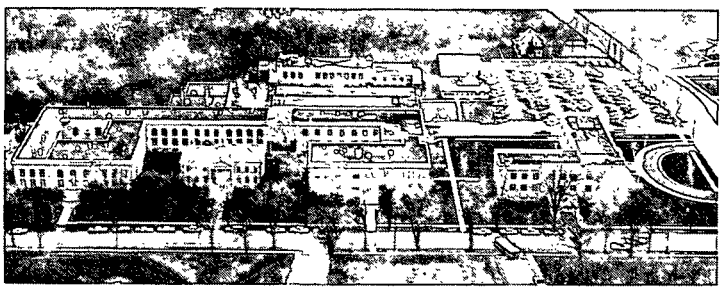

THE INSTITUTE OF PAPER CHEMISTRY, APPLETON, WISCONSIN

IPC TECHNICAL PAPER SERIES
NUMBER 258

DEVELOPMENT AND CHARACTERIZATION OF IN VITRO EMBRYOGENIC SYSTEMS IN CONIFERS

M. R. BECWAR, S. R. WANN, M. A. JOHNSON,
S. A. VERHAGEN, R. P. FEIRER, AND R. NAGMANI

SEPTEMBER, 1987

Development and Characterization of in vitro Embryogenic Systems in Conifers

M. R. Becwar, S. R. Wann, M. A. Johnson, S. A. Verhagen, R. P. Feirer, and R. Nagmani

This manuscript is based on results of work done on IPC Project 3223. This paper has been submitted for publication in the Proceedings of the International Union of Forestry Research Organization, Somatic Cell Genetics of Woody Plants, Martinus Nijhoff Publ., Dordrecht, The Netherlands

DEVELOPMENT AND CHARACTERIZATION OF IN VITRO EMBRYOGENIC SYSTEMS IN CONIFERS

BECWAR, M.R., WANN, S.R., JOHNSON, M.A., VERHAGEN, S.A., FEIRER, R.P., AND NAGMANI, R.

The Institute of Paper Chemistry, Appleton, WI 54912

1. ABSTRACT

Our progress is reviewed on development of somatic embryogenesis in conifers for mass propagation. A distinct embryogenic callus (EC) phenotype, white, mucilaginous, and rapidly growing, has been initiated on modified MS media with 2,4-D or NAA (2-5 mg/L) and BA(0-1 mg/L) from immature embryos of Norway spruce (Picea abies), white spruce (Picea glauca), loblolly pine (Pinus taeda), pond pine (Pinus serotina), and white pine (Pinus strobus). EC has also been initiated from mature embryos of Norway spruce and maintained as rapidly growing (48 hour doubling) liquid suspensions. Initiation of EC in Picea and Pinus differ markedly in several ways. Precotyledonary embryos were optimal in Pinus and EC originated from the suspensor region. In Picea EC originated from the hypocotyl and cotyledon region of predominantly post-cotyledonary embryos. Biochemically, EC of Picea and Pinus were similar and distinctly different from nonembryogenic callus (NEC) in terms of ethylene evolution rates (EC low and NEC high), level of total reductants, including glutathione (EC low and NEC high), and protein synthesis rates (EC high and NEC low). Conifer somatic embryos contained proplastids closely resembling those found in early zygotic embryos. On proliferation medium in the light, EC was white and maintained the proplastid morphology, whereas, NEC was green and contained mature chloroplasts with grana. These biochemical and ultrastructural differences served to both verify and predict embryogenic potential.

With Norway spruce somatic embryos, maturation frequencies as high as 25% have been attained. Germination frequencies as high as 82% (mean 56%) have been obtained. Twenty-nine percent of the somatic embryo plantlets survived transfer to the greenhouse, set a dormant terminal bud, overwintered to -5°C, and renewed vegetative growth synchronously with control seedlings. This is the first report of overwintering and renewed vegetative growth from resting buds of conifer somatic embryo plants.

DEVELOPMENT AND CHARACTERIZATION OF IN VITRO EMBRYOGENIC SYSTEMS IN CONIFERS

BECWAR, M.R., WANN, S.R., JOHNSON, M.A., VERHAGEN, S.A., FEIRER, R.P., AND NAGMANI, R.

The Institute of Paper Chemistry, Appleton, WI 54912

1. INTRODUCTION

The long life cycle of conifers slows genetic improvement via the traditional sexual breeding process. Clonal (vegetative) propagation techniques show considerable promise for achieving more rapid tree improvement and increased productivity (7,15,20,29). There are three distinct methods of clonal propagation that are applicable to forest trees: macropropagation, micropropagation, and somatic embryogenesis. Macropropagation, the rooting of stem cuttings, is widely used in certain hardwoods as Eucalyptus spp. and softwoods as Norway spruce (Picea abies). Micropropagation involves regeneration from small pieces of tissue from either preexisting meristems, e.g., axillary buds, or from adventitious buds. Although micropropagation has proven utility in the clonal propagation of several commercially important forest trees, including Eucalyptus spp. and radiata pine (Pinus radiata), its primary limitation for mass propagation is the high production cost of individual propagules. Recent advances in regeneration via adventitious bud culture suggest that the technique may be economical for radiata pine (1).

A more promising clonal propagation technique for the economical production of large numbers of propagules is somatic embryogenesis, the production of embryolike structures from somatic tissue under in vitro conditions. Somatic embryos can be produced from cells, thus making highly efficient liquid cell culture techniques available for maintenance and production purposes. Furthermore, somatic embryos can be encapsulated to form artificial seeds for highly efficient delivery to existing tree nursery programs (27).

Somatic embryogenesis has been reported for several coniferous species, including Norway spruce (16), European larch (Larix decidua) (24), radiata pine (28), sugar pine (Pinus lambertiana) (13), loblolly pine (Pinus taeda) (14), and white and black spruce (Picea glauca and P. mariana) (19). In our laboratory we have initiated embryogenic cultures of Norway spruce (3), white spruce (25), loblolly pine, pond pine (Pinus serotina), and white pine (Pinus strobus) (5,35).

Here we review our progress on development and characterization of in vitro embryogenic systems in conifers. Emphasis is placed on similarities and differences between embryogenic systems in Picea and Pinus.

2. INITITATION OF EMBRYOGENIC CALLUS IN CONIFERS

2.1 Picea

2.1.1. Origin of embryogenic callus. Immature embryos of Norway spruce and white spruce produce both an embryogenic callus (EC) and a nonembryogenic callus (NEC) when cultured on basal medium supplemented with auxin and cytokinin (16,19). Recent investigations in our laboratory have provided detailed information on the nature and site of origin of the EC (25). Immature embryos of Picea cultured on callus induction medium produced two

types of white to translucent calli that were phenotypically distinct. The callus that proliferated from the hypocotyl region was white to translucent, glossy, mucilaginous, and embryogenic. The epidermal and subepidermal tissue of the hypocotyl gave rise to the mucilaginous callus. After about 10 days in culture this callus gave rise to early stage somatic embryos consisting of an embryonal initial and suspensor initial cells. These results showed that in Picea a callus phase preceded somatic embryogenesis. The other type of white callus originated from the radicle and was non-embryogenic. Other investigations (19) have provided corroborative evidence for the hypocotyl origin of EC in Picea.

