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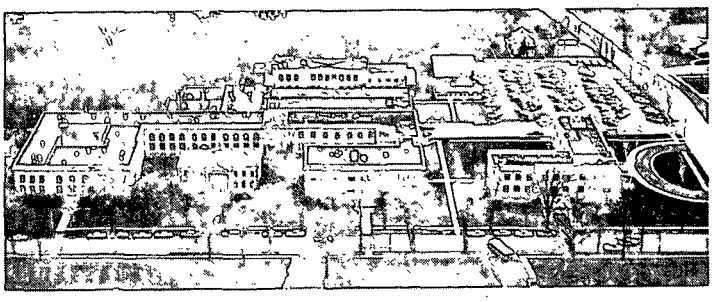

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BIOCHEMICAL DIFFERENCES BETWEEN EMBRYOGENIC AND NONEMBRYOGENIC CALLI OF CONIFERS

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

Additional key words - Pine, spruce, larch, glutathione, ethylene, protein

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Introduction

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

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

Abbreviations - GSH, reduced glutathione; GSSG, oxidized glutathione; IEF, isoelectric focusing; PMSF, phenylmethyl sulfonyl fluoride

Materials and methods

Initiation of embryogenic and nonembryogenic calli

Embryogenic calli of Pinus taeda L., Pinus strobus L., Picea abies (L.) Karst, and Picea glauca (Moench) Voss were initiated from immature embryos. In Picea spp., initiation of embryogenic callus was by the method of Hakman et al. (1985). In Pinus taeda and Pinus strobus, initiation occurred on a wide variety of media in the dark, in some cases similar to conditions described by Gupta and Durzan (1986). In Pinus serotina Michx., embryogenic callus was extruded from the archegonial end of cultured intact ovules, similar to the method described by Smith et al. (1985). Embryogenic cultures of Larix decidua Mill were generously provided by J. M. Bonga and were initiated as described by Nagmani and Bonga (1985). Some of the details of the initiation processes for the various conifers studied are depicted in Table 1. Nonembryogenic callus was obtained under most of the conditions listed above by the culture of embryos of much later stages of development than are required for the initiation of embryogenic callus.

(Table 1 here)

Biochemical assays

Biochemical analyses of embryogenic and nonembryogenic calli were conducted as previously described (Wann et al. 1986, 1987). Briefly, ethylene was determined by gas chromatography; apparent protein synthesis was measured by tritiated leucine incorporation into trichloroacetic acid precipitable protein; GSH was measured by the rereduction of oxidized GSH (GSSG) by commercial GSSG reductase; and total reductants were measured by ferric ion reduction. In Picea spp., embryogenic and nonembryogenic calli of the same genotype and age cultured under

the same nutritional, environmental and hormonal conditions were assayed. Isogenic analyses of embryogenic and nonembryogenic calli were not conducted in Pinus and Larix, although the conditions used to initiate both phenotypes were similar.

The extracts used for the isozyme analyses were prepared by the freeze/thaw technique of Berger et al. (1985). The isoperoxidases were resolved by isoelectric focusing (IEF) for 35 min at 25 watts on a pH 3.5-9.5 agarose gradient using an LKB Multiphor 2117. The stain was a modification of that used by Gove and Hoyle (1975) and consisted of 45 mM guaiacol, and 3 mM H_2O_2 in 0.2M acetate buffer, pH 5.0. Some bands are subject to fading as is well known for the guaiacol reaction (Maehly and Chance 1954). This is not a major problem in this agarose IEF system, although any photographs should be taken without undue delay. For the SDS-PAGE analyses, soluble proteins were extracted from embryogenic or nonembryogenic calli by homogenizing the tissue in cold 50 mM HEPES pH 7.5 containing 1 mM PMSF (phenylmethylsulfonyl fluoride). After a five minute centrifugation in an Eppendorf microfuge, protein in the supernatant was quantified by the Bradford procedure (Bradford 1976) and loaded onto standard 7.5-15% gradient SDS-polyacrylamide gels (Laemmli 1970). Equal amounts of protein (2-3 µg) were loaded onto each lane. Visualization was by a modified silver stain (Oakley et al. 1980).

Results and discussion

Biochemical characterization of embryogenic vs. nonembryogenic calli of several conifers is presented in Table 2. All but the larch embryogenic callus were initiated in this laboratory, and that was started by one of the authors before

she joined this laboratory. Data of this nature for Norway spruce have been published previously, but some is presented here for reference. As can be noted in Table 2, for Norway spruce and white spruce we have had the luxury of comparing embryogenic and nonembryogenic calli that originated from a single explant. Only one comparison of an embryogenic with a nonembryogenic callus is presented for each species. Although more data are extant for additional cell lines of each species, those presented are typical of calli with established embryogenic competence or incompetence.

(Table 2 here)

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

Whereas the full significance of the foregoing observations is not yet appreciated, several important statements can be made about them. For these biochemical parameters, the embryogenic state in conifers is the same irrespective of species. The embryogenic condition in conifers may also be described as a state function, i.e., it is independent of the path by which it is reached. "Path" in

this context includes explant, culture conditions and even ploidy level (the Larix embryogenic callus is haploid, others are diploid). Biochemical characterization of the embryogenic state in conifers takes on increased importance because, morphologically, the embryogenic condition in the calli of these trees is only nominally similar and is path dependent. For example, when the embryogenic calli are grown under proliferative conditions, the extent of somatic embryo development varies with species. In pines, embryogenic callus may be dominated by preembryogenic masses or very early stage embryos. However, in spruce and larch the somatic embryos reach a fairly advanced state of development without removal of the embryogenic calli from proliferation media (Fig. 1).

