acids in the process of differentiation was also apparent by the lowered embryo production in the presence of 2,4-D. In the case of GSH, the inhibition of embryo proliferation could be understood in terms of the requirement for some type of de- or undifferentiated event to produce a proembryo. If BSO reduces GSH levels to the extent that this cannot occur, embryogenesis will be reduced. Following this reasoning, it would seem that adding GSH to 2,4-D supplemented medium would promote more embryo proliferation, but the type of proliferation promoted does not necessarily lead to embryogenesis [Table 35, (NS1)13]. A precedent exists for this in wild carrot, as GSH concentrations are similar for carrot growing proliferatively or in an organized fashion during the early stages of embryogenesis.[36] However, no such precedent has been investigated for AOPP inhibition of PAL, and the decline in somatic embryogenesis exhibited when AOPP is added to the proliferation medium is unexplained. Note that the cell line in which AOPP promoted embryo development [(NS1)2] could be considered a marginally embryogenic cell line (control = 170 embryos/g) compared to (NS1)12, which was unaffected by the addition of AOPP. Therefore, it appears that treatment with AOPP can be used to boost embryogenesis in poor lines, but good lines remain unaffected. This was not observed in additions of BSO, where embryogenesis was enhanced in a line that was already highly embryogenic, (NS1)5.

The enhancement of embryo development by BSO was further demonstrated in studies in which somatic embryos were held on embryo development medium for longer (4 weeks) time intervals (see Table 37). At 10^{-5}M, BSO resulted in a

Table 35. Effect of BSO and GSH on somatic embryo proliferation (HM 2/1) and development (HM I/A).*

Treatment	Somatic Embryo Density, embryos/g		
	HM 2/1	HM I/A	
Cell Line	(NS1) 13	(NS1)1	(NS1)5
Control	820[a]	460[a]	510[b]
10^{-5}M BSO	470[b]	500[a]	610[ab]
10^{-4}M BSO	320[bc]	510[a]	810[a]
5 x 10^{-3}M GSH	200[c]	230[b]	470[b]

*Means followed by a common superscript are not significantly different as determined by ANOVA with Duncan's New Multiple Range Test ($p \leq 0.05$).

Table 36. Effect of AOPP on somatic embryo proliferation (HM 2/1) and development (HM I/A).*

Treatment	Somatic Embryo Density, embryos/g		
	HM 2/1	HM I/A	
Cell Line	(NS2) 14	(NS1)2	(NS1)12
Control	450[a]	170[b]	460[a]
10 µM AOPP	330[ab]	210[b]	360[a]
20 µM AOPP	300[b]	340[a]	430[a]

*Means followed by a common superscript are not significantly different as determined by ANOVA with Duncan's New Multiple Range Test ($p \leq 0.05$).

Table 37. Effect of BSO and GSH on embryogenic and embryo maturation in Norway spruce.*

Treatment	Embryo Density, embryo/g	Maturation Frequency, %
Control	400[a]	12[b]
10^{-5}M BSO	330[a]	25[c]
10^{-4}M BSO	760[b]	0.21[a]
5 x 10^{-3}M GSH	200[a]	0.41[a]

*Means followed by a common superscript are not significantly different as determined by ANOVA with Duncan's New Multiple Range Test ($p \leq 0.05$).

doubling of the maturation frequency, without a reduction in the embryo density. However, at a high level (10^{-4}M) there was an "explosion" of embryogenesis resulting in 100% increase in the embryo density but with an extremely low maturation rate. It is apparent that by altering BSO levels the desired response (enhanced density or maturation) can be achieved. It should be noted that BSO has not been observed to improve embryo development in cell lines in which poor maturation rates are the norm. This observation has been especially borne out in cell lines derived from mature NS embryos for which a developmental protocol has yet to be formulated.

The preliminary results with AOPP and BSO imply that the wealth of data accumulated over the years by Project 3223 on wild carrot somatic embryogenesis will be directly applicable to conifers. Much of this data will find an application not in the induction of morphogenesis in unresponsive cultures, but more in an increase in the efficiency of development of somatic embryos from competent cultures. Currently, the low embryo maturation rate is perhaps the most serious problem hindering the use of somatic embryogenesis in conifers as a plant propagation system.

EXPLORATORY RESEARCH

ORIGIN AND DEVELOPMENT OF SOMATIC EMBRYOS IN NORWAY SPRUCE AND WHITE SPRUCE

Introduction

Somatic embryogenesis in tissue cultures of conifers is a recent event and was first described in Norway spruce[1] and European larch.[16] Since then, sugar pine,[11] radiata pine (personal communication, Smith, 1986) and white spruce[49] have been added to the list of coniferous species that are capable of asexual reproduction *in vitro* via somatic embryogenesis. In all these reports, however, information regarding the origin and development of somatic embryos is lacking. In this paper we report the single cell origin and development of somatic embryos in Norway and white spruce.

Materials and Methods

Callus Initiation

Immature female cones of *Picea abies* (L.) Karst. (Norway spruce) and *P. glauca* (Moench) Voss (white spruce) were collected from trees located near Appleton, WI, as described by Becwar *et al.*[5] Seeds were removed from cones and surface sterilized in commercial bleach (20% V/V) for 15 min and rinsed three times with sterile distilled water. Immature embryos were dissected from female gametophytic tissue and cultured on basal medium. The basal medium contained macro- and micronutrients and vitamins as reported by Hakman *et al.*[1] and gelled with 0.5% Bactoagar (Difco). The sugars used were 3.4% sucrose, arabinose (150 mg/L), glucose (180 mg/L) and xylose (150 mg/L). The growth regulators were 2 mg/L 2,4-D and 1 mg/L BAP.

The cultures were incubated at 23°C with 16 h irradiance (15-50 μ E m^{-2} sec^{-1} at culture level) from cool white fluorescent and incandescent lights.

Histological Techniques

The cultures were examined at 24 h intervals for a period of two weeks through a dissecting scope illuminated with fiber optic lights at 15X. Additionally, the entire explant along with the induced callus was placed on a glass slide and stained with 0.5% toluidine blue in glycerin and pressed gently with a cover glass. The entire preparation was then observed under a Zeiss photomicroscope fitted with phase contrast optics to follow the cellular origin and development of somatic embryos.

Results

Origin of Somatic Embryos

The immature zygotic embryo consisted of three distinct regions: a pointed radicle, and a cylindrical hypocotyl terminating in a ring of 6-8 cotyledons (Fig. 36). A mass of loose white callus was initiated at the radicle end of the embryo after 48-96 h of culture (Fig. 37). The cells of this callus were long, narrow and coiled with dense cytoplasmic contents. Formation of the callus at the radicle end was followed by proliferation of the outer 2-4 layers of the hypocotyl region of the explant. This resulted in the sloughing off of the hypocotyl portion from the rest of the explant. The outer cell layers of the hypocotyl divided profusely to form a mass of callus which was phenotypically different from the callus of radicle origin. The hypocotyl callus was white to translucent, glossy, and mucilaginous, (Fig. 37). The cells of this callus were broader (Fig. 38) than the cells of the radicle callus.

After 10 days of culture some of the cells of the mucilaginous hypocotyl callus became distinct from the surrounding cells. Figure 39 represents a 2-celled proembryo formed by a quantal or unequal cell division in an embryogenic cell. This division gave rise to a distal, small semicircular embryonal

initial (ei) cell with dense cytoplasmic contents and proximal large suspensor initial (si) cell (Fig. 39).