Numerous immature and mature embryos of Picea have been cultured on a variety of callus-inducing media, and initiation of EC from the radical region as reported by Gupta and Durzan (12) was not observed. We have observed initiation of EC at very low frequencies from cotyledons of 10-14 day old germinants of Norway spruce. This was reported earlier by Krogstrup (22). Recently, Lelu (23) initiated EC from 23% of cotyledons of 3-7 day old germinants of Norway spruce.

2.1.2. Optimum initiation window. In Norway spruce EC was initiated from immature embyros on the basal medium of von Arnold and Eriksson (31) supplemented with 2,4-D and BA (2 and 1 mg/L, respectively). All Picea cultures were initiated and maintained at 23°C with 16 hr irradiance (15-50 $\mu E \cdot m^{-2} \cdot sec^{-1}$) from cool-white fluorescent and incandescent lights. Initiation frequencies as high as 75% were attained from embryos collected in Wisconsin during July, 1985 (3). EC was also initiated from a different source tree during the summer of 1986 and the results are summarized in Table 1. During the four week period from June 30 to July 21, initiation of EC was significantly higher than earlier or later collections. During the period of high initiation, embryo explants were predominantly cotyledonary. A 14-day cold (4°C) storage period prior to culture initiation did not significantly increase initiation (Table 1). Hakman and von Arnold (17) reported that a cold pretreatment of cones for two months increased EC initiation. The 14-day cold pretreatment period we used may not have been long enough to significantly increase initiation. Regardless, these results demonstrate the utility of cold storage for extending the time available for initiation from immature embryos of Picea.

TABLE 1. Frequency of initiation of EC from immature embryo explants of freshly collected and cold stored cones of Norway spruce.

Collection time month/date	Explant length (mm)	Explants with cotyledons, (%)	Embryogenic callus initiation (%) from explants[1]	
			fresh[2]	cold stored
6/23	0.2 ± 0.1	0	6 a	22 a
6/30	0.5 ± 0.1	0	59 b	34 b
7/7	0.9 ± 0.4	64	44 b	6 a
7/14	2.3 ± 0.4	83	62 b	72 b
7/21	3.4 ± 0.2	100	55 b	65 b
8/4	--	100	0 a	--

[1]Twenty-five explants per each treatment, 5 explants per plate. Data are mean values among plates. Means within rows followed by the same letter are not significantly different (p = 0.05).

[2]Means followed by the same letter are not significantly different.

The optimum window for initiation of EC from immature embryos of white spruce has also been determined (Figure 1). During the summer of 1986 a total of 388 immature embryo explants of white spruce were cultured on a modified von Arnold and Eriksson (31) basal medium supplemented with 2,4-D and BA (2 and 1 mg/L, respectively). Culture conditions were identical to those used for initiation from immature Norway spruce embryos (3). Cotyledonary embryos of about 1.5 to 2.0 mm were the most effective explants for initiation of EC. Thus, embryo length and the stage of development of cotyledonary primordia were useful indices for identifying the optimum stage for initiating EC from white spruce immature embryos.

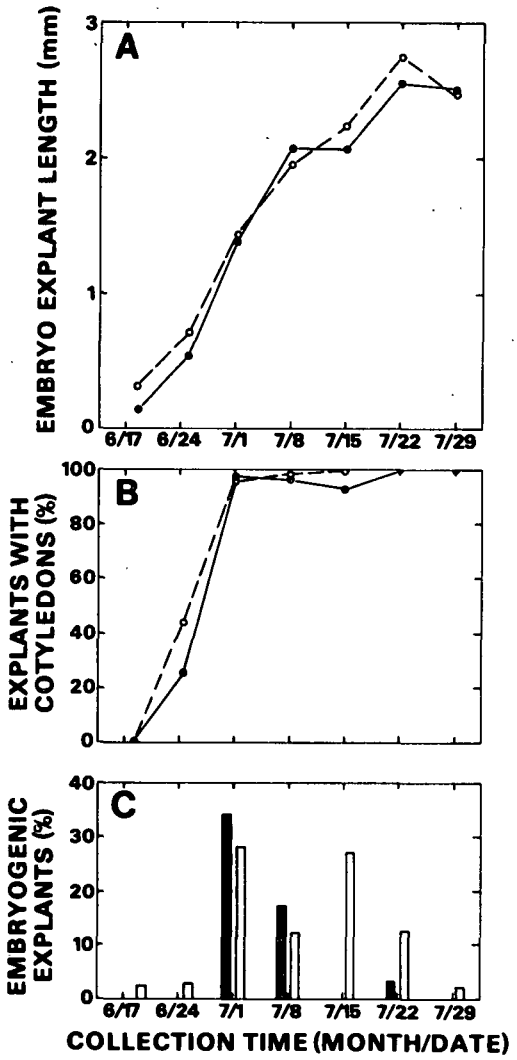

FIGURE 1. The stage of development of white spruce immature embryo explants: (A & B) and the frequency of initiation of EC(C). Solid symbols, tree 1 and open symbols, tree 2.

The window for initiation of EC in Norway spruce has been extended to mature enbryos by utilizing a modified basal medium, BLG. This medium as described by Amerson (2), is a modified MS medium. The modifications include replacement of NH_4NO_3 with 10 mM glutamine; reduction of KNO_3 from

1900 to 100 mg/L, $MgSO_4$ from 370 to 320 mg/L, sucrose from 3 to 2%; and the addition of 100 mg/L asparagine and 745 mg/L of KCl. The results are fully summarized elsewhere (6). In brief, the results showed that by culturing mature embryos in the light (16 hr photoperiod) on half-strength BLG with either 2,4-D or NAA and BA, approximately 25% of the explants intitiated EC and a majority of the lines were successfully maintained. In contrast, initiation of EC from mature embryos was achieved at a very low frequency (< 3%) on the protocol used for immature embryos, and none of these lines could be maintained. Furthermore, the change to full-strength BLG did not result in initiation of EC from mature embyros. These results point out the importance of the levels and interactions of several components of the BLG medium for extending the initiation of EC to mature embryos. Von Arnold and Hakman (32) have been able to initiate EC from mature embryos of Norway spruce cultured in the dark on the von Arnold and Eriksson (31) medium by reducing the sucrose level from 3.4 to 1%. Taken collectively, our results on initiation from mature embryos and those of von Arnold (30) demonstrate that changes in and optimization of medium components can significantly affect initiation and therefore play a major role in extending the initiation window to more mature tissues.

2.1.3. Quantification of embryogenic capacity. A method for quantitative determination of the level of somatic embryogenesis in conifer EC has been developed (4). EC of Norway spruce was dispersed in liquid by agitation and plated in a thin layer of medium containing 0.6% low melting point agarose. The density of somatic embryos ranged from 200 to 1500 per gram of EC among 11 lines surveyed. Thus, the somatic embryo counting technique was useful for identifying highly embryogenic lines among those with similar phenotypes. This technique has also proven useful for evaluating the effectiveness of biochemical treatments aimed at enhancing the level of embryogenesis (33).

