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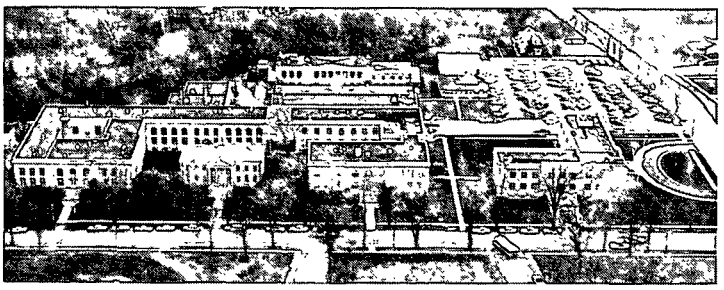

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NORWAY SPRUCE AS A MODEL SYSTEM FOR SOMATIC EMBRYOGENESIS IN CONIFERS

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ABSTRACT

Embryogenic and nonembryogenic cultures of Norway spruce were initiated from the same genotype and cultured under identical conditions. Biochemical, metabolic, and ultrastructural analysis of the two tissue types revealed many differences, some of which may be considered as characteristic of an embryogenic or nonembryogenic state in conifers. The parameters measured might be employed as markers for embryogenesis, or alternatively, to identify metabolic pathways critical for the further growth and development of conifer somatic embryos into plants.

INTRODUCTION

Until 1985, no confirmed examples of somatic embryogenesis in conifers existed in the literature. However, just within the past year, high frequency somatic embryogenesis has been reported in Picea abies (L.) Karst. (Norway spruce) (1) and Larix decidua L. (European larch) (2). These reports of somatic embryogenesis in two coniferous subfamilies suggest the process is generic to the family Pinaceae. Nevertheless, somatic embryogenesis in the commercially important genus Pinus remains elusive. To the extent that the process of somatic embryogenesis is similar among conifers, modeling somatic embryogenesis in spruce or larch may assist in obtaining embryogenesis in pines.

In this report, we have taken advantage of the observation that both embryogenic and nonembryogenic callus of Norway spruce are initiated from the same culture when the explant (immature embryo) is cultured near the time of cotyledon differentiation (1). Embryogenic and nonembryogenic callus were manually separated from each other and maintained independently (but under identical culture conditions) to provide pairs of cell lines of the same age and genotype that were homogeneous with respect to callus type. Biochemical, metabolic, and ultrastructural comparisons of the two callus types revealed many significant differences, some of which might be considered as characteristic of an embryogenic (or nonembryogenic) state in conifers. Knowledge of the metabolic and biochemical state of the embryogenic cell type can be used not only to develop simple yet definitive biochemical assays for detecting embryogenic tissues, but also to define metabolic pathways critical to the growth and development of somatic embryos.

MATERIALS AND METHODS

Cell Cultures

Immature ovate cones of Norway spruce [Picea abies (L.) Karst.] were collected at weekly intervals throughout the month of July from a site in Appleton, WI.

Cones were cold treated, seeds were sterilized, and cultures were initiated from immature embryos by the method of Hakman et al. (1). Briefly, immature embyos were cultured (and subsequently maintained) on a solidified basal medium (0.5% agar; Bacto, Difco) described by Hakman containing 2% sucrose and 2 mg/L 2,4-D and 1 mg/L BA. Cultures were incubated under a 16 h photoperiod of 1,200 lux cool-white fluorescent and 800 incandescent at 22°C. After 4-6 weeks both embryogenic and nonembryogenic tissues were initiated from the majority of the embryos. Manual separation of the two callus types with a forceps afforded the establishment of both embryogenic and nonembryogenic cell lines from the same explant. All biochemical data were recorded after the two callus types had been separated and cultured independently for at least 4 months. Somatic embryo development was accomplished by the procedure of Becwar et al. (3). Briefly, pieces of embryogenic callus (100-200 mg fresh weight) were transferred to basal medium lacking growth regulators but containing 1% activated charcoal. After 1 week, callus pieces were transferred to basal medium containing 1 µM each IBA and ABA. All environmental conditions were as described above. After two weeks, the level of somatic embryogenesis (expressed as the number of somatic embryos per fresh weight) was determined by the dispersal method described by Becwar et al. (3).