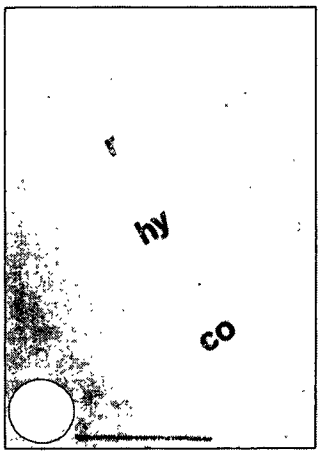


Figure 36. Immature zygotic embryo explant with radicle (r), hypocotyl (hy), and cotyledons (co). Scale bar = 1 mm.

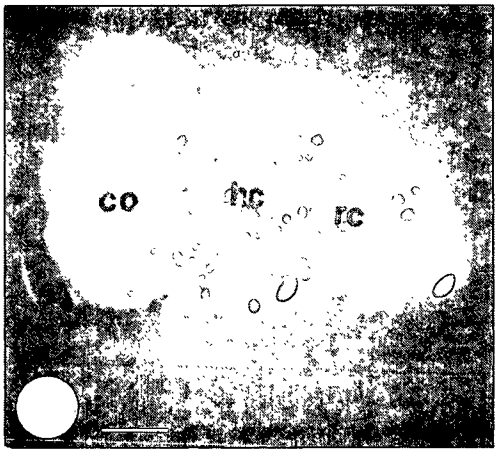


Figure 37. Initiation of callus from the radicle (rc) and the hypocotyl (hc). Scale bar = 1 mm.

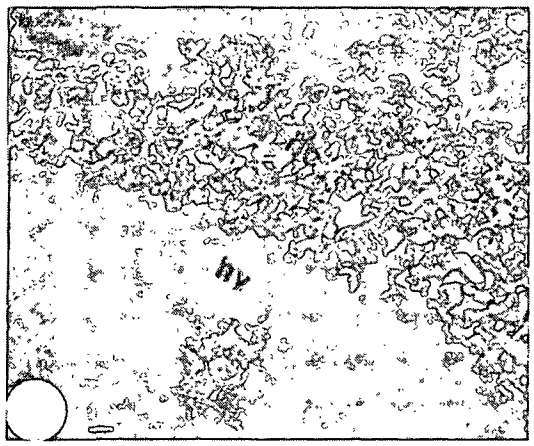


Figure 38. Section of the hypocotyl (hy) proliferating to form hypocotyl callus (hc). Scale bar = 10 µm.

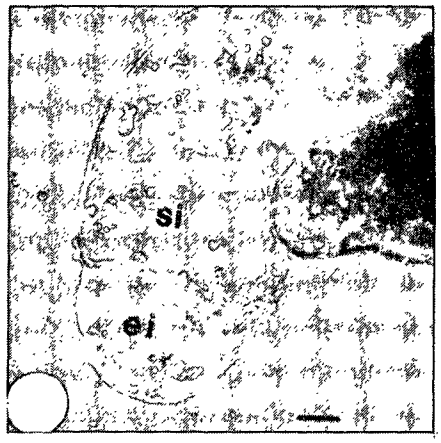


Figure 39. Two-celled somatic proembryo with embryonal initial (ei) and suspensor initial (si). Scale bar = 10 µm.

Development of Somatic Proembryos

The term somatic proembryo is used here in accordance with the terminology used for the description of zygotic conifer embryo development.[50]

The embryonal initial cell divided further by both periclinal and anticlinal divisions, resulting in an early somatic proembryo consisting of 4-16

cells arranged in 1-3 tiers, constituting the embryonal tier (et) (Fig. 40, 41). The embryonal tier of cells was subtended by 2-4 broad suspensor cells. Cell divisions in the suspensor tier (st) occurred later than in the embryonal tier, resulting in the formation of a somatic proembryo with an embryonal mass (em) of 16-32 cells which were densely cytoplasmic with prominent, centrally located nuclei, subtended by 2 or 4 broad suspensor cells with prominent nuclei. The cells of the suspensor were considerably enlarged and vacuolated with sparse peripheral cytoplasm (Fig. 42).

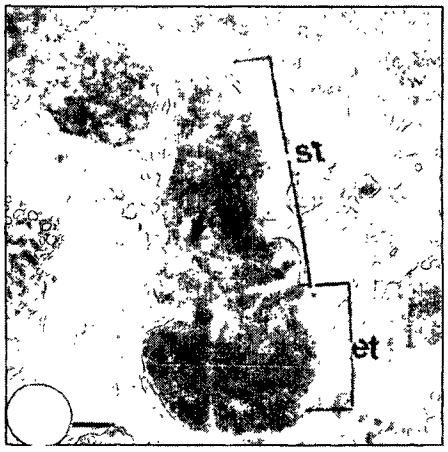


Figure 40. Four celled somatic proembryo with embryonal tier (et) and suspensor tier (st). Arrow points to cell wall between two suspensor cells. Scale bar = 10 µm.

Figures 43 and 44 represent progressive stages of somatic embryo development, where cells of the st divided repeatedly, accompanied by elongation of the suspensor segments. This resulted in a long-coiled suspensor (Fig. 43). Simultaneously, the cells of the em divided further to produce an embryonal mass characteristic of conifer embryogeny (Fig. 44). The events in somatic embryogenesis of both Norway and white spruce essentially followed the same sequence

and were completed in about two weeks. Fully differentiated somatic embryos with 6-8 cotyledons appeared very similar to conifer zygotic embryos both in external morphology (Fig. 45) and internal anatomy.[5]

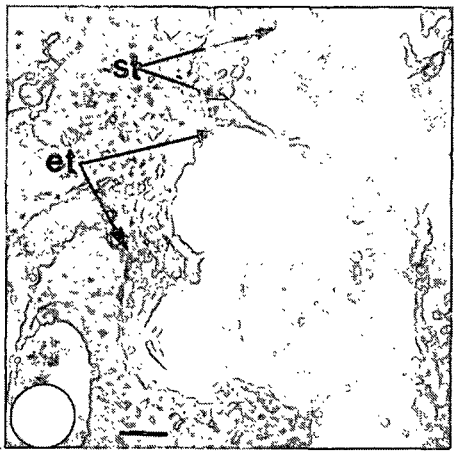


Figure 41. Somatic proembryo with 2 to 3 embryonal tiers (et) subtended by suspensor tier (st). Scale bar = 10 µm.

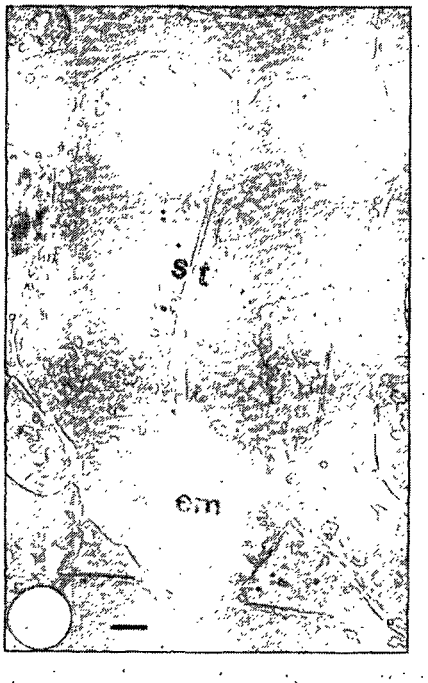


Figure 42. Somatic proembryo with an embryonal mass (em) and broad cells of suspensor (st). Scale bar = 10 µm.

