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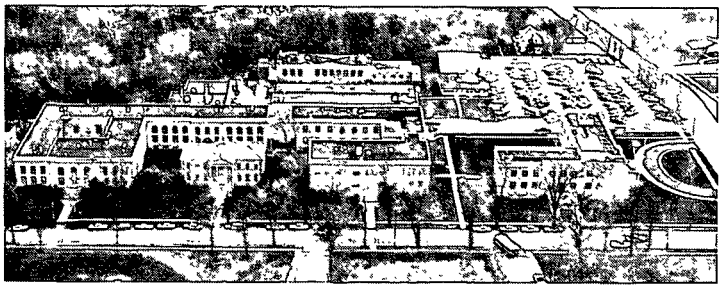

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SOMATIC EMBRYO DEVELOPMENT AND PLANT REGENERATION FROM EMBRYOGENIC NORWAY SPRUCE CALLUS

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SOMATIC EMBRYO DEVELOPMENT AND PLANT REGENERATION FROM EMBRYOGENIC NORWAY SPRUCE CALLUS

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ABSTRACT

Somatic embryogenesis, the production from tissue cultures of embryo-like structures that can grow into plants, has only recently been discovered in conifers. We have established callus cultures from immature Norway spruce embryo explants that are highly embryogenic; a gram of callus yields over 10^3 somatic embryos. The somatic embryos "germinate" similarly to seed derived embryos, developing green cotyledons and shoot growth, a hypocotyl, and a primary root. Potential applications of this conifer regeneration system are discussed relative to forest biotechnology.

INTRODUCTION

Until recently coniferous species were absent from the list of plants in which plant regeneration via somatic embryogenesis had been attained. Somatic embryogenesis is the production of embryo-like structures containing both root and shoot primordia from tissue or callus cultures. This *in vitro* method of asexual propagation has the potential of being an efficient and economical method of plant multiplication. The first reports of embryogenic callus in coniferous species with the capacity to regenerate plantlets were in Norway spruce (1,2) and European larch (3). The explants used were immature embryos and female gametophytes, respectively. Recently von Arnold and Hakman (4) have also reported the initiation of embryogenic callus from mature embryos of Norway spruce. Although several previous workers had reported finding somatic embryo-like structures in conifer cell and tissue cultures, none of these structures developed further into plantlets (5).

This paper reviews our progress in establishing a conifer somatic embryogenesis regeneration system in Norway spruce. We also describe methods developed to quantitatively determine the level of embryogenesis among Norway spruce callus lines, enabling the identification of highly embryogenic lines. Lastly, the potential usefulness of this somatic embryogenesis regeneration system is discussed relative to mass propagation and genetic modification of conifers.

METHODS

Immature female cones were collected from open pollinated *Picea abies* (L.) Karst trees in Appleton and Argonne, Wis. Explants and resulting callus lines derived from these cones are hereafter identified by codes according to collection site and date (Table 1). 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 aseptically dissected out from female gametophyte tissue and cultured on basal medium (BM) described by Hakman *et al.* (1). All BM contained 0.5% agar and 3.4% sucrose (w/v). For initiation and maintenance of callus the BM was supplemented with 2 mg/L 2,4-dichlorophenoxy acetic acid (2,4-D) and 1 mg/L 6-benzyl amino purine (BAP). Cultures were subcultured every two weeks and maintained at 23°C with 16 hr irradiance (15 to 50 $\mu E\ m^{-2}\ sec^{-1}$ at culture level) from cool-white fluorescent and incandescent lights.

We used the protocol outlined in Fig. 1 to obtain somatic embryo development. The indole-3-butyric acid (IBA) level used (1 µM) is similar to that used to obtain soybean somatic embryo development (6). Abscisic acid (ABA) has been reported to suppress abnormal somatic embryo formation in carrot (7), and was included in our somatic embryo development protocol.