2.2. Pinus

Two culture protocols were used to initiate EC in Pinus spp. The first protocol used fertilized ovules as an explant, i.e., the female gametophyte with the intact embryo-suspensor complex. This technique was first reported effective for initiation of EC in radiata pine (28). The basal medium used, MSG, was a modified MS basal with NH_4NO_3 replaced by 1450 mg/L (10 mM) glutamine, the KNO_3 level reduced from 1900 to 100 mg/L, and the addition of 745 mg/L of KCl. The basal medium was supplemented with 1% activated charcoal. The second protocol used was the culture of isolated immature embryos. The basal media used were MSG and DCR1. DCR1 was a modified DCR medium used by Gupta and Durzan (11) with the glutamine level increased from 50 to 250 mg/L. Various levels of 2,4-D and BA were added to the basal media on which immature embryos were cultured. All cultures were grown in the dark at 23°C.

2.2.1. Pond pine. Figure 2A shows extruded callus from a loblolly pine ovule. Pond pine extruded callus was phenotypically similar. The frequency at which a white to translucent and mucilaginous callus was extruded from the archegonial end of ovules of pond pine is shown in Table 2. Although up to 12% of the ovules initiated the extruded callus phenotype, only some of these cultures could be maintained (Table 2). Initiation, as used here, refers to initial formation of the extruded callus, whereas maintenance refers to lines established in culture for over one year. To determine the origin of the extruded callus, ovules were cut open when the callus was removed from the ovule for subculture. In all cases the primary embryo had neither developed or atrophied to a significant degree, and

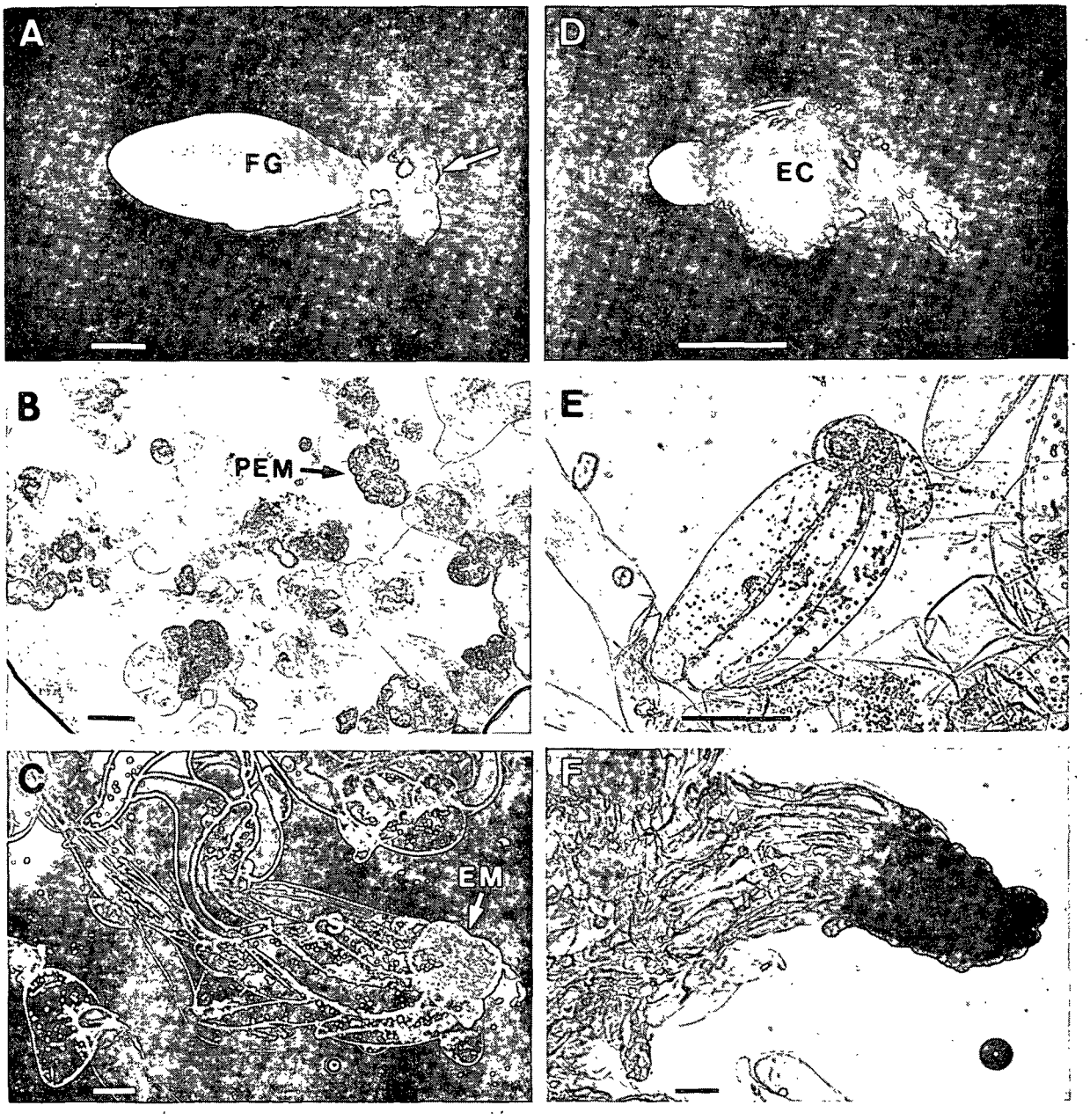

FIGURE 2. Initiation of somatic embryogenesis in Pinus. A: White-mucilaginous callus extruded (arrow) from female gametophyte (FG) of loblolly pine. B: Micrograph of extruded callus of pond pine showing pre-embryonal masses (PEM). C: Early stage somatic embryos of pond pine showing embryonal mass (EM) and attached suspensor-like cell(S). D: Origin of embryogenic callus (EC) from suspensor region of loblolly pine embryo. E: Early stage somatic embryo of loblolly pine. F: White pine somatic embryo. Scale bars: 1 mm in A and D; 100 μm in B, C, E, and F.

callus proliferation was confined to the suspensor region. Histological examination of the extruded callus revealed a mixture of unaggregated suspensor-like cells and globular clumps of densely cytoplasmic cell which resembled pre-embryonal masses (Figure 2B). The majority of the globular structures appeared similar to somatic embryos lacking suspensors. Further histological examination of the extruded callus revealed the presence of early stage somatic embryos, containing an embryonal mass and attached suspensor-like cells (Figure 2C).

TABLE 2. Initiation and maintenance of mucilaginous callus extruded from ovules of pond pine.

Collection time (month/date)	Germination[1] (%)	Extruded callus	
		Initiation[2] frequency (%)	Lines maintained
7/11	0	12	0
7/18	8	2	2
7/25	68	5	1
8/1	96	2	1
8/8	--	1	0

[1]Germination efficiency of embryos cultured on basal medium.
[2]One-hundred ovules cultured per collection.

Smith (28) reported that the frequency at which the extruded callus was formed was dependent on the stage of embryo development. Specifically, his results showed that as embryos matured within the ovule (and embryo germination was possible) the initiation frequency dropped off. In pond pine the extruded callus was initiated from both ovules of early collections which contained predominantly pregerminable embryos and ovules of collections as late as August 1, which contained germinable embryos (Table 2).

2.2.2. Loblolly pine. The time course of embyro development in loblolly pine during the period in which cultures were initiated is shown in Figure 3. Note that embryo explants cultured on July 14 to 28 were predominantly precotyledonary and had a mean length of 1 mm or less, whereas embryos cultured during August were mostly cotyledonary and 1 mm or larger.