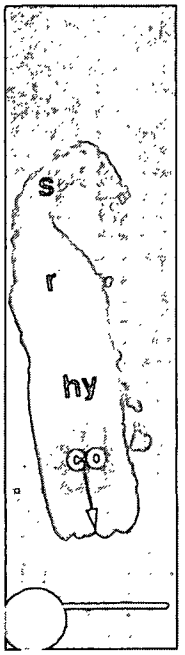


Figure 45. Fully developed somatic embryo with radicle (r), hypocotyl (hy), a ring of cotyledons (co) and remnants of suspensor (s). Scale bar = 1 mm.

Discussion

The explants used for obtaining somatic embryos in conifer species vary. In Norway[1] and white spruce,[49] immature embryos were used as explants, whereas in European larch[16] the explant source was immature female gametophytic tissue. In sugar pine[11] the mature embryo from 5-year old seeds and in radiata pine (personal communication, Smith, 1986) the entire fertilized ovule were used as explants. The origin of somatic embryos may vary depending on the type of explant material used. In particular, do somatic embryos arise directly from the explant or via callus formation? The present study shows that in both Norway and white spruce callus phase precedes somatic embryogenesis.

The part of the explant from which callus originates appears to be the epidermal or subepidermal tissue of the hypocotyl. In angiosperms, hypocotyl

epidermal tissue has also been reported to be an important source of adventitious embryos or embryogenic tissue.[51] A recent report of somatic embryogenesis from excised cotyledons of 7-day old seedlings[25] of Norway spruce suggests that epidermal or subepidermal tissue of the entire embryo (except the radicle) may be embryogenic under appropriate conditions. Also in pearl millet and several other graminaceous species, the histological studies of cultured immature embryos have shown that single subepidermal cells at the periphery of the scutellum undergo internal segmenting divisions resulting in discrete groups of richly cytoplasmic cells. Continued divisions in these groups of cells resulted in the formation of somatic embryos.[52,53]

In our study we make a further distinction between two types of callus arising from the cultured embryos. Callus arising from the radicle part of the zygotic embryo is nonembryogenic, while that from the hypocotyl is embryogenic in nature. Similarly, cereal tissue cultures are also heterogeneous, and visual selections can be extremely important in recognizing embryogenic callus.

The origin of somatic embryos in both Norway and white spruce could be traced to single cells of the callus mass produced from the hypocotyl region of the zygotic embryo. These single cells did not undergo any early free nuclear divisions that are characteristic of early zygotic embryogenesis in spruce.[54] Instead, the single cells divided by quantal cell division that produced two unequal daughter cells with an unequal distribution of cytoplasmic contents forming embryonal and suspensor initials.

ULTRASTRUCTURAL STUDIES OF EMBRYOGENIC CONIFER CULTURES

Introduction

Perhaps the most distinguishing feature of embryogenic Norway spruce callus is its white or translucent appearance, even when grown in the light.

The nonembryogenic callus is characteristically green when grown under these conditions. Embryogenic larch callus, obtained from Dr. Jan Bonga's lab, also exhibits a pale, translucent phenotype. In addition to biochemical studies of the two callus types, morphological and ultrastructural examinations of the cells are being carried out to better characterize embryogenic conifer callus. We hoped to determine if the lack of green coloration of the embryogenic calli was due to a lack of chloroplasts, a deficiency of chlorophyll within the chloroplasts, or the presence of another form of plastid such as a proplastid that is typically found in early zygotic embryos or meristems. Mature chloroplasts arise from progenitor plastids in meristems and embryos via a defined developmental pathway progressing through eoplasts, amyloplasts, amoeboid plastids (all three of which may be termed proplastids) to mature chloroplasts. Although the terminology and order of the progression through the proplastid forms is subject to some variation in the literature, the general scheme outlined above appears to be well accepted[55] (see Fig. 46). Our ultrastructural studies of cultured and intact tissues have concentrated on the morphology of plastids. To date, chloroplasts (plastids) in Norway spruce white and green calli, somatic embryos, zygotic embryos and seedlings have been examined. Observations of other conifer species (and carrot in conjunction with thesis research by Russell Feirer) are also being carried out, some of which will appear below. Total chlorophyll levels in the tissues (chlorophyll a + chlorophyll b) as well as estimates of protochlorophyll have also been determined.

Methods

Samples were prepared for transmission electron microscopy (TEM) as follows: Tissue was fixed in 3% glutaraldehyde (in 0.05M sodium cacodylate buffer, pH 7.0) for 2-3 h, washed briefly with buffer, and postfixed in 1% OsO_4

in the same buffer for 2-3 h at room temperature. After a brief wash, samples were slowly dehydrated in ethanol at 4°C, followed by slow infiltration with SPURR at room temperature, and embedded by polymerization at 70°C for 6 h. The tissues were sectioned at 90 nm and stained with uranyl acetate [5% in 50% (v/v) ethanol] followed by lead citrate (0.5%, pH 12). To insure penetration/infiltration of fixative into seedling cotyledon sections and zygotic embryos, the tissues were subjected to several cycles of vacuum/atmospheric pressure while in fixative. (Photomicrograph legend: bar = 1 micron, G = grana, M = mitochondrion, N = nucleus, P = proplastid, Pb = prolamellar body, S = starch grain).

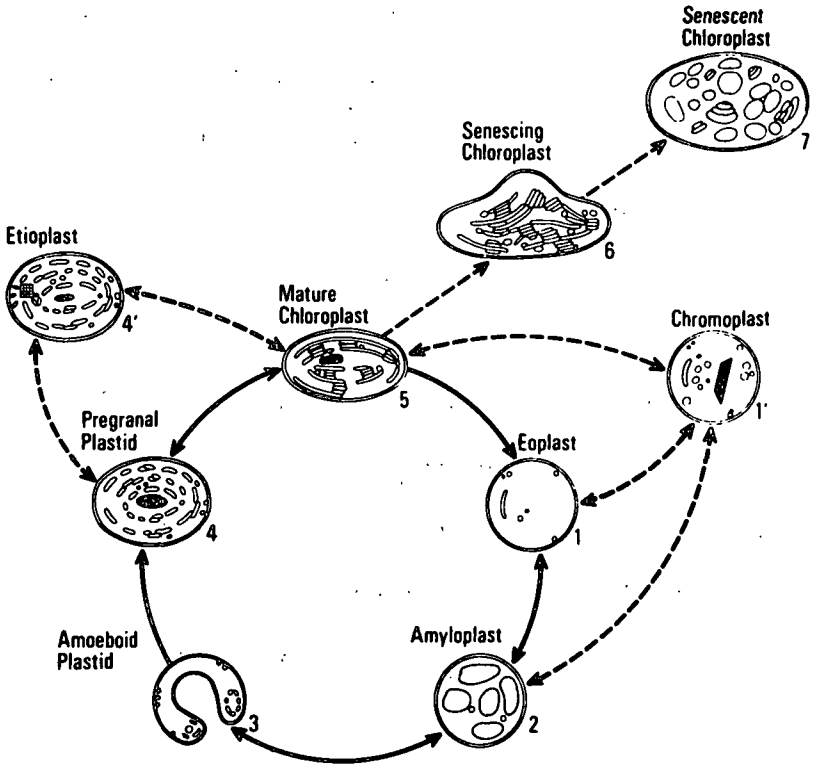


Figure 46. Plastid developmental cycle (taken from Thomson[55]).