Ethylene Determination

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 stoppered with a rubber stopper containing a cylindrical half-hole rubber septum. After 24 hours, 1 mL of head gas 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 a detector temperature of 200°C the average retention time for ethylene was 1.45 minutes.

Glutathione (GSH) and Total Reductants Determination

Callus pieces ranging from 30 to 110 mg fresh weight were extracted with 10 mL of Type I water in a Ten Broeck homogenizer for GSH analysis. 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 (4) as recently described (5).

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For assay of "total" reductants (of sufficiently negative potential to reduce ferric ion), callus pieces 10-30 mg in fresh weight were employed, but the assay could be reliably performed on 1-2 mg of callus as well. The tissue was extracted with water as above, centrifuged at low speed, and reductants were assayed in the supernatant by the method of Singh et al. (6), i.e., equal volumes of extract and freshly prepared ferric chloride-potassium ferricyanide reagent were mixed, incubated 1 hr 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 A_{760}/g fresh weight. Where protein was determined on the aqueous extracts, the Bradford reagent (7) was used.

Protein Synthesis Rate Determination

Callus pieces of about 100 mg fresh weight were placed in wells of multiwell plates (24 wells/plate; 2 mL/well) to which was added 300 µL liquid basal medium containing the growth regulators used for culture maintenance. To this was 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 h, the tissue was collected, rinsed with 10 mM (unlabelled) leucine, and homogenized in 500 µL buffer (50 mM HEPES, pH 7.6). After centifugation in an Eppendorf microcentrifuge, the supernatant was treated with 3 mL cold 10% TCA, mixed, and stored at 0°C for 30 min. A portion of this supernatant was also used for protein determination by the method of Bradford (7). Upon standing at 0°C, a precipitate was collected on Whatman GF/A glass filter pads, dried, and measured for radioactivity.

Microscopy

Samples of embryogenic and nonembryogenic tissue were prepared for transmission electron microscopy as follows: Material was fixed in 3% gluteraldehyde (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 0.1 µm and stained with uranyl acetate [5% in 50% (v/v) ethanol] followed by lead citrate (0.5%, pH 12).

Inhibitor Studies

To test the effects of the specific enzyme inhibitors buthione sulfoximine (BSO) and α-aminooxy-β-phenylpropionic acid (AOPP) on somatic embryogenesis 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) 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 (3). 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.

RESULTS AND DISCUSSION

Marker Development

Embryogenic callus of Norway spruce can be readily distinguished from nonembryogenic callus by its color and texture. This macroscopic difference in appearance is apparently reflected at the cellular level by the biochemical differences shown in Table 1. It should be stressed that these differences are in all likelihood physiological, as the unique mode of differentiation of the two callus types permits comparisons in which genotype, age, and nutrional, hormonal and environmental influences are all constant. Comparative situations between embryogenic and nonembryogenic tissues with the above features held constant are unusual in plant tissue culture even outside of conifers, and in this regard Norway spruce provides an excellent system for the development of biochemical markers for somatic embryogenesis.

Table 1 Biochemical differences between embryogenic (E) and nonembryogenic (NE) Norway spruce callus[1]

Assay	Callus Type NE	Callus Type E	Ratio, NE/E
Ethylene evolution rate, mL/mg fresh weight/day	1.1-1.8	0.01-0.09	19-117
GSH, nmoles/µg protein	1.7-2.2	0.27-0.35	5.3-7.1
Total reductants, A_{760}/g fresh weight	438-535	22-32	17-20
Protein synthesis rate, cpm ^{3}H-leu/µg protein[2]	32-60	688-764	0.05-0.08

[1]All results statisically significant as determined by one-way ANOVA with Duncan's New Multiple Range test ($p = 0.05$); results based on analysis of three genotypes (except where noted) in which embryogenic and nonembryogenic callus lines were available.
[2]Two genotypes assayed.