The stage of explant development was one of the most critical factors for successful initiation of EC from immature embryos of Pinus. The results of our initial experiments (Table 3) showed that EC was only established from precotyledonary embryos which were less than 0.3 mm in length. Subsequent experiments have verified that the precotyledonary immature embryo stage is optimum for initiation and establishment of EC. Initial results also showed that low levels of 2,4-D were effective for initiation of EC, whereas higher levels (10 mg/L) were ineffective (Table 3). The EC originated from the suspensor region of the loblolly pine embryos (Figure 2D), in agreement with the site of origin of EC reported for sugar pine (13). Histologically, the EC callus initiated from immature embryos of loblolly pine was similar to the extruded EC callus of pond pine, i.e., a heterogeneous mixture of unaggregated suspensor-like cells, globular clumps of highly cytoplasmic cells, and early stage somatic embryos (Figure 2E).

2.2.3. White pine. The time course of embryo development during culture initiation in white pine is shown in Figure 4. Note that embryo explants derived from the first two collections of cones (July 2 and 9) were mostly precotyledonary and less than 1 mm in length, whereas those from the last three collections were mostly cotyledonary and greater than 2 mm.

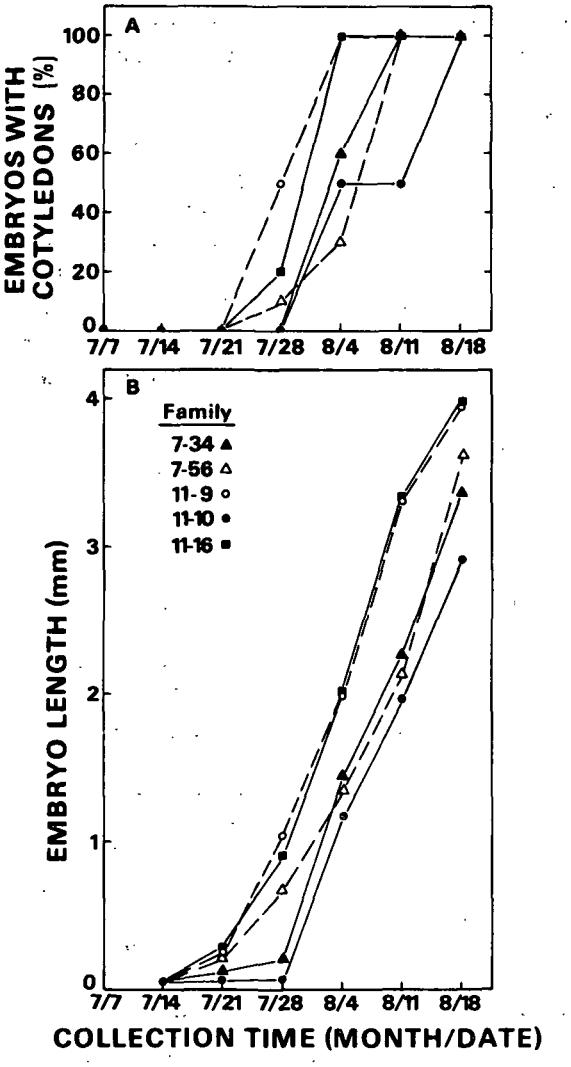

FIGURE 3. The stage of development of immature embryo explants of five families of loblolly pine. A: Percentage of embryos with cotyledonary primordia. B: Embryo length.

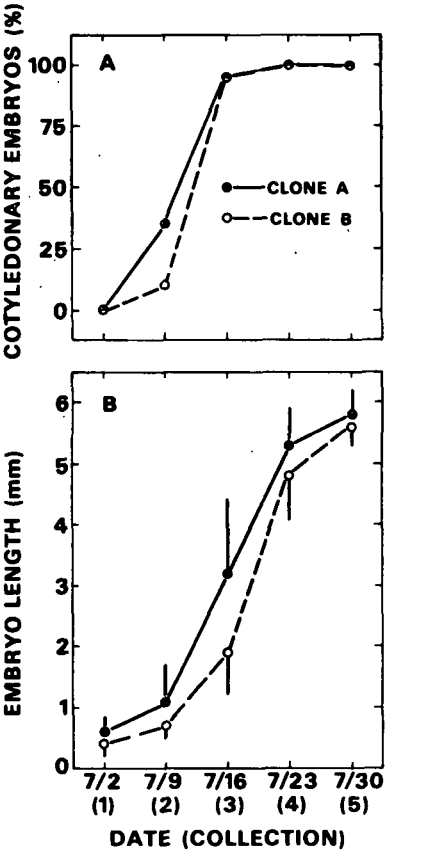

FIGURE 4. The stage of development of immature embryo explants of white pine collected during July 1986 in Wooster, OH. A: Percentage of embryos with cotyledonary primordia. B: Embryo length.

The results in Table 4 show that precotyledonary embryos of white pine (e.g., embryos from the first two collections) were effective explants for initiation of EC, whereas cotyledonary embryos were ineffective. The EC originated from the suspensor region of the immature embryo explants. Thus, initiation of EC in white pine was similar to loblolly with respect to optimal stage of explant development and site of origin. Although EC was initiated on both MSG and DCR basal medium supplemented with 2,4-D and BA (Table 4), no EC was initiated on the DCR 3/0 treatment (data not

shown), suggesting a cytokinin requirement for initiation. A white pine somatic embryo is shown in Figure 2F.

TABLE 3. Effect of the stage of explant development and media modifications on establishment of EC from immature embryo explants of loblolly pine.

Stage of explant development[1]			Frequency (%) of embyrogenic lines maintained			
			Basal medium (A/C mg/L)[3]			
embryo size (mm)	embryos with cotyledons (%)	N[2]	DCR 10/.5	DCR 3/.5	MSG 10/1	MSG 2/1
0.1-0.3	0	130	0	2.3	0	1.5
0.7-1.2	37	85	0	0	0	0

[1]Embryo explants derived from cones collected July 21 to Aug. 4, 1986 from five families of loblolly pine. See Figure 3.
[2]N = number of explants cultured per each medium treatment.
[3]Refer to text for description of basal medium. A/C refers to auxin and cytokinin levels.

TABLE 4. Effect of the stage of embryo development on initiation of EC in white pine.

Collection (month/date)	Embryos with Cotyledons (%)	N[2]	Embryogenic callus[1] initiation frequency (% explants)	
			Medium	
			MSG 2/1	DCR 3/.5
1 (7/2)	0	150	--	2.7
2 (7/9)	23	230	1.3	2.2
3 (7/16)	95	150	0	0

[1]Initiation from cones stored at 4°C for 2-3 months. Cones were derived from five trees: clones A and B (Figure 4) collected in Wooster, OH and three from Freedom, WI.
[2]N = number of explants cultured per each medium treatment.

2.3. Comparison of *in vitro* embryogenesis in *Pinus* and *Picea*

A visual inspection of EC in *Pinus* and *Picea* suggests phenotypic similarity. They are both white to translucent and mucilaginous. Further comparative observations of *Pinus* and *Picea* EC show marked differences in initiation and growth characteristics.