Cotyledons were collected from seedlings germinated under normal greenhouse conditions (light grown) or in complete darkness (dark grown; all handling and extract preparation using these tissues was performed under a dim green safelight) 2-3 weeks after the seeds were sown.

Total chlorophyll was estimated by determining the A_{652} of an 80% acetone extract of the tissues. The O.D. was then multiplied by a constant to calculate µg chlorophyll.[56]

The relative ratios of protochlorophyll to chlorophyll were determined by examining emission peaks on a spectrofluorometer spectrum. A Perkin-Elmer 650-10s spectrofluorometer was used to generate an emission spectrum of the 80% acetone extract derived from the tissues. With an excitation wavelength of 436 nm, emission peaks are observed at 630 and 668 nm for protochlorophyll or chlorophyll, respectively.[57,58]

Results

The chloroplasts of light grown conifers are typical of those found in most higher plants. The organelles, usually 2 to 10 microns in length, contain starch grains, protein bodies and internal thylakoid membranes organized into grana (Fig. 47). The organization of the thylakoid membranes is dependent, however, upon the age and physiological state of the tissue.

The plastids in the embryogenic spruce callus exhibit a unique morphology at the ultrastructural level. The plastids, which appear more darkly stained (more electron dense) than mitochondria in our photographs, lack the internal organization of a mature chloroplast. At higher magnification some mottling is evident, which may reflect the presence of thylakoid membranes which are in a disorganized state (Fig. 48a,b). Some of these plastids contain small starch grains, although they are not nearly as large as the starch grains present in mature chloroplasts in leaf or cotyledon tissue. The plastids present in the green, nonembryogenic spruce tissues, on the other hand, appear to be similar to a typical chloroplast (Fig. 48c,d). Thylakoid membranes, some organized into grana, are found in all of the chloroplasts. Large starch grains are also prominent in some of the chloroplasts.

Zygotic embryos excised from mature Norway spruce seeds were also examined. As shown in Fig. 49a,b, the plastids resemble those observed in embryogenic spruce calli. Although a significant number contain prominent starch grains, they also lack organized thylakoid membranes.

In most higher plants, including conifers, several aspects of chloroplast development are regulated by light. If seeds are germinated in the dark or if other tissues are grown in darkness, chloroplast development may be altered such that etioplasts are formed. Etioplasts are colorless; therefore the tissues do not appear green and contain thylakoid membranes that are not organized into grana. A prominent prolamellar body is normally present, which may represent a temporary storage site of the thylakoid membrane components. Upon further development, such as that stimulated by light, thylakoid membranes are formed and appear to radiate from the prolamellar bodies to form grana (Fig. 50). It is obvious that the morphology of the chloroplasts in dark grown, pale colored Norway spruce and larch seedlings are notably different from those in the embryogenic calli. These facts provide further evidence that the plastid types observed in the somatic embryos are proplastids as found in zygotic embryos, and not etioplasts.

When total chlorophyll levels were estimated in acetone extracts of the tissues, the results obtained were not surprising and simply confirmed, on a quantitative basis, visual observations that had been described earlier (Table 38). Both embryogenic spruce and larch tissues contained significantly less chlorophyll than the corresponding nonembryogenic tissues. Somatic embryos of carrot, in early stages of development, exhibited low levels of chlorophyll whether grown in the light or darkness. As the embryos developed further (later torpedo and beyond) greening of the cotyledons did become pronounced. Chlorophyll synthesis in angiosperms is dependent upon light, with light being required in two steps in chlorophyll biosynthesis. Gymnosperms, according to limited treatment in most biochemistry and physiology texts, do not require light for either of these steps. While the data for carrot and spruce in Table 38 illustrate these facts, the chlorophyll levels in the dark germinated larch seedlings were

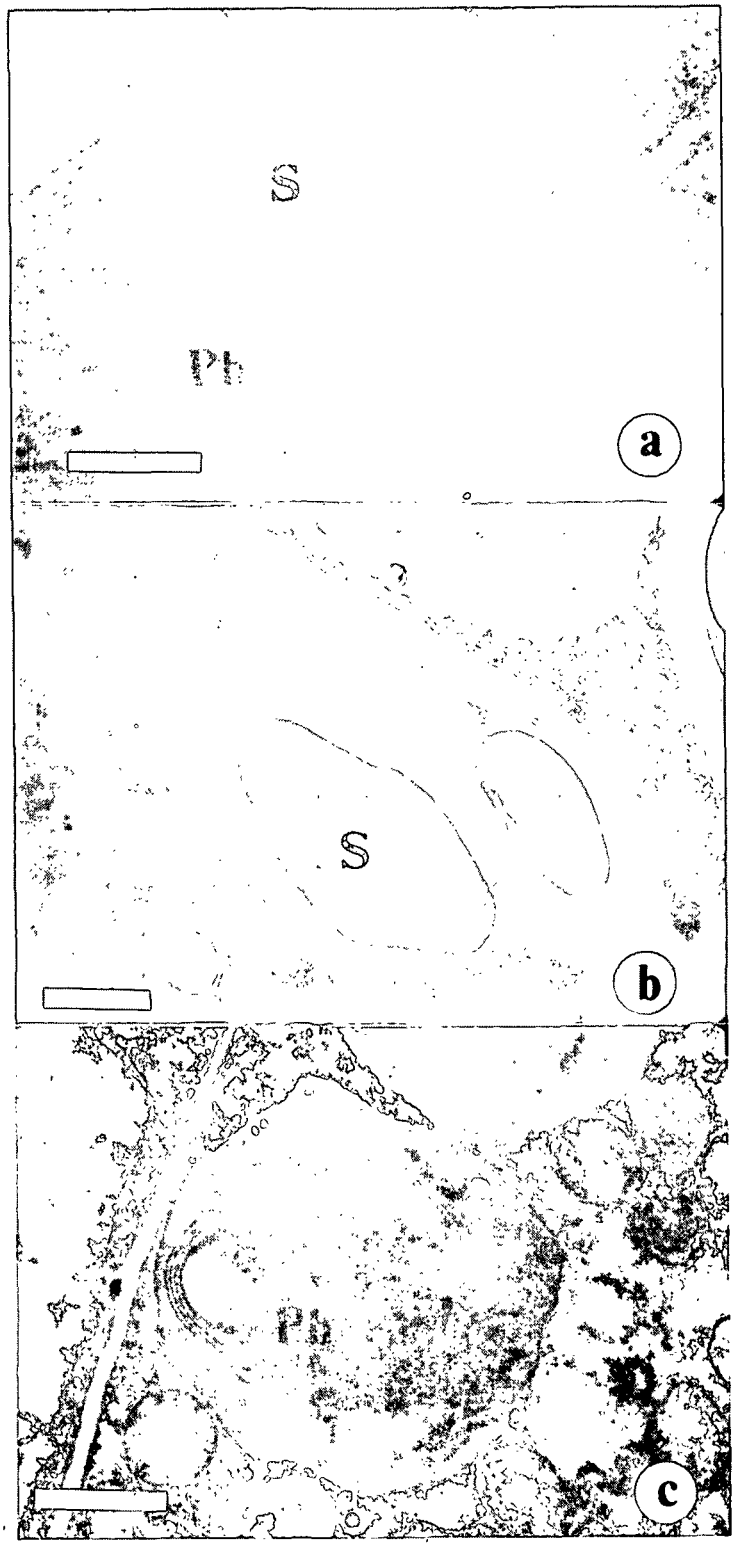


Figure 50. Chloroplasts from Norway spruce (a) and larch seedlings germinated in darkness (b,c).

significantly lower than those grown in the light. Whether or not chlorophyll biosynthesis in larch seedlings is light dependent remains to be resolved at this time.