In order for the measurement of a biochemical, metabolic or ultrastructural feature to find utility as a marker for embryogenic potential it should meet several requirements. Aside from being unambigously characteristic of an embryogenic state for conifers in general, any feature under consideration as a marker should be easily and rapidly measured and require small amounts of tissue. These requirements are needed because often the

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deterministic events for embryogenesis occur shortly after culture initiation and even then only in localized sectors of the primary culture. However, potential markers do not have to reflect causal events in the process of embryogenesis, but need only be indicative of features associated with an embryogenic condition.

All of the determinations in Table 1 fit the above mentioned criteria for markers of somatic embryogenesis in Norway spruce. In all assays except for GSH, differences at least an order of magnitude were detected between the two callus types. The large differences between the two callus types suggest that the substances measured in Table 1 are characteristic of an embryogenic (or nonembryogenic) state in Norway spruce.

All assays were rapid (usually less than 24 h) and tissue requirements ranged from less than 10 mg for the determination of total reductants to 30-100 mg for the remaining assays. In addition, analysis of ethylene enjoys the distinct advantage of being nondestructive to the sample, enabling its reuse in other experiments. While the biochemical assays shown in Table 1 are diagnostic of an embryogenic state in Norway spruce, it is not known if any of the substances measured had a role in determining whether or not this state was achieved. Ethylene, as is the case with most plant growth regulators has been shown to have both inhibitory and stimulatory effects on morphogenesis. For example ethylene has been shown to inhibit somatic embryogenesis in wild carrot (8) but stimulate bud formation on lily scale explants (9). The observation that nonembryogenic Norway spruce callus endures considerable amounts of ethylene has also been reported in nonmorphogenic cell suspensions of loblolly pine (10), suggesting that high ethylene evolution rates might be a general feature of nonembryogenic tissues in conifers.

Elevated levels of GSH have been previously shown to be associated with undifferentiated plant tissue compared to differentiated tissue (11). Accordingly, as differentiation proceeds endogenous GSH levels decline in the tissue. An indication of the universality of the inverse relationship between GSH and the extent of differentiation is that it has been observed in such diverse systems as slime mold (12), wild carrot (5) and malignant animal cell lines (13). In light of the observation that embryogenic Norway spruce callus contains numerous somatic embryos in various stages of development it is not unexpected that this tissue would fit the above relationship as well. The utility of GSH concentrations as a marker therefore may depend on the presence of differentiated structures, which might not be present in tissues that are just beginning to differentiate, or, tissues that have the capacity for differentiation but are inhibited from doing so by the culture conditions.

On the other hand, the measurement of the total reducing power of the tissue may find practical application not as marker for the competency for embryogenesis but rather as an indication of a lack of embryogenic potential. A characteristic exhibited by most plant tissue cultures including conifers is that proliferative (callus) growth can be maintained by auxin. However, in conifers, unlike Umbelliferous plants, removal of the auxin in attempts to stimulate organized growth results in a cessation of growth and a rapid decline in tissue as indicated by the extensive browning. This browning, attributable to the formation of condensed phenolic compounds (tannins) is a principal feature of most conifer callus, irrespective of the species of original explant. Nonembryogenic callus of Norway spruce is no different from other conifers in this respect. Although Table 1 shows that the level of reducing agents is substantially higher in nonembryogenic callus, a survey of compounds that react with the single reagent (Table 2) reveals that phenolic compounds are in all likelihood responsible for reduction of the reagent. These phenolic compounds are precursors for flavonoid and lignin biosynthesis and probably do not function as reducing agents in the cell. However, compounds such as those depicted in Table 2 are likely responsible for the browning exhibited upon transfer to auxin-free medium by nonembryogenic callus and these compounds are apparently lacking in embryogenic callus. Therefore, it may be inferred that the presence of phenolic compounds in conifer callus indicates a situation that is not conducive to morphogenesis and thus represents a "negative" marker for embryogenic potential.