The most striking differences in initiation include differences in the optimum stage of explant development, the site of origin, and the frequency of initiation of EC. In *Picea* postcotyledonary embryos were optimum and the EC originated from the hypocotyl (25) and cotyledon regions (22,23). In *Pinus* precotyledonary embryos were optimum and EC originated from the suspensor region. Initiation frequencies were relatively high in *Picea*, 40 to 75% from immature embryos and 25% from mature embyros. In contrast,

initiation frequencies were consistently low in Pinus, typically less than 3%. It is possible that changes in medium components may increase initiation frequencies in Pinus and enable initiation from more mature tissues. As indicated previously, media modifications were effective in extending the window to mature embryos in Norway spruce. Another difference between Pinus and Picea was that auxin and cytokinin were obligatory for initiation in Picea but were not required for initiation of EC from fertilized ovules of pond pine.

Proliferative growth of EC also differs considerably between Pinus and Picea. In Pinus spp., EC was often dominated by pre-embryonal masses (see Figure 2B) or very early stage somatic embryos (see Figure 2C and E). However, in Picea spp. somatic embryos reached a more advanced stage of development on proliferation medium.

3. BIOCHEMICAL CHARACTERIZATION OF EMBRYOGENIC CONIFER CALLUS

Biochemical analyses of Picea and Pinus EC and NEC were conducted as previously described (33-35). Briefly, ethylene was determined by gas chromatography, protein synthesis was measured by 3H-leucine uptake, glutathione (GSH) was measured by the rereduction of oxidized GSH by commercial GSH reductase, and the level of total reductants was measured by the ability to reduce potassium ferricyanide. In Picea spp., EC and NEC assayed were derived from the same genotype (explant) and were cultured under identical conditions. In Pinus spp., although EC and NEC were initiated and maintained under similar or identical conditions, the callus types were derived from different embryo explants, and were therefore genotypically different.

Significant differences in biochemical parameters were detected between EC and NEC in both Pinus and Picea spp. (Table 5). These differences were of sufficient magnitude to suggest that they are indicative of an embryogenic condition in conifers. Although interspecific differences were detected, relative to NEC, EC (1) evolved less ethylene, (2) contained lower amounts of GSH and other nonspecific reducing agents, and (3) synthesized or "turned over" protein at a faster rate. Similar trends in protein synthesis rates and level of total reductants between EC and NEC of European larch have also been observed (data not shown). These results suggest that EC callus in conifers is biochemically and metabolically similar irrespective of species, and distinctly different from NEC. While these conclusions are broadly stated for conifers, white spruce stood out as not exhibiting clear biochemical differences between EC and NEC. This may in part be due to the fact that when grown under proliferative conditions, somatic embryos of white spruce can contain anthocyanins which would reduce potassium ferricyanide.

The biochemical assays performed were all rapid, convenient, and required small amounts of tissue. In the case of ethylene, the assay was nondestructive, enabling reuse of the tissue in other experiments. These attributes make the assays attractive candidates for markers of embryogenic potential. However, biochemical markers may have limited utility in conifers because the EC phenotype can be easily recognized. It seems that biochemical assays of EC will be of greater utility in identifying key metabolic pathways involved in growth and development of somatic embryos into plants.

4. ULTRASTRUCTURAL CHARACTERIZATION OF EMBRYOGENIC CONIFER CALLUS

The chloroplasts of light grown conifers are typical of those found in most higher plants. The organelles, usually 2-10 μm in length, contain

starch grains, protein bodies and internal thylakoid membranes organized into grana. The organization of the thylakoid membranes is dependent, however, upon the age and physiological state of the tissue.

TABLE 5. Biochemical differences between EC and NEC of conifers.

Species	Ratio of measured parameter (NEC/EC)[1]			
	Protein synthesis rate	Total reductants	GSH	Ethylene evolution rate
loblolly pine	0.03	4	9	345
white pine	0.02	13	3	127
pond pine	0.4	10	8	14
Norway spruce	0.03	17	3	12
white spruce	0.4	2	1.2	2.4

[1]Only white spruce ratios not significantly different ($p = 0.05$) from 1.0.

In contrast, plastids in EC of spruce had a unique morphology at the ultrastructural level (Figure 5A & B). The plastids, appeared more darkly stained (more electron dense) than mitochondria, lacked the internal organization of a mature chloroplast. Some of these plastids contained small starch grains, although they were not nearly as large as the starch grains present in mature chloroplasts in leaf or cotyledon tissue. The plastids present in the green NEC of spruce, on the other hand, appeared similar to a typical chloroplast (Figure 5C & D). Thylakoid membranes, some organized into grana, were found in all the chloroplasts. Large starch grains were also prominent in some of the chloroplasts.

Zygotic embryos excised from mature Norway spruce seeds were also examined. The plastids resembled those observed in EC of spruce. That is, they also lacked organized thylakoid membranes (photographs not shown). In order to determine if the plastid morphology observed in zygotic and somatic embryos was unique to Norway spruce, EC of loblolly pine, pond pine, white pine, and European larch were examined (10). EC of these species all contained plastids that closely resembled those in EC of spruce (photographs not shown). In addition, the plastids observed in somatic embryos of carrot exhibited the same morphology as those in EC of conifers.

Further investigations showed that as Norway spruce somatic embryos matured (cotyledons begin to form and green) chloroplasts with thylakoids and grana became evident. We have observed the same phenomenon in carrot, where somatic embryos beyond the torpedo stage, if grown in the light, contained green mature chloroplasts. Thus, it appears that proplastids are indicative of early stages of embryonic development, whether *in vivo* or *in vitro*. These findings provide evidence in support of our previous biochemical studies (9,33,34) that many aspects of somatic embryogenesis mimic or correspond to *in vivo* embryogenesis (21). In summary, chloroplast morphology may be one of the best indicators that embryogenesis in *Pinus* and *Picea* is occurring and proceeding "normally" *in vitro*.

5. CONIFER EMBRYOGENIC SUSPENSION CULTURES

For the purposes of mass propagation, a liquid embryogenic culture system is desirable for several reasons, including the following. Liquid cultures are easier and more economical to maintain than callus cultures. They have potential for higher growth rates (decreased cell doubling