Table 38. Chlorophyll content of various tissues (µg chlorophyll/gram fresh weight).

Tissue	Light Grown	Dark Grown
Norway Spruce		
Embryogenic 21(NS 1)8	15 ± 12	N.D.
Nonembryogenic 19(NS1)8R	96.1 ± 1	N.D.
Larch		
Embryogenic 15(1-18)5	8 ± 12	N.D.
Nonembryogenic 25(LD12-253-2)	135 ± 68	N.D.
Carrot (B 0493)	12 ± 3	13 ± 5 n.s.
Norway spruce cotyledons (3223-53)	1449 ± 596	1097 ± 148 n.s.
Larch cotyledons (XLD 6-82)	1757 ± 263	398 ± 219
Carrot cotyledons (B-0493)	1566 ± 633	299 ± 247

n.s. = indicates that the light vs. dark grown values are not significantly different.
N.D. = not determined.

The conversion of protochlorophyll (protochlorophyllide) to chlorophyll, one of the last steps in chlorophyll biosynthesis, is light dependent in angiosperms. Relative levels of these two compounds are easily determined by spectrofluorometric methods, with protochlorophyll and chlorophyll having emission maxima of 630 and 670 nm, respectively. These analyses showed that dark grown carrot and larch seedlings had major (carrot) or significant fractions (larch) of protochlorophyll. Chlorophyll predominated in dark grown tissues of spruce,

loblolly pine and Douglas-fir. Most importantly, however, chlorophyll predominated in the embryogenic spruce callus (no protochlorophyll was detected), suggesting that the white, translucent nature of the embryogenic Norway spruce calli is not due to a simple block in the final step in the biosynthesis of chlorophyll.

Discussion

The white or translucent appearance characteristic of embryogenic Norway spruce callus has served as a visual, primary marker of embryogenic or promising conifer calli. The finding that total chlorophyll levels are lower in the embryogenic tissues as compared to the nonembryogenic calli simply expresses our earlier visual observations on a quantitative basis. Two steps in the synthesis of chlorophyll in angiosperms are regulated by light, these being logical points of control of pigment biosynthesis by environmental or other influences. Since protochlorophyll does not accumulate in the embryogenic tissues, the lack of color in the white calli is probably not due to a block in the final steps of chlorophyll biosynthesis.

The morphology of the plastids seen in our embryogenic spruce and larch calli was at first both exciting and puzzling. To have an aspect of the ultrastructure of embryogenic tissue be so distinctive met our requirements of a marker of somatic embryogenesis in conifers. Troubling, however, was the fact that the plastid types observed in the embryogenic tissues did not resemble chloroplasts usually described in greening tissues, where chloroplasts are derived from etioplasts containing limited thylakoid membranes and/or prolammelar bodies. Further search of the literature revealed that the plastids observed in our tissues closely resemble descriptions and photographs of proplastids found in

developing zygotic embryos. In perhaps the most thorough ultrastructural examination of zygotic embryogenesis reported in the literature, the embryonic development of Capsella (shepherd's purse) was followed from the egg, 2-cell, 3-cell, heart and torpedo stages through the mature embryo.[59,60] In these reports, the one-cell zygote through the heart stage embryos were found to contain proplastids that appear very similar to those in our tissues. From comparisons of the micrographs and descriptions of the proplastids in these and zygotic embryos of other species, it is now clear that the proplastids found during zygotic embryogenesis are very much like those in our somatic embryos. During the heart and torpedo stages of Capsella embryogenesis, the proplastids further developed into green chloroplasts containing thylakoid membranes and grana. Especially notable was the observation that the proplastid stage persisted in the suspensor cells and the procambium of the developing embryo. Whether this persistence of proplastids in the suspensors is related to the nature of the responsive tissues in immature conifer seeds (suspensors or basal end of embryo) is an interesting question. Other, less rigorous ultrastructural examinations of zygotic embryogenesis have been performed in barley and nasturtium, where proplastids are again similar to those observed in our tissues.[61,62] The description of "colorless plastids with an electron-dense matrix and electron transparent tubes oriented in various directions" could certainly be used to describe the proplastids in some of our preparations.[61] Proplastids have also been observed in suspensor tissues of geranium.[63]

Among the numerous reports describing chloroplast morphology in greening or germinating conifer seedlings, only green chloroplasts or plastids containing prolamellar bodies are noted[64] (see also Fig. 50). Zygotic embryos of black pine (P. nigra) and jack pine (P. banksiana) have been examined at the ultrastructural level, although the authors either stated that proplastids were difficult to

recognize before germination[58] or simply identified the plastids as proplastids and did not elaborate on their presence or significance.[65] Proplastids similar to those noted in our embryogenic calli and zygotic embryos are evident in the micrographs in both of these reports, however.

There are few reports of the occurrence of proplastids in somatic embryos and cultured tissues. In early studies by Steward's group, the ultrastucture of carrot cultures was examined with particular attention given to plastid morphology.[66] In root explants and cells derived from the explants through 16 days of culture, a conversion of leukoplasts (old term for proplastids) to green chloroplasts was observed. The plastids described in that study originated in carrot root, however, and may not represent the condition found in zygotic or somatic embryos. Steward's group also characterized the ultrastructure of early plantlets grown in the light.[67] Mature, green chloroplasts were observed in the developing shoot, and leukoplasts were noted in the root. The proplastids did resemble those in our somatic embryos. Proplastids, not chloroplasts, were found in our tissues, due probably to the immature nature of the somatic embryos and state of the calli. Recent, preliminary work has shown that when our Norway spruce somatic embryos further develop (cotyledons beginning to green) chloroplasts having thylakoids and grana become evident. We have observed the same phenomenon in carrot, where somatic embryos beyond the torpedo stage, if grown in the light, contain green mature chloroplasts. It appears, then, that proplastids are especially indicative of early stages of embryonic development, whether in zygotic or somatic embryos. Proplastids have also been found in tobacco cultures grown in the dark.[68] The plastids from these dark grown tissues look similar to embryonic proplastids, but whether or not those cultures were competent was not discussed in that report.