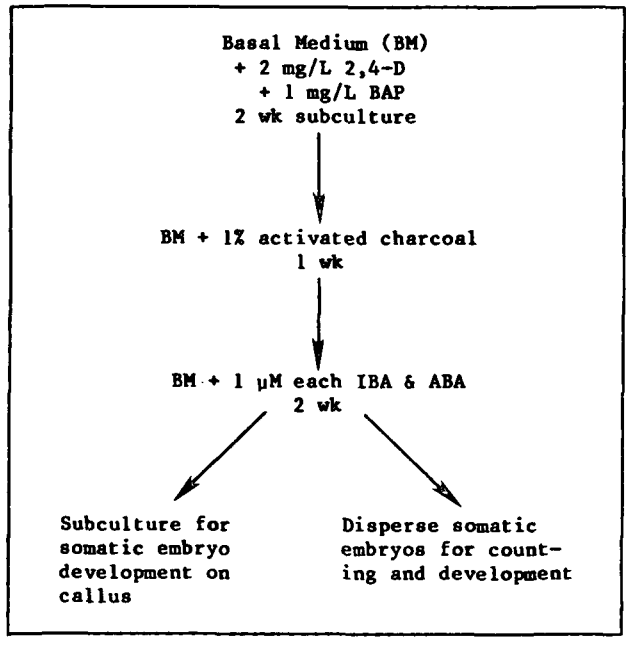

Fig. 1 Norway spruce somatic embryo development protocol.

As indicated in Fig. 1 somatic embryos developed either directly from the subcultured callus or after being plated in a thin layer of agarose medium. The somatic embryo dispersion and counting technique is described in Fig. 2. This agarose plating method was developed from the protoplast culture technique of Shillito *et al.* (8). 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.

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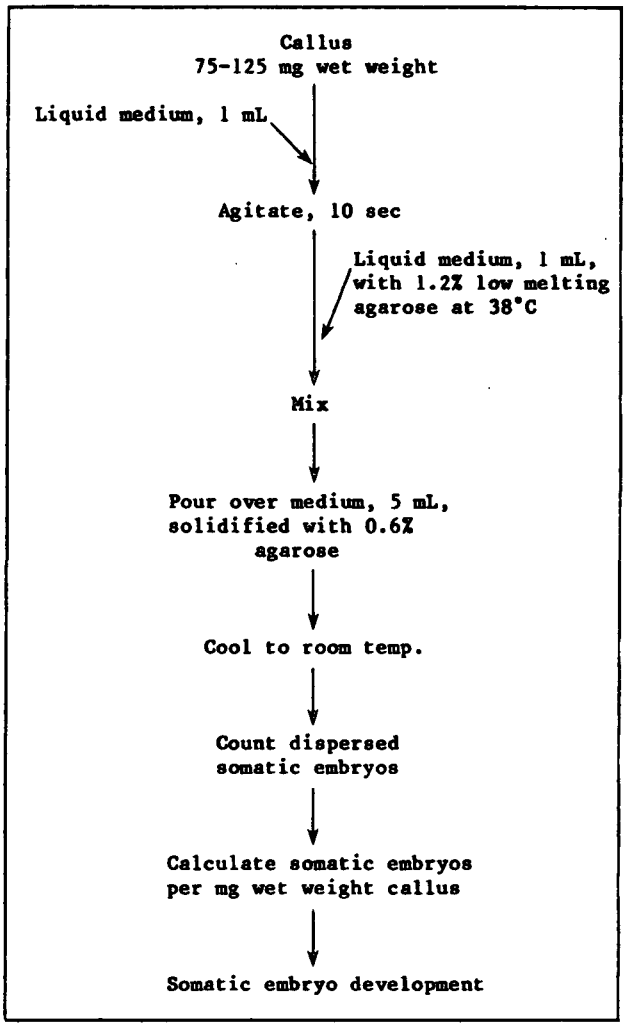

Fig. 2 Somatic embryo dispersion and counting technique. Liquid medium is BM with 1 µM each IBA and ABA.

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

RESULTS AND DISCUSSION

Initiation of Embryogenic Callus

Norway spruce callus cultures were maintained as lines derived from individual immature zygotic embryo explants. The callus which developed from the explants sectored into two distinct phenotypes: a white to translucent (Fig. 3A) and a green (Fig. 3B). By separating these callus phenotypes each could be maintained through subculturing. Dispersion of the callus revealed that the white phenotype was comprised of somatic embryos, elongated suspensor-like cells, and dense cell clusters (Fig. 3C). The green callus was comprised of only cells and cell clusters (Fig. 3D).