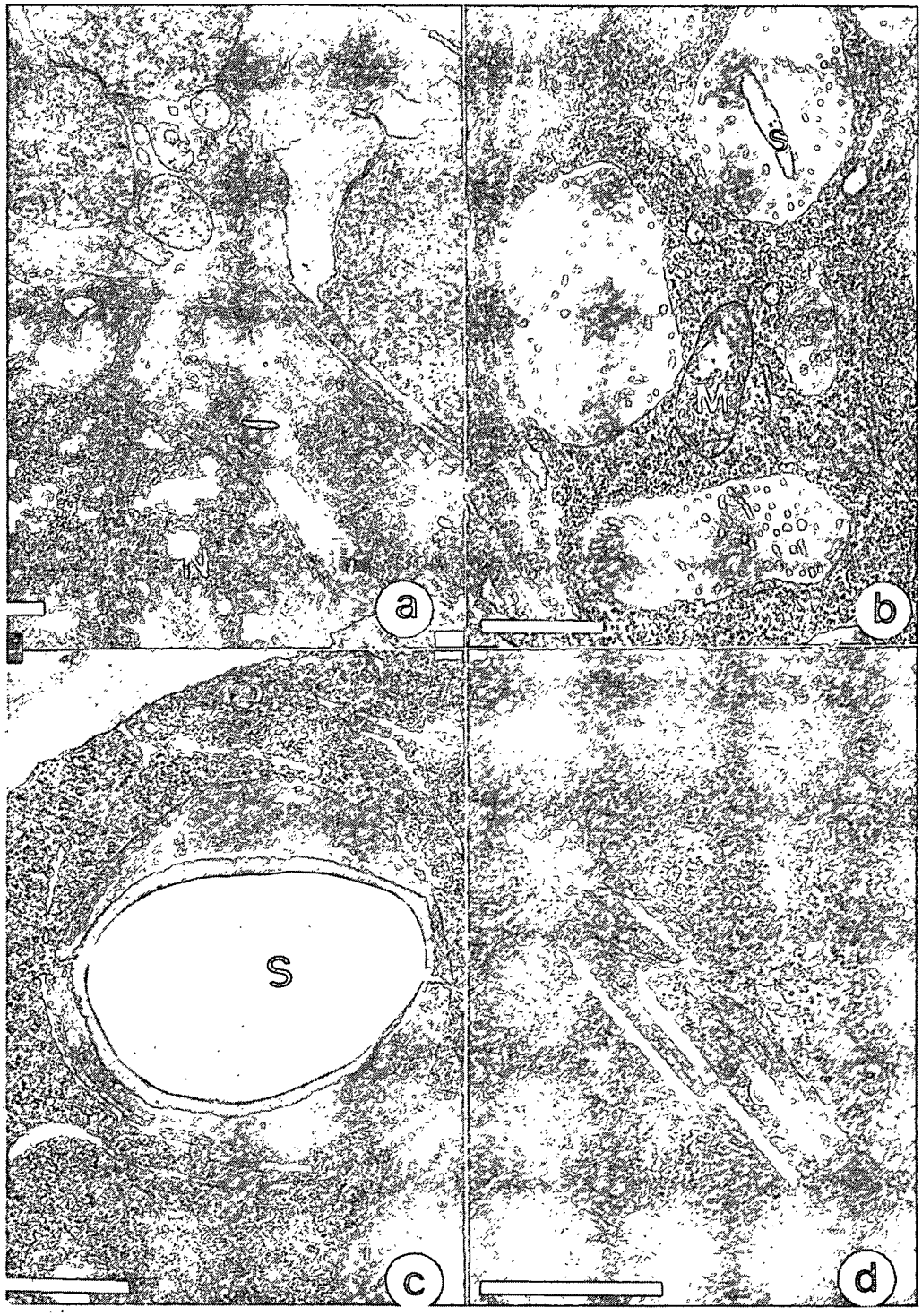

FIGURE 5. Proplastids in embryogenic (A & B) and chloroplasts in non-embryogenic (C & D) callus of Norway spruce. P = proplastid. M = mitochondria. S = starch grain. G = grana. N = nucleus. Scale bars = 1 μ m.

times), thus eliminating the need for maintaining large stocks of callus. Lastly, liquid culture systems are amenable to automation of both somatic embryo proliferation and development en masse (e.g., large bioreactor culture systems). In addition to their utility for mass propagation, liquid cultures serve as an ideal source for cells and protoplasts for genetic modification experiments.

Although establishment of embryogenic suspension cultures has been re-reported in both Picea and Pinus (14,18), quantitative information has not been presented on growth characteristics and somatic embryo yield. We have established rapidly growing embryogenic suspensions of Norway spruce. The cultures were derived from EC initiated from mature embryo explants on half-strength BLG with 2 mg/L 2,4-D or NAA and 1 mg/L BA as previously described.

During the linear phase of growth, the liquid cultures had a doubling time of about 48 hours and reached a somatic embryo density of about 100/mL (Figure 6). Rapid growth rates were maintained by repeated subculture at 10-14 day intervals (1:10 dilution with fresh medium).

With both high growth rates and somatic embryo yield, the Norway spruce suspension cultures show promise as an ideal system for mass production of somatic embryos. Continued efforts are directed at obtaining maturation of the somatic embryos while still in the liquid culture system.

6. DEVELOPMENT OF CONIFER SOMATIC EMBRYOS TO PLANTS

The Norway spruce somatic embryos can be converted to phenotypically normal plants (Figure 7). Somatic and seedling plants overwintered and renewed vegetative growth synchronously. A complete description of the frequency of plant regeneration is presented elsewhere (6). Based on optimum maturation frequencies (25%), germination (56%), and conversion to plants (29%), the highest mean efficiency of 4% was attained. Because of the exceedingly long regeneration cycles of conifers, it is of prime importance to verify at an early stage that the somatic plants are uniform and genetically "true-to-type." Current efforts are directed toward production of large numbers of Norway spruce somatic plantlets for quantitative analysis of uniformity and growth characteristics.

7. COMPARISON OF IN VIVO AND IN VITRO CONIFER EMBRYOGENESIS

Polyembryony, the formation of multiple embryos, is common in vivo in conifers. There are two types of polyembryony, simple and cleavage (8,26) (Figure 8). Simple polyembryony, the fertilization of more than one egg per female gametophyte, occurs in both Picea and Pinus. Therefore, the resulting embryos may be genotypically different. In addition to simple polyembryony, cleavage polyembryony occurs in Pinus (Figure 8). Cleavage embryos result from the division of apical tier cells of an individual proembryo and are, therefore, genetically identical.

Observations of in vitro embryogenesis in both Picea and Pinus have suggested that one mechanism of embryo formation may be a cleavage type of process. That is, multiple embryos were found with common suspensors. Although further investigations are needed to fully characterize possible types of in vitro embryogenesis in conifers, the following suggest the possibility of differences between Picea and Pinus. As indicated previously, in Picea a callus phase preceded initiation of somatic embryos (25). Therefore, in Picea besides cleavage formation of embryos in vitro, another type of embryo initiation mechanism is operative. In Pinus initiation of embryogenesis in vitro may simply be a reinitiation of the cleavage process (which occurs in vivo).

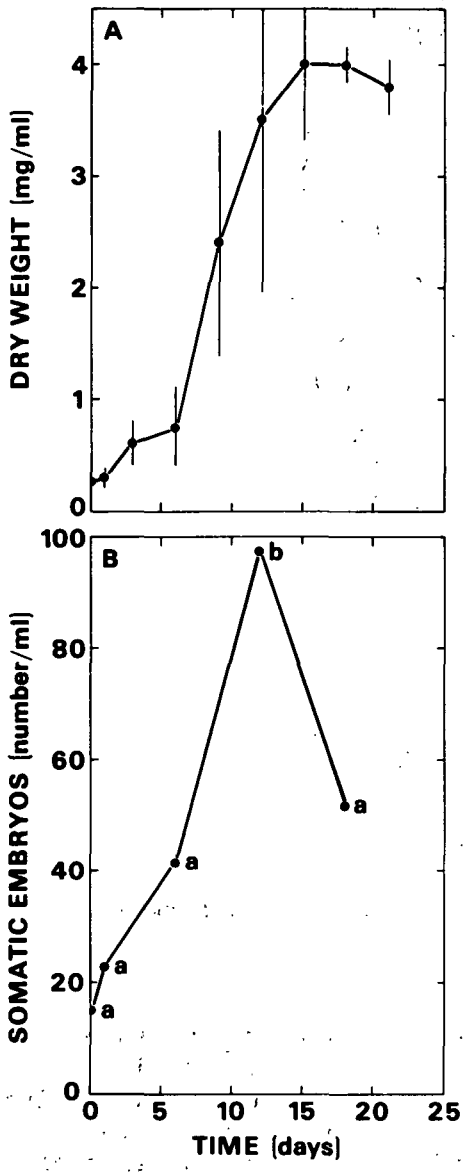

FIGURE 6. Growth curve (A) and somatic embryo density (B) of liquid suspension cultures of Norway spruce grown on half-strength BLG with 2 mg/L NAA and 1 mg/L BA. Vertical bars in A are ± standard deviation among triplicate samples. Means of triplicate counts in B followed by unlike letters are significantly different (p = 0.05).