In conclusion, available data suggest that the proplastids observed in the embryogenic spruce and larch calli are of the type found in zygotic embryos. The argument that the somatic embryos contain proplastids characteristic of zygotic embryos or meristems is strengthened by our observation of proplastids in conifer zygotic embryos as well as carrot zygotic and somatic embryos. These findings provide evidence in support of our earlier biochemical studies, which showed that many aspects of somatic embryogenesis mimic and correspond to in vivo zygotic embryogenesis. It is important to note, as did Steward's group, however, that the morphology of the plastids is most probably a consequence, and not a cause of somatic embryo development.[67] Chloroplast morphology may still be one of our best indicators that somatic embryogenesis is occurring and proceeding normally in our cultures.

Additional ultrastructural examinations of spruce and other conifer tissues continue in an effort to expand our findings to other proven and putative embryogenic cultures. Comparisons of developing zygotic and carrot somatic embryos of different stages and origins will be done in conjunction with student research.

SCREENING PINE SOURCES FOR MORPHOGENETIC POTENTIAL

Introduction

The study described in this section was based on the hypothesis that a correlation exists between organogenic and embryogenic potential of loblolly pine families. Because conifers are highly heterozygous (Bonga[9], p. 387-412) there may be considerable variation in their in vitro response among different families. Indeed, research by the North Carolina State University tree improvement program bears this out; families such as 9-17 have a higher organogenic

potential than numerous other families tested. Reports in the literature have suggested or provided evidence for a relationship between organogenic and embryogenic potential.[69]

In 1986 we undertook a survey of ten full-sib crosses of loblolly pine and five pitch pine families to identify seed sources with high organogenic potential. The ultimate goal of this work was to obtain explant material from the same seed sources for evaluation of embryogenic potential. We would thus be able to test the hypothesis of a correlation between organogenic and embryogenic potential.

Methods

Seeds for this study were derived from full-sib crosses of loblolly pine and families of pitch pine grown in Westvaco Corp. seed orchards in Summerville, SC. Seeds were collected from mature cones either in the fall of 1984 or 1985 and used in the study during January and February of 1986. Table 39 contains a list of seed sources used in the study.

The cotyledonary regeneration protocol of North Carolina State University was used to survey for organogenic potential.[17] The technique is outlined in Table 40.

Cotyledons from individual embryos were cultured in individual wells of six-well culture plates. At 17 days the percentage of the cotyledons from each embryo that appeared swollen and green was determined (Step 5 in Table 40). After an additional 28 days the percentage of cotyledons from each embryo that contained organogenic buds was also determined (Step 7 in Table 40).

Table 39. Seed sources for organogenic survey.

I. Loblolly pine (LP) full-sib crosses

1. LP 11 - 1135 x 18 - 1206
2. LP 11 - 1135 x 11 - 1017
3. LP 11 - 1052 x 11 - 1011
4. LP 11 - 297 x 11 - 283
5. LP 11 - 297 x 11 - 274

6. LP 11 - 1049 x 8 - 1003
7. LP 11 - 268 x 11 - 297
8. LP 11 - 268 x 11 - 283
9. LP 11 - 268 x 11 - 229
10. LP 11 - 268 x 11 - 274

II. Pitch pine (PP) families

1. PP 62
2. PP 65
3. PP 71
4. PP 75
5. PP 80

Table 40. Loblolly and pitch pine cotyledon regeneration protocol.

1. Germinate nicked seeds in 1% H_2O_2, 30°C, 4 days (exchange H_2O_2 daily), cool white fluorescent light, 24 $\mu Em^{-2}\ s^{-1}$.
2. Sterilize gametophyte 5 minutes with 15% Hi-lex.
3. Excise embryos and place cotyledons on BLG medium with 10 mg/L BAP.
4. Incubate for 17 days, 23°C, cool white fluorescent, 24 $\mu Em^{-2}\ s^{-1}$ and incandescent, 14 $\mu Em^{-2}\ s^{-1}$.
5. Determine % green cotyledons.
6. Transfer explants to 1/2 GD medium with charcoal. Incubate 28 days as in step 4.
7. Determine % organogenic cotyledons.

Results and Discussion

The results for the loblolly pine survey are presented in Table 41. The seed sources are ranked according to the percentage of organogenic cotyledons per embryo. There was a high level of variation in both the percentage of green cotyledons and organogenic cotyledons among cotyledons derived from different embryos within seed sources. This is evident by the results of statistical comparisons. Even so, the seed source which ranked highest (LP 268 x 229) was significantly more organogenic than the five lowest ranking seed sources.

It is also of interest how well the data on percentage of green cotyledons as determined at 17 days agreed with the later determination of organogenic potential (Table 41). Seed sources that ranked high at 17 days in terms of green cotyledons ranked high in their organogenic response.

Table 41. Comparison of the frequency of green loblolly pine explants after 17 days in culture to the frequency of organogenesis.

Full-sib Seed Source	Mean % Cotyledons per Embryo*		
	Green**	Organogenic***	N****
LP 268 x 229	94	96[a]	30
LP 268 x 297	88	83[ab]	30
LP 297 x 274	87	82[ab]	29
LP 268 x 283	82	72[abc]	27
LP 1052 x 1011	84	71[abc]	29
LP 268 x 274	70	63[bc]	24
LP 297 x 283	72	55[bcd]	28
LP 1049 x 1003	67	45[cd]	25
LP 1135 x 1017	66	44[cd]	29
LP 1135 x 1206	61	31[d]	26

*Means followed by a common superscript are not significantly different as determined by ANOVA with Duncan's New Multiple Range Test ($p < 0.05$).
**Determined after 17 days on induction medium, BLG with 10 mg/L BAP.
***Determined after 17 days on induction medium and an additional 28 days on 1/2 GD with charcoal.
****N is number of embryos from which cotyledons were excised, 5-6 cotyledons per embryo.

It should also be noted that the parent lines 268 and 297 were common to the top four organogenic seed sources, and that the parent line 1135 was in the least organogenic seed sources. These results, although preliminary, suggest that genetic differences may exist among the parental lines evaluated for organogenic capacity.

The results of the pitch pine survey of organogenic potential are presented in Table 42. Pitch pine family 62 ranked highest in percentage of green and organogenic cotyledons and ranked statistically higher than the three lowest families. There was complete agreement between the ranking of the five pitch pine families by percentage of green and organogenic cotyledons.

Table 42. Comparison of the frequency of green pitch pine explants after 17 days in culture to the frequency of organogenesis.

Half-sib Seed Source	Mean % Cotyledons per Embryo*		
	Green**	Organogenic***	N****
PP 62	90[a]	85[a]	17
PP 65	77[ab]	72[ab]	30
PP 75	64[b]	49[b]	27
PP 80	61[b]	49[b]	30
PP 71	57[b]	45[b]	28

*Means followed by a common superscript are not significantly different as determined by ANOVA with Duncan's New Multiple Range Test ($p \leq 0.05$).
**Determined after 17 days on induction medium BLG with 10 mg/L BAP.
***Determined after 17 days on induction medium and an additional 28 days on 1/2 GD with charcoal.
****N is number of embryos from which cotyledons were excised, 5-6 cotyledons per embryo.

In conclusion we did observe differences in organogenic response among genetically different seed sources of loblolly and pitch pine. Although the number of repetitions per seed source was high in our experiments, usually 25 to

30, the level of variation within seed sources was extremely high. The experiment would need to be repeated to verify that seed sources which ranked high (e.g., LP 268 x 229 and PP 62) do so in subsequent evaluations.