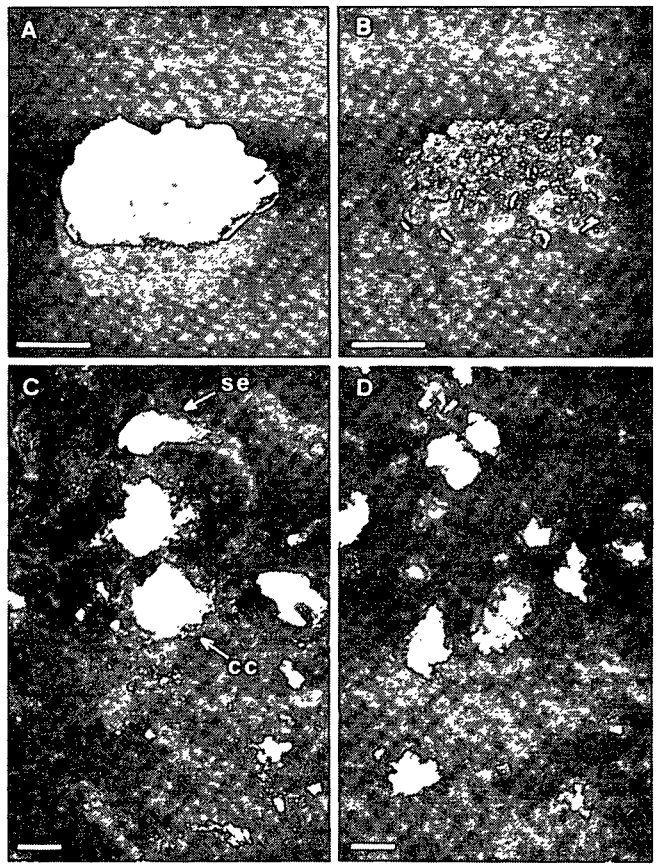

Fig. 3 Callus phenotypes derived from one Norway spruce immature embryo explant, (NS1)5, and grown under identical cultural conditions. A) White to translucent embryogenic callus. B) Green callus with little regeneration potential. C) White embryogenic callus dispersed and plated in a thin agarose layer. Note somatic embryos (SE) and cell clusters (CC). D) Green callus dispersed and plated as in C. Scale bars A & B = 5.0 mm, C & D = 0.5 mm.

The frequency of immature embryo explants forming the white embryogenic callus was higher at early collection dates (Table 1). Over three-fourths of the explants (14 of 18) collected on July 12, 1985 in Appleton, Wis initiated white embryogenic callus. Four of these formed only the white embryogenic callus. The remaining ten developed green callus initially, and after several subcultures the green sectored into the white and green phenotypes. All other callus lines which had had both phenotypes (e.g., two NS2 lines, eleven NS3 lines, eleven NS4 lines, and four NS8 lines) developed in this manner; that is, green callus originated from the explant and white callus later developed from it. Embryo explants collected in Appleton after 7/26 did not form the white callus, but rather only the green callus. Explants derived from trees in Argonne, Wis. (located approximately 200 km north of Appleton) responded similarly (Table 1). That is, the earlier the collection date, the higher the frequency of initiation of the

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desirable white callus. These results indicate the importance of time of explant collection, and suggest a developmental "window" exists in which immature Norway spruce embryos are highly responsive to forming embryogenic callus.

Table 1 Identification of codes and summary of callus lines initiated in Norway spruce

Explant Collection			Number	Callus Lines Initiated[a]		
Code	Site	(1985 date)	of Explants	W Only	(Both)	G Only
NS1	Appleton	7/12	18	4	(10)	1
NS2	Appleton	7/19	10	1	(2)	5
NS3	Appleton	7/26	20	0	(11)	9
NS4	Argonne	8/2	18	1	(11)	6
NS5	Appleton	8/2	18	0	(0)	16
NS6	Appleton	8/9	25	0	(0)	25
NS7	Appleton	8/16	45	0	(0)	45
NS8	Argonne	8/26	88	4	(4)	75

[a]W = white embryogenic callus. G = green callus. Value in parentheses indicates number of lines having both callus types derived from the same explant genotype.