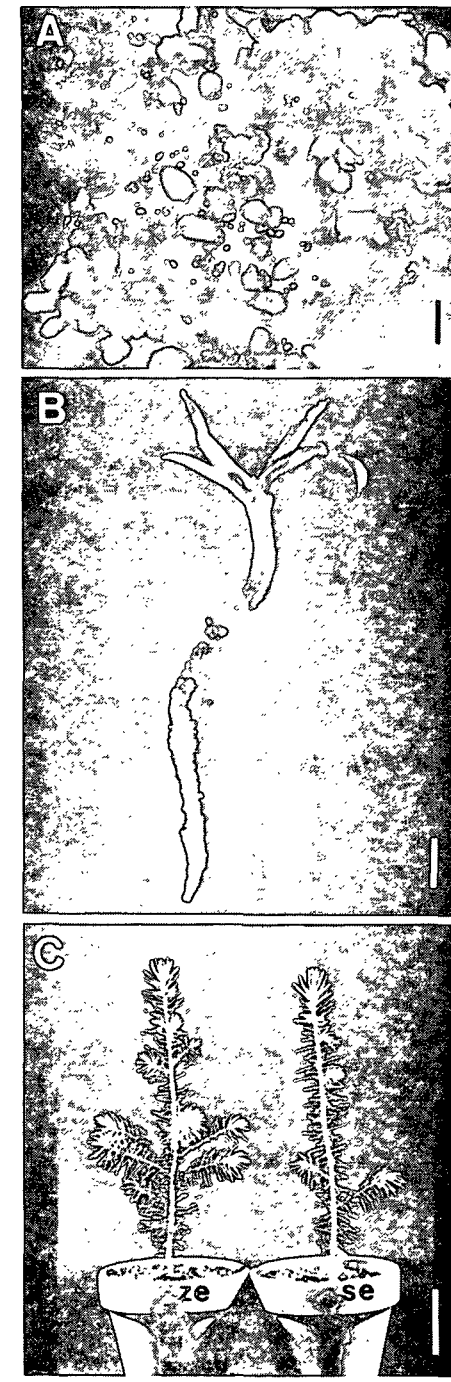

FIGURE 7. Maturation (A), "germination" (B), and conversion (C) of Norway spruce somatic embryos to plants. C: Comparison of somatic embryo plant (se) and control plant grown from zygotic embryo (ze). Scale bars: 1 mm in A & B; 5 cm in C.

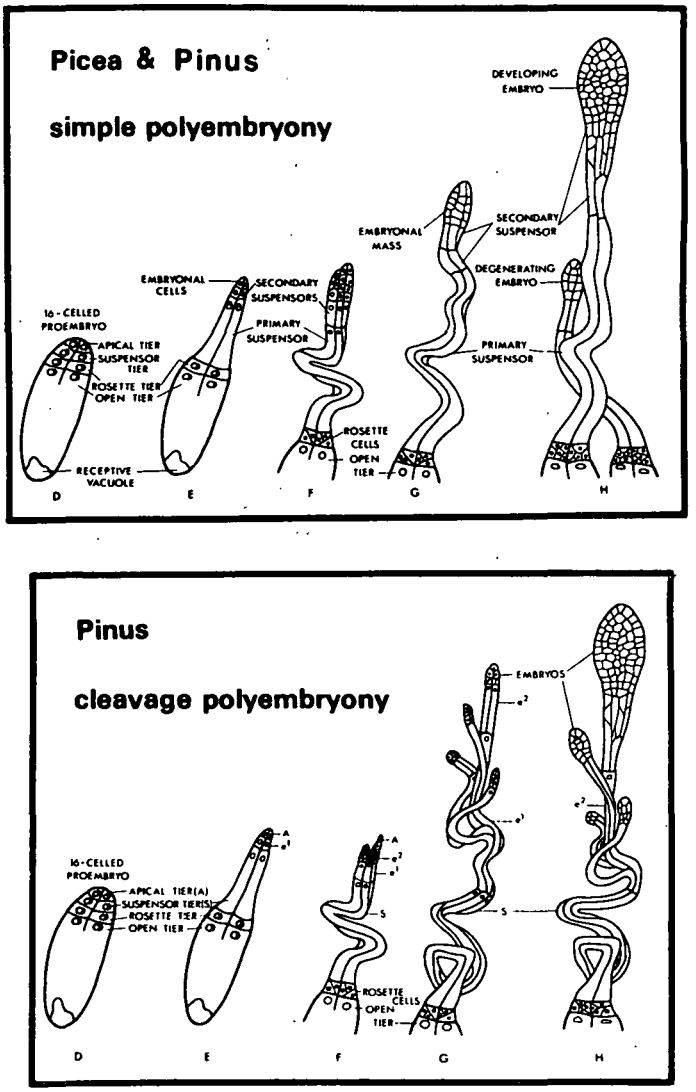

FIGURE 8. In vivo embryogenesis in Picea and Pinus. Used with permission from Owens and Blake (26).

8. SUMMARY

Much progress has been made on the development of in vitro embryogenic systems in conifers since the first reports of somatic embryogenesis in Norway spruce (16) and European larch (24) in 1985. The embryogenic callus phenotype, white to translucent and mucilaginous, has been similar in all conifers. Furthermore, our biochemical studies have provided evidence of the physiological similarity of embryogenic conifer callus among species. Although similar physiologically, EC in Picea and Pinus have different optimum initiation windows and sites of origin from immature embryo explants. These differences may relate to inherent differences in embryology between Picea and Pinus.

Considerable progress has also been made on regeneration of plants from conifer somatic embryos. Several reports have verified that conifer somatic embryos reached the germination stage (3,12-14,17,19,24,30). Our results (6) indicate that Norway spruce somatic embryo plants were similar to seedling derived plants in terms of physiological response to changing

environment. This is the first demonstration of overwintering and renewed vegetative growth from resting buds of conifer somatic embryo plants.

9. ACKNOWLEDGMENTS

We wish to acknowledge all members of the IPC Forest Biology Division who participated in this research. In particular, Debbie Hanson, Lynn Kroll, Judy Wyckoff, John Carlson, Julian Conkey, Gary Wyckoff, Robert Arvey, and Egon Hummenberger. We also thank the following individuals for supplying conifer cones: Lee Handley (Westvaco Corp., Summerville, SC), George Lowertz (Union Camp Corp., Rincon, GA), and Howard Kriebel (Ohio St. Univ., Wooster, OH).

10. REFERENCES

1. Aitken-Christie J, Singh AP, and Davies H: A new tissue culture system for radiata pine. In: Genetic Manipulation of Woody Plants. Eds., Hanover JH and Keathley DE. Plenum Press, NY. 1987.