Furthermore, we have learned through discussion with R. Mott and H. Amerson of North Carolina State University that best results are obtained when only seeds with radicles protruding from the seed coat are used. In the studies we reported here, we used all seeds regardless of whether or not root radicles had emerged.

The finding of a good correlation between percentage of green cotyledons at 17 days and the percentage of organogenic cotyledons could be of value as a screening technique for evaluating large numbers of genetically different seed sources.

In summary this study served as the initial step in evaluating a possible relationship between organogenic and embryogenic potential. Currently, our primary emphasis is on initiating embryogenesis in loblolly pine. The study of the relationship between embryogenesis and organogenesis has been given a lower priority. If significant differences in embryogenic potential are found among families, it would then be prudent to evaluate the organogenic potential of the same families.

NEAR-TERM DIRECTIONS

A new conceptual plan is being developed to guide the course and content of efforts in the coming year and to lay out directions for the next several years. The plan will build upon recent IPC advances, place more weight on tracking developments elsewhere, provide for faster midcourse corrections, and include a strategy for replacing the two Industrial Research Fellows now in residence.

Norway spruce callus cultures derived from developing seed will continue to be used as a model system. Recent successes, however, will permit shifting greater resources to work directly on the target species - loblolly pine and Douglas-fir. Work on white pine will continue as it is more closely related to and behaves more like the target species. Efforts involving other hard pines will continue at a modest level; results are expected to apply to loblolly.

Results from investigations of the "juvenility window" last summer will be used to govern timing of cone collections more precisely, thereby reducing workloads and increasing efficiency. In addition, cones will be secured from South American and New Zealand sources to permit year-round experimentation. Taken together, these measures should increase both the number and variety of experiments undertaken in the future.

Work on the aforementioned species will focus on refining procedures for initiating callus and inducing somatic embryogenesis. Modified and new protocols will be tested in the model system, with the most promising ones applied to the target species. In Douglas-fir and white pine, special attention will be given to increasing the frequency and reproducibility of embryogenesis. Biochemical work will continue in direct support of cell and tissue culture efforts.

Markers now available will be used routinely to screen for competence and to determine the utility of changes in media composition and growth regulators. Biochemical assays will also be done to develop leads for further media modification.

In the model system, increased effort will be devoted to increasing the number of somatic embryos that form and mature and the ease with which they can be converted to plantlets and transferred to soil. Biochemical comparisons with developing zygotic embryos will be performed to enlarge our understanding of developmental processes. Results will be used to develop procedures for enhancing development and maturation of somatic embryos. Morphological, anatomical, biochemical, and molecular assays will also be done to verify that plantlets derived from somatic embryos are normal and behave like regular seedlings.

Recently, somatic embryogenesis was obtained with explants from fully developed Norway spruce seed. Work with this and other more mature material will be accelerated. Continued success will provide a stepping stone to work with tissues or cells from trees old enough to have been proven genetically superior, and will also permit year-round experimentation.

Some small portion of the total effort will be devoted to exploratory research: developing alternative culture systems suitable for mass production, regenerating from single cells and protoplasts, and adapting procedures for effecting genetic transformation. Increased emphasis will be placed on student involvement in these and related undertakings.

RELATED STUDENT RESEARCH

COMPLETED IN 1986

Brent Earnshaw - Ph.D. Program, Biochemical orientation, entitled "The role of cellular antioxidants (glutathione and ascorbic acid) in the growth and development of wild carrot suspension cultures."

Rene Kapik - M.S., Independent Study, entitled "Phenolic components of the primary cell wall and their possible role in the regulation of growth."

IN PROGRESS

Michael Bogenschutz - M.S., Independent Study, entitled "Electroporation-mediated genetic transformation of Norway spruce cells."

Tyrone Cornbower - M.S., Independent Study, entitled "Response of white spruce to mechanical pulping following hemicellulose hydrolysis."

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

Luke Nealey - Ph.D. Program, Organic chemistry orientation, entitled "Isolation and characterization of xyloglucan from suspension cultured loblolly pine cell medium."

Robert Sell - M.S., Independent Study, entitled "Somaclonal variation in conifer tissue cultures."

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. St. Norbert College - A cooperative study with Dr. John Phythyon concerning techniques for isolating and characterizing DNA from conifer chloroplasts.
3. Yale University; Williams - A cooperative study with Dr. Robert Slocum who is assisting in efforts to characterize polyamine metabolism in cultured plant cells.
4. Ohio State University, Wooster - A joint effort with Dr. Howard B. Kriebel involving his constructing cloned cDNA sequences from embryogenic and nonembryogenic callus of Norway spruce. In addition, Dr. Kriebel has supplied immature white pine cones for our work on somatic embryogenesis.

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GLOSSARY

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.

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.

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.

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.

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.

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.

Gene - One of the units of inherited material carried on a chromosome; arranged in a linear fashion and indivisible.

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

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.

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.

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.

In vitro - Outside the living organism.

In vivo - Within the living organism.

Isozymes - Multiple forms of a single enzyme.

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.

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.

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.

Proembryo - Embryo in very early precotyledonary stages of development.

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

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.

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.

SEM - Scanning electron microscope.

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.

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 - Chain of cells which produces at its extremity the developing 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.

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

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.

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.

MOLECULAR BIOLOGY GLOSSARY

Agrobacter 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.

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 abreviated 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.

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.

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.

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.

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

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.

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

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.

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

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

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

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.

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.

Probe - A radioactive DNA or RNA molecule used to detect the presence of its complementary strand on an electrophoretic "gel" by hybridization and autoradiography

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

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 R1).

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-labelled 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.

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 (promotors) 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.

Sequence - The order of the nucleotides in the DNA or RNA chain.

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

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.

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

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.

CODES

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

All cell lines in excess of one year old:

Example: 20(NS 384-1)2E

20 = subcultured 20 times

NS = Norway spruce

384 = research plan (RP384)[a]

-1 = time of initiation or treatment identification

2 = line or genetic source, e.g., seedling No. 2

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

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

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	Explant Codes
LP - loblolly pine	C = cotyledon
DF - Douglas-fir	H - hypocotyl
PP - pitch pine	B - bud
PO - pond pine	E - immature embryo
NS - Norway spruce	M - mature embryo
WP - white pine	N - nucellus
WS - white spruce	G - gametophyte

CONE SOURCES

Species	Tissue Culture Code	Source	Industrial Codes
Douglas-fir	DF A	Weyerhaeuser	WTC-167
	DF B	Federal Way, WA	WTC-168
	DF C		WTC-169
	DF D		WTC-170
	DF E		WTC-171
	DF F		WTC-195
	DF G		WTC-196
	DF H		WTC-205
	DF I		WTC-207
Loblolly pine	LP A	Union Camp	10-1003 D-22 HQI
	LP B	Rincon, GA	10-1007 F-21 HQI
	LP C		10-1011 C-20 HQI
	LP D		10-1018 B-16 HQI
	LP E		10-1019 C-14 HQI
	LP F	Westvaco	7-34
	LP G	Summerville, SC	7-56
	LP H		11-9
	LP I		11-10
	LP J		11-16
Norway spruce	NS	Greenville, WI	--
	NSA	U. Arkansas Fayetteville, AR	
Pitch pine	PP A	Westvaco	1-417
	PP B	Summerville, SC	65
Pond pine	PO A	Union Camp	2-1011 R7
	PO B	Rincon, GA	10-760 Q6
	PO C		10-762 S9
	PO D		22-403 S10
White pine	WP A	Ohio State	1588
	WP B	Wooster, OH	1590
	WP I	Freedom, WI	--
	WP II		--
	WP III		--
White spruce	WS A	Greenville, WI	--
	WS B		--