Quantification of Embryogenesis

Callus lines derived from 23 different immature embryo explants and maintained under identical cultural conditions for 7 months were evaluated for embryogenic capacity using the procedure outlined in Figs. 1 and 2. The callus lines are ranked according to somatic embryo density in Table 2. There were significant differences in the level of somatic embryogenesis among the white 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 highly embryogenic callus lines 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 2 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). In light of the results in Table 1, this suggests that the developmental stage of the embryo explant effects both the initiation frequency of embryogenic white callus and the subsequent embryogenic capacity of the resulting callus lines.

Eight green callus lines were surveyed for the presence of somatic embryos (Table 2). Somatic embryos were only found in one line, (NS5)17. Although this occurred at a very low frequency (two somatic embryos counted in one of the four dispersed callus pieces). It 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. As previously described, this was how the white callus lines such as (NS1)1, (NS1)5, and (NS1)8 were initially derived, that is, the green callus sectored into both white and green types.

Table 2 Differences in somatic embryogenesis among Norway spruce callus lines

Callus[a] Line	Callus[b] Phenotype	Somatic Embryos[c] 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

[a]Code in parenthesis identifies explant collection. Number following parenthesis identifies explant from which callus derived.
[b]W = white embryogenic callus. G = green callus.
[c]Mean value of four observers on each of four callus pieces per each line. Means followed by common letters are not significantly different as determined by an analysis of variance with Duncan's new Multiple Range Test ($p = 0.05$).

In summary the white embryogenic callus lines we tested all had a similar phenotype based on visual observations. 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 suspensor-like cells, and cell clusters all embedded in a mucilaginous matrix (personal communication, R. Nagmani).

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. It should be possible to

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determine the importance of genetic, physiologic, or other components of embryogenesis among Norway spruce callus lines. This technique has already proven useful for evaluating the effectiveness of biochemical treatments aimed at enhancing the level of embryogenesis (9).

Somatic Embryo Development and Plant Regeneration

The developmental sequences of Norway spruce somatic embryos plated in a thin layer of agarose is shown in Fig. 4A-C. Somatic embryos were initially embedded in the agarose layer (Fig. 4A). Somatic embryo counts were taken at this stage. Elongation of the suspensor cells pushed the embryonal head up and out of the agarose layer (Fig. 4B). This is similar to early zygotic embryo development in vivo in that suspensor elongation pushes the embryonal head through the erosion cavity of the female gametophyte (10). Further development of somatic embryos, hypocotyl elongation and cotyledon formation, occurred on somatic embryos protruding above the agarose layer (Fig. 4C). A similar developmental sequence occurred on somatic embryos on callus which had been transferred through the Fig. 1 developmental protocol. Somatic embryos were easily removed with a forceps (Fig. 4D), from either callus or the agarose layer. The developing ring of cotyledonary leaves was prominent at this stage (Fig. 4E). The internal anatomy of somatic embryos appeared similar to conifer zygotic embryos that had developed in vivo (Fig. 4F). Development of a primary root and needles is shown in Fig. 4G. Several somatic embryo derived plants have been transferred to soil.

POTENTIAL FOREST BIOTECHNOLOGY APPLICATIONS

A summary diagram of the conifer somatic embryogenesis process and how it could be utilized for asexual propagation and cellular genetic modification is shown in Fig. 5. Currently, only juvenile explant tissue of conifers has been induced to form embryogenic callus. This includes immature embryos and female gametophytic tissues. The report of embryogenic callus derived from mature embryo explants of Norway spruce is significant in this regard (4). Although mature embryos are juvenile, it demonstrates that their cells retain