2. Amerson HV, Frampton LJ, McKeand SE, Mott RL, and Weir RJ: Loblolly pine tissue culture: laboratory, greenhouse, and field studies. In: Tissue Culture in Forestry and Agriculture. Eds., Henke, R.R., et al. Plenum Press, NY. pp. 271-287, 1985.

3. Becwar MR, Noland TL, and Wann SR: Somatic embryo development and plant regeneration from embryogenic Norway spruce callus. Tappi J. 70(4):155-160, 1987.

4. Becwar MR, Noland TL, and Wann SR: A method for quantification of the level of somatic embryogenesis among Norway spruce callus lines. Plant Cell Reports 6:35-38, 1987.

5. Becwar MR, Wann SR, and Kriebel HB: Initiation of embryogenic callus in Pinus strobus (eastern white pine) from immature embyro explants (abstract). In: Genetic Manipulation of Woody Plants. Eds., Hanover JH and Keathley DE. Plenum Press, NY. 1987.

6. Becwar MR, Verhagen SA, and Wann SR: The frequency of plant regeneration from Norway spruce somatic embryos. Proceedings, 19th Southern Forest Tree Improvement Conf., College Station, TX, June 16-18. The National Technical Information Service, Springfield, VA. 1987.

7. Bonga JM: Tree tissue culture applications. In: Advances in cell Culture, Vol. 5. Ed., Maramorosch K. Academic Press, Inc., NY, p. 209-39, 1987.

8. Buchholz JT. Origins of cleavage polyembryony in conifers. Bot. Gaz. 81:55-71, 1926.

9. Feirer RP, Wann SR, and Einspahr DW: The effects of spermidine synthesis inhibitors on in vitro plant development. Plant Growth Regul. 3: 319-327, 1987.

10. Feirer RP, Wann SR, Becwar MR, Nagmani R, and Carlson J: A comparison of embryogenic calli: chloroplasts ultrastructure and protein

synthesis (abstract). In: Genetic Manipulation of Woody Plants. Eds., Hanover JH and Keathley DE. Plenum Press, NY. 1987.

11. Gupta PK and Durzan DJ: Shoot multiplication from mature trees of Douglas-fir (Pseudotsuga menziesii) and sugar pine (Pinus lambertiana). Plant Cell Reports. 4:177-179, 1985.

12. Gupta PK and Durzan DJ: Plantlet regeneration via somatic embryogenesis from subcultured callus of mature embryos of Picea abies (Norway spruce). In Vitro. 22:685-688, 1986.

13. Gupta PK and Durzan DJ: Somatic polyembryogenesis from callus of mature sugar pine embryos. Bio/Tech. 4:643-645, 1986.

14. Gupta PK and Durzan DJ: Biotechnology of somatic polyembryogenesis and plantlet regeneration in loblolly pine. Bio/Tech. 5:147-151, 1987.

15. Haissig BE, Nelson ND, and Kidd GH: Trends in the use of tissue culture in forest improvement. Bio/Tech. 5:52-57, 1987.

16. Hakman I, Fowke LC, von Arnold S, and Eriksson T: The development of somatic embryos in tissue cultures initiated from immature embryos of Picea abies (Norway spruce). Plant Sci. 38:53-59, 1985.

17. Hakman I and von Arnold S: Plantlet regeneration through somatic embryogenesis in Picea abies (Norway spruce). J. Plant Physiol. 121: 149-158, 1985.

18. Hakman I and Fowke LC: An embryogenic cell suspension culture of Picea glauca (white spruce). Plant Cell Repts. 6:20-22, 1987.

19. Hakman I and Fowke LC: Somatic embryogenesis in Picea glauca (white spruce) and P. mariana (black spruce). Can. J. Bot. 65:656-659,1987.

20. Hasnain S, Pigeon R, and Overend RP: Economic analysis of the use of tissue culture for rapid forest improvement. The Forestry Chronicle, 6(4):240-245, Aug., 1986.

21. Johnson MA, Carlson JA, Conkey JH, and Noland TL: Biochemical changes associated with zygotic pine embryo development. J. Expt. Bot. 38: 518-524, 1987.

22. Krogstrup P: Embryo-like structures from cotyledons and ripe embryos of Norway spruce (Picea abies). Can. J. For. Res. 16:664-668, 1986.

23. Lelu M, Boulay M, and Arnaud Y: Formation of embryogenic calli from cotyledons of Picea abies collected from 3 to 7 day old seedlings. C.R. Acad. Sci. Paris. 305(series III):105-109, 1987.

24. Nagmani R and Bonga JM: Embryogenesis in subcultured callus of Larix decidua. Can. J. For. Res. 15:1088-1091, 1985.

25. Nagmani R, Becwar MR, and Wann SR: Single-cell origin and development of somatic embryos in Picea abies (Norway spruce) and P. glauca (white spruce). Plant Cell Reports 6:157-159, 1987.

26. Owens JN and Blake MD: Forest tree seed production. A review of the literature and recommendations for future research. Information report PI-X-53. Petawawa Nat. Forestry Inst., Can. For. Serv., Chalk River, Ontario. 161 pp, 1985.

27. Redenbaugh K, Paasch BD, Nichol JW, Kossler ME, Viss PR, and Walker KA: Somatic seeds: Encapsulation of asexual plant embryos. Bio/Tech. 4: 797-801, 1986.

28. Smith DR, Singh AP, and Wilton L: Zygotic embyros of Pinus radiata in vivo and in vitro. Int. Conifer Tissue Culture Work Group. Abstracts. Ed. Smith, D.R., New Zealand Forest Service, Rotorua, New Zealand, 1985.

29. Timmis R, Abo El-Nil MM, and Stonecypher RW: Potential genetic gain through tissue culture. In: Cell and tissue culture in Forestry. Eds., Bonga JM and Durzan DJ. Martinus Nijhoff Publ. Dordrecht, The Netherlands. Vol. 1, p. 198-215, 1987.

30. von Arnold S: Improved efficiency of somatic embryogenesis in mature embryos of Picea abies (L.) Karst. J. Plant Physiol. **128**:233-244, 1987.

31. von Arnold S and Eriksson T: In vitro studies of adventitious shoot formation in Pinus contorta. Can. J. Bot. **59**:870-874, 1981.

32. von Arnold S and Hakman I: Effect of sucrose on initiation of embryogenic callus from mature zygotic embryos of Picea abies (Norway spruce). J. Plant Physiol. **122**:261-265, 1986.

33. Wann SR, Feirer RP, Johnson MA, and Noland TL: Norway spruce as a model system for somatic embryogenesis in conifers. Proc. TAPPI Res. and Devel. Conf., Forest Biotech. Raleigh, NC, Sept. 28-Oct. 1, TAPPI Press, Atlanta, GA. pp. 131-135, 1986.

34. Wann SR, Johnson MA, Noland TL, and Carlson JA: Biochemical differences between embryogenic and nonembryogenic callus of Picea abies. Plant Cell Reports **6**:39-42, 1987.

35. Wann SR, Johnson MA, Feirer RP, Becwar MR, and Nagmani R: Biochemical differences between embryogenic and nonembryogenic callus of conifers (abstract). In: Genetic Manipulation of Woody Plants. Eds., Hanover JH and Keathley DE. Plenum Press, NY. 1987.