STATISTICS

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

AMINO ACIDS ABBREVIATIONS

ala	alanine
arg	arginine
asn	asparagine
asp	aspartic acid
cit	citrulline
cys	cysteine
γ-aba	aminobutyric acid
gln	glutamine
glu	glutamic acid
gly	glycine
his	histidine
hyp	hydroxyproline
ile	isoleucine
leu	leucine
lys	lysine
met	methionine
orn	ornithine
phe	phenylalanine
pro	proline
ser	serine
thr	threonine
trp	tryptophan
tyr	tyrosine
val	valine

CUMULATIVE LIST OF ABBREVIATIONS

AA	Ascorbic acid
2,4-D	2,4-Dichlorophenoxyacetic acid
ABA	Abscisic acid
ACC	1-Aminocyclopropane-1-carboxylic acid
ADC	Arginine decarboxylase
ADP	5'-Adenosine diphosphate
AMP	5'-Adenosine monophosphate
ANOVA	Analysis of variance
AOA	Aminooxyacetic acid
AOAA	Aminooxyacetic acid
AOPP	α-Aminooxy-β-phenylpropionic acid
ATP	Adenosine triphosphate
AVG	Aminoethoxyvinylglycine
BA	Benzylaminopurine = benzyl adenine
BAP	Benzylaminopurine = benzyl adenine
BLG	Brown and Lawrence medium + gln
BSO	Buthionine sulfoximine
cAMP	3',5'-Cyclic adenosine monophosphate
CBM	Bornman medium
C/N	Carbon/nitrogen
D	Dark
DCR	Durzan sugar pine medium
DF	Douglas-fir
DFMA	α-difluoromethylarginine
DFMO	α-difluoromethylornithine
DCHA	Dicyclohexylammonium sulfate
DHA	Dehydroascorbic acid
dSAM	Decarboxylated SAM
DW	Dry weight
E	Embryogenic
E_c	Embryogenic callus
EDTA	Ethylenediaminotetraacetic acid
E_i	Embryonal initial
FAA	Free amino acid(s)
FTIR	Fourier transform infrared
FW or fr.wt.	Fresh weight
G-1-P	Glucose-1-phosphate
GA	Gibberellic acid (gibberellin)
GC	Gas chromatography
GC/MS	Gas chromatography/mass spectrometry
GD	Gresshof and Doy medium
GSH	Glutathione (reduced)
GSSG	Glutathione (oxidized)
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid
HFBI	Heptafluorobutyrylimidazole
HFSE	High frequency somatic embryogenesis
HM	Hakman medium
HPLC	High performance liquid chromatography
IAA	Indoleacetic acid

IBA	Indolebutyric acid
IPA	Isopentenylaminopurine = 2iP
L	Larch, light or liter
LFSE	Low frequency somatic embryogenesis
LM	Litvay medium
LP	Loblolly pine
lx	Lux
MEOI	Methyleneoxindole
MES	Morpholinoethane sulfonic acid
MOI	Methyloxindole
MOPS	Morpholinopropane sulfonic acid
MGBG	Methylglyoxal bis-guanyl hydrazone
MS	Murashige and Skoog medium
NAA	Naphthalene acetic acid
NAD^+	Nicotinamide adenine dinucleotide (oxidized)
$NADP^+$	Nicotinamide adenine dinucleotide phosphate (oxidized)
NE	Nonembryogenic
NBT	Nitrobluetetrazolium
NOAA	Naphthoxyacetic acid
NS	Norway spruce
OBHA	o-benzylhydroxylamine
ODC	Ornithine decarboxylase
P	Putrescine or phosphate
PAL	Phenylalanine ammonia lyase
pcv	Packed cell volume
PEG	Polyethylene glycol
PO	Pond pine
PP	Pitch pine
PPi	Pyrophosphate
ProA	Proanthocyanidin
RP	Red pine or research plan
S	Suspensor
SAM	S-adenosylmethionine
Sd	Spermidine
S_e	Somatic embryo
S_i	Suspensor initial
SIM	Selective ion monitoring
Sp	Spermine
TLC	Thin-layer chromatography
TrpAM	Tryptamine
2iP	Isopentenylaminopurine
UDP	Uridine diphosphate
UDPG	Uridine diphosphate glucose
UTP	Uridine triphosphate
WC	Wild carrot
WCM	Wild carrot medium
WP	White pine
WS	White spruce

APPENDIX

BASAL MEDIA FORMULATIONS - COMPARISON[a]

Components, mg/L	MS	MSG	BLG	DCR	HM	CBM	GD
NH_4NO_3	1650	--	--	400	1200	--	--
$(NH_4)_2SO_4$	--	--	--	--	--	--	200
KNO_3	1900	100	100	340		2000	1000
$MgSO_4 \cdot 7H_2O$	370		320			375	250
KH_2PO_4	170				340	200	--
$CaCl_2 \cdot 2H_2O$	440			85	180		150
$Ca(NO_3)_2 \cdot 4H_2O$	--	--	--	556	--	--	--
$Na_2HPO_4 \cdot 7H_2O$	--	--	--	--	--	--	300
$NaH_2PO_4 \cdot H_2O$	--	--	--	--	--	--	56.3
KCl	--	745	745	--	--	--	90
KI	0.83				0.75	0.825	0.75
H_3BO_3	6.2				0.63	6.25	3.0
$MnSO_4 \cdot H_2O$	16.9			22.3	2.2	17	10.0
$ZnSO_4 \cdot 7H_2O$	8.6				2.87	8.75	3.0
$Na_2MoO_4 \cdot 2H_2O$	0.25				0.025		0.25
$CuSO_4 \cdot 5H_2O$	0.025			0.25	0.0025		0.25
$CoCl_2 \cdot 6H_2O$	0.025				0.0025		0.25
$NiCl_2 \cdot 6H_2O$	--	--	--	0.025	--	--	--
$FeSO_4 \cdot 7H_2O$	27.8				13.9	13.9	
Na_2EDTA	37.3				18.65	18.65	
Inositol	100			200		90	10.0
Glycine	--	--	--	2	--	--	
Nicotinic acid	0.5				2	0.625	0.1
Pyridoxine	0.1			0.5	1	1.03	
Thiamine HCl	0.1			1.0	5	8.75	1.0
Sucrose	30000		20000		34200		20000
Glucose	--	--	--	--	180	--	--
Xylose	--	--	--	--	150	--	--
Arabinose	--	--	--	--	150	--	--
Glutamine	--	1450	1500	250[b]	--	1500	--
Asparagine	--	--	100	--	--	--	--
MES	--	--	--	--	--	220	--
Casein hydrolyzate	--	--	--	500	--	--	--
AGAR	0.8%		0.7%		0.5%	--	
Agarose	--	--	--	--	--	0.5%	
pH	5.8				5.5	5.5	5.5

[a]Media components are the same as MS levels unless stated.
[b]DC1 = DCR with 50 mg/L glutamine.