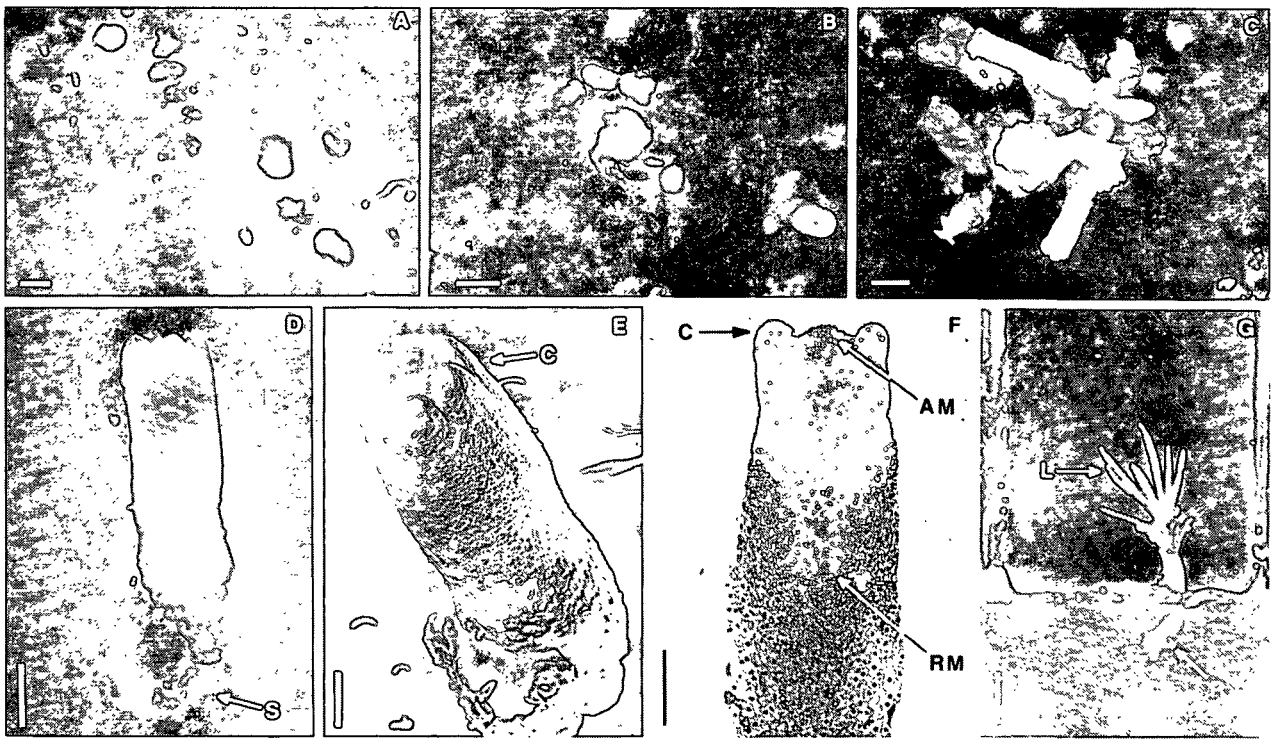

Fig. 4 Plant regeneration via somatic embryogenesis in Norway spruce. A through C shows the developmental sequence of somatic embryos cultured in a thin layer of agarose medium. A: At time of dispersal, several somatic embryos with dense embryonal heads and elongated suspensor tails. B: Somatic embryos 14 days later. Note suspensor cells push embryonal head up and out of agarose layer. C: Cotyledons of somatic embryos are visible and green 14 days later. D: Fully developed somatic embryo removed from agarose layer with elongated suspensor (S) attached. E: Scanning electron micrograph of somatic embryo showing developing cotyledonary leaves (C). F: Photomicrograph of thin section of somatic embryo stained with toluidine blue. AM = apical meristem, RA = root apex. G: Norway spruce plant regenerated from somatic embryo. Note development of true leaves (L) and growth of primary root (R). Scale bars; A-C = 1.0 mm, D = 0.5 mm, E & F = 0.25 mm, and G = 5.0 mm.

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the capacity to form embryogenic callus well beyond the narrow "window" of time during early embryo development. Because the zygotic embryo arises from the sexual fusion of gametes, it is genetically dissimilar from the maternal tree. Therefore, it is not the preferred explant for "true-to-type" cloning. The importance of finding regeneration from somatic embryos of Norway spruce, regardless of the explant, is that it demonstrates that conifers are not unlike numerous other herbaceous and woody plant species in having the capacity to undergo embryogenesis *in vitro*.

In order to develop somatic embryogenesis as a viable method for mass propagation of conifers it will be necessary to obtain embryogenic callus from explants which are more likely to result in "true-to-type" regenerates. One possibility is mature vegetative tissue, such as vegetative bud meristems or newly developed leaf fascicles (Fig. 5, upper right). This is a formidable task as most mature conifer tissues have limited capacity for either conventional propagation or *in vitro* morphogenesis (11). An alternative is to use juvenile explant tissue that does not arise from sexual gametic fusion. Nucellar tissue is an example. It is diploid and genetically the same as the maternal tree. Furthermore, there are several reports of nucellar derived somatic embryogenesis in woody plants (12), including species other than *Citrus*, such as *Vitis* and *Ribes* that are naturally monoembryonic.