Table 2 Specificity of total reductant test

Compound	A_{760}/m mole
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

Little in the way of significance can currently be attached to the observations on the rate of protein synthesis or the chloroplast structure of the two callus types. The high rate of protein synthesis occurring in the embryogenic callus may be a consequence of the diversity of processes required to maintain a system that is both differentiating and dedifferentiating simultaneously. The low level of phenolic compounds present in the embryogenic callus may reflect a level of competition between phenolic compound and protein synthesis. Presumably, the common substrate phenylalanine could be incorporated into protein, or be deaminated by phenylalanine ammonia lyase (PAL) to give the phenolic precursor, trans-cinammic acid. High protein synthesis rates that might be coupled with a low PAL activity in embryogenic callus may effectively limit the amount of phenolic compounds synthesized by this tissue.

The most obvious and striking difference between the two callus types is their color. Nonembryogenic Norway spruce callus grown in the light is the typical green color characteristic of other light-grown conifer callus. Embryogenic callus remains white to translucent even when cultured in the light. Nevertheless, both callus types contain chloroplasts (Fig. 1 and 2). In nonembryogenic callus, the chloroplasts are not altogether atypical and are highly differentiated, with well formed

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thylakoid membranes and grana. However, cloroplasts in embryogenic tissue represent an unusual type, which cannot be ascribed as an intermediate state in the normal process of chloroplast development.

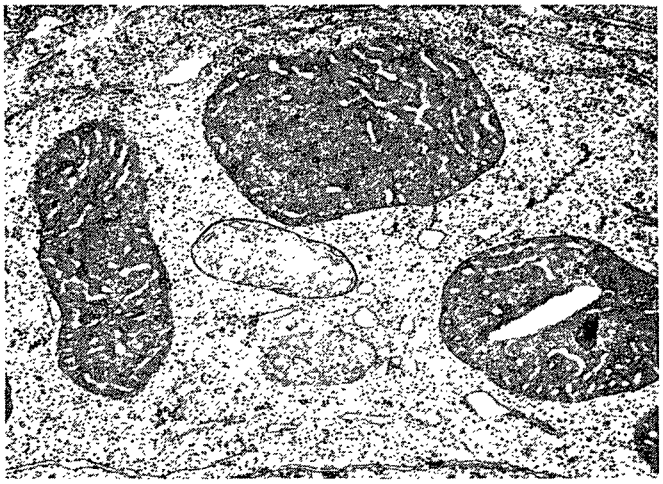

Fig 1 Chloroplasts in embryogenic Norway spruce callus (X 19,000).

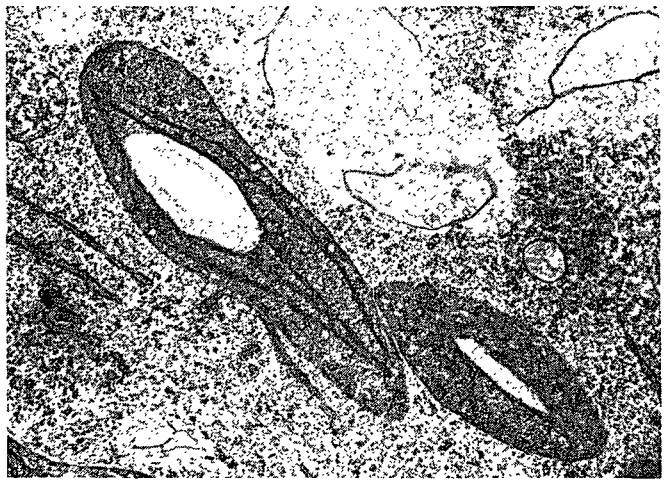

Fig 2 Chloroplasts in nonembryogenic Norway spruce callus (note well formed grana encircling starch grain, X14,000).

With the observation of well formed chloroplasts and the detection of lignin and flavonoid precursors (phenolic) in nonembryogenic callus, it appears that this tissue may be more cytologically differentiated than embryogenic callus. The observation of a "window" in the capacity for production of embryogenic callus from immature embryos (1) suggests that an immature zygote is best able to return to a state from which embryogenic redetermination can occur. Apparently, the capacity to dedifferentiate to this extent is lost rapidly by the developing zygote. As a result, most explants are capable of achieving only a certain level of dedifferentiation - apparently a state in which a cytologically differentiated callus cell is produced.