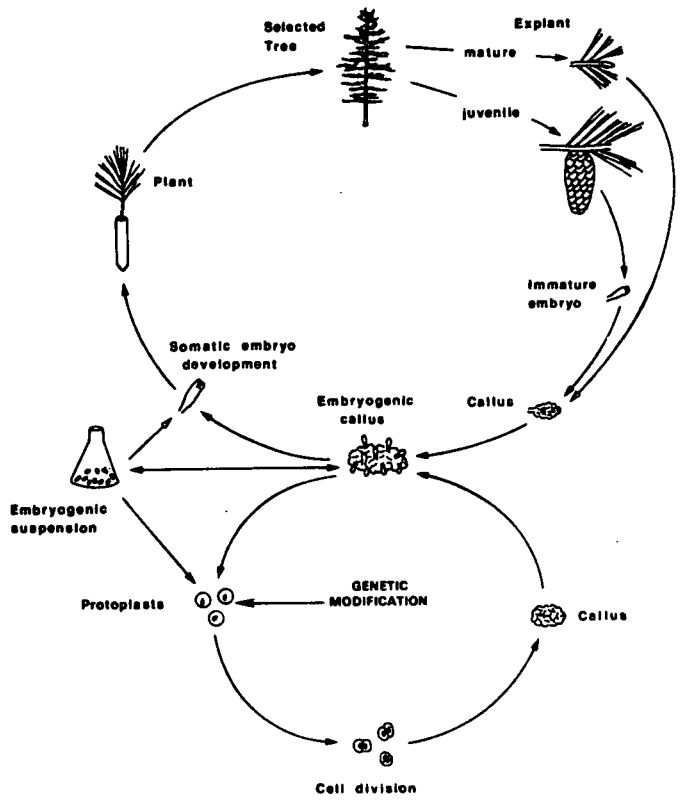

Fig. 5 Potential forest biotechnology application of a conifer somatic embryo regeneration system.

Also shown in Fig. 5 are two cell culture techniques, maintenance of liquid embryogenic suspensions and isolation of protoplasts, that will be important for development of cellular genetic modification procedures. Protoplasts, cells in which the cell wall has been enzymatically removed, are more amenable to genetic modification techniques than are cells with intact cell walls. We are currently developing liquid suspensions from Norway spruce embryogenic callus. Both liquid embryogenic suspensions and embryogenic callus cultures are ideal sources for obtaining cells for isolating protoplasts (Fig. 6). In several cereal crops, protoplast isolation from rapidly growing embryogenic suspensions was required to obtain subsequent protoplast division, callus formation, and plantlet regeneration (13-15). Several techniques for direct cellular genetic modification are currently available and should be applicable to conifer protoplasts. These include polyethylene glycol induced DNA uptake into protoplast (16), and electroporation - a brief electrical pulse applied to a protoplast suspension to permeabilize the plasma membrane and increase uptake of DNA (17). Both of these transformation techniques have resulted in stable integration of foreign DNA into the genomic DNA of the plant cells.

CONCLUSIONS

Of the potential applications of somatic embryogenesis, mass propagation and cellular genetic modification, our program emphasis is to first develop this *in vitro* technique for mass propagation of commercially important loblolly pine and Douglas-fir. Having an operational conifer somatic embryogenesis regeneration system in Norway spruce is an important step toward this goal in several ways. First and perhaps most importantly, differences between embryogenic and nonembryogenic conifer cells can now be characterized and embryogenic markers developed (9). Furthermore, as discussed previously, the morphology of embryogenic callus in both Norway spruce and European larch is similar. This gives support to the hypothesis that embryogenic callus in other conifers will be of similar quality. An important question that remains to be answered before mass propagation of conifers via somatic embryogenesis can be fully developed, is the uniformity of regenerated plants. It should now be possible for the first time in conifers to answer this question and determine the degree of somaclonal variation among somatic embryo derived plants.

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