Inhibitor Studies

In addition to providing markers for somatic embryogenesis, biochemical studies of embryogenic and nonembryogenic callus also serve to identify metabolic pathways that are at least associated with, if not critical to somatic embryogenesis. By perturbation of these pathways, followed by determination of the effect on somatic embryogenesis, some knowledge of the importance of certain aspects of metabolism may be gained. Given the apparent association of high concentrations of GSH and phenolics with a nonembryogenic condition, attempts were made to inhibit their synthesis in embryogenic callus with BSO, and AOPP, respectively. Both of these chemicals are specific enzyme inhibitors, with BSO inhibiting glutamylcysteine synthetase (14) and AOPP inhibiting PAL (15). To address the question of whether or not these metabolic pathways play a deterministic role in somatic embryogenesis, the inhibitors were added to the medium in which the embryos are differentiated (i.e., embryo proliferation medium). On the other hand, if these metabolic pathways are critical to the further development of somatic embryos into seedlings, this can be tested by the addition of the inhibitors to the embryo development medium.

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 to the inhibitors to embryo proliferation medium always resulted in a decrease in embryogenesis (Tables 3 and 4). Therefore, it seems that both GSH levels and total reductants (i.e., phenolics) are associated with an embryogenic condition, but probably in the developmental aspects of somatic embryogenesis. However, the role of these inhibitors in the process of differentiation was also apparent by their inhibition of embryo production. In the case of GSH, the inhibition of differentiation 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. 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 (5). However, no such precedent exists 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 = 0.17 embryos/mg) 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.

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Table 3 Effect of BSO and GSH on somatic embryogenesis in Norway spruce[1]

Treatment	Somatic Embryo Density, embryos/mg		
	Site of Treatment Application		
	Proliferation Medium	Developmental Medium	
	(NS1)13[2]	(NS1)1	(NS1)5
Control	0.82a	0.46a	0.51b
10^{-5} mM BSO	0.47b	0.50a	0.61ab
10^{-4} mM BSO	0.32bc	0.51a	0.81a
5×10^{-3} mM GSH	0.20c	0.23b	0.47b

[1]Within a column, means followed by a common letter are not significantly different as determined by one-way ANOVA with Duncan's New Multiple Range test ($p = 0.05$).
[2]Cell line designation, see Ref. (3).

Table 4 Effect of AOPP on somatic embryogenesis in Norway spruce

Treatment	Somatic Embryo Density, embryos/mg		
	Site of Treatment Application		
	Proliferation Medium	Developmental Medium	
	(NS2)4	(NS1)2	(NS1)12
Control	0.45a	0.17b	0.46a
10 µM AOPP	0.33ab	0.21b	0.36a
20 µM AOPP	0.30b	0.34a	0.43a

[1]Within a column, means followed by a common letter are not significantly different as determined by one-way ANOVA with Duncan's New Multiple Range test ($p = 0.05$).
[2]Cell line designation see Ref. (3).

CONCLUSIONS

It is not clear at this point whether the substances discussed here play a causal role or are merely associated with somatic embryogenesis in Norway spruce, as the inhibitor studies would seem to indicate. In either event, a descriptive approach to analysis of nonembryogenic and embryogenic callus types is all that is required for marker development. This descriptive approach resulted in the additional benefit of identifying metabolic pathways that are, at the very least, associated with somatic embryogenesis. The contribution that biochemical analysis can make toward both marker development and an understanding of embryo development serves to illustrate the utility multidisciplinary approach to plant tissue culture.

Although it cannot be stated with certainty that somatic embryogenesis in pines will be the same as in spruce, the similarity between the nonembryogenic conditions among conifers is worth noting. In this regard, the markers developed here can be deployed at least to assist in the early identification of a nonembryogenic state in other coniferous species.

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