(Fig. 1 here)

The magnitudes of the biochemical differences between the two phenotypes were such that the assays could be utilized as markers for embryogenic callus in conifers. This was demonstrated with the total reductants assay which predicted embryogenic potential months in advance of the appearance of somatic embryos in loblolly pine and pond pine. In pines, embryogenic potential was predicted at a time when the calli were dominated by what turned out to be preembryogenic masses (Fig. 2).

(Fig. 2 here)

Whereas the foregoing conclusions apply to all of the conifers that were investigated, white spruce (Picea glauca) stands out as not exhibiting clear biochemical differences in these parameters between embryogenic and nonembryogenic callus. The lack of significant differences in total reductants can be easily understood in light of the observation that, even when growing under proliferation conditions with 2,4-D, white spruce embryogenic calli sometimes

exhibit a pink coloration indicative of anthocyanins which can reduce ferric ion in the test. In other words, unlike the other conifers, white spruce somatic embryos occasionally show precocious secondary product accumulation. The lack of significant differences in the other markers for white spruce might be related to the observation that it takes several subculture intervals to separate the two callus phenotypes of spruce (Wann et al. 1987). Thus, for a period, apparently nonembryogenic callus will continue to contain localized regions where embryogenic tissue will emerge. It is quite possible that when the two callus phenotypes of white spruce were assayed, complete segregation of embryogenic and nonembryogenic callus had not yet been completed even though visibly so. It should be noted that the trends in each of these parameters for the two phenotypes are the same in white spruce as for the other conifers. It is likely that with greater replication white spruce would also show statistically significant differences in these parameters for the two phenotypes.

Both isozyme patterns and SDS-PAGE separations of soluble proteins have been useful in distinguishing embryogenic from nonembryogenic calli as well. Fig. 3 shows that three different Norway spruce cell lines each exhibit distinctive guaiacol isoperoxidase banding patterns for embryogenic versus nonembryogenic callus extracts. Isozyme patterns of other enzymes such as acid phosphatase (not shown) are also capable of sorting the two phenotypes. The proteins separated and visualized by SDS-PAGE showed clear differences between embryogenic and nonembryogenic calli with the exception of white spruce (Fig. 4). White spruce presents difficulties for the other markers also as discussed above. The most obvious difference observed on the protein gels is the prominence of a protein of approximately 18-20 kd in nonembryogenic tissues of all but the white spruce. The use of isozymes and total protein patterns to iden-

tify embryogenic tissue is becoming common, e.g., see Everett et al. (1985) and Sung and Okimoto (1981).

(Fig. 3 and 4 here)

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

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Table 1. Summary of procedures used to initiate embryogenic calli in various spruce and pine species.

Species	Explant	Medium	Growth Regulators, mg/L	Culture	Reference
Picea abies *Picea glauca*	Immature cotyledonary embryos	LP[a]	2 2,4-D; 1 BA	Light	Hakman *et al.* 1985
Pinus taeda	Immature precotyledonary embryos	MSG[b]	2 2,4-D; 1 BA	Dark	Gupta and Durzan, 1986
Pinus strobus	Immature precotyledonary embryos	DCR[c] MSCG[d]	3 2,4-D; 0.5 BA 5 2,4-D	Dark	Gupta and Durzan, 1986
Pinus serotina	Fertilized ovules	MSG[b]	None	Light, 1% char coal	Smith *et al.* 1985

[a]von Arnold and Eriksson, 1981. Can. J. Bot. 59:870-874.
[b]Amerson *et al.* 1985. *In*: Tissue Culture in Agriculture and Forestry, Henke, Hughes, Constantin, and Hollander (eds.), Plenum Press, NY. p. 274.
[c]Gupta and Durzan 1985. Plant Cell Rep. 4:177-179.
[d]MSG medium supplemented with casein hydrolyzate 1000 mg/L and glutamine 500 mg/L (R. Nagmani, unpublished).

Table 2. Biochemical differences between embryogenic (E) and nonembryogenic (NE) conifer callus.

Species	Cell Line	Embryo-genic?	Protein Synthesis,[a] cpm/µg	Total Reductants,[a] A_{700}/g fwt	GSH,[a] nmol/g fwt	Ethylene,[a] nL/g fwt
Pinus taeda (loblolly pine)	(LP12F)1	Yes	6474 ± 220	7 ± 1	59 ± 10	2 ± 2
	(LP10DE)	No	193 ± 28	28 ± 2	510 ± 40	689 ± 151
P. strobus (E. white pine)	(WP2II)1	Yes	8172 ± 853	13 ± 2	140 ± 19	6 ± 6
	(WP5B)3	No	186 ± 45	172 ± 32	378 ± 43	759 ± 61
P. serotina (pond pine)	(PO12Ao)1	Yes	7684 ± 878	8 ± 1	88 ± 16	8 ± 1
	(PO10Ag)3	No	2997 ± 2642	83 ± 22	704 ± 86	111 ± 35
Picea abies (Norway spruce)	(NS1)8	Yes	5061 ± 1614	32 ± 2	120 ± 28	0.15 ± 0.04
	(NS1)8	No	157 ± 77	535 ± 10	325 ± 40	1.75 ± 0.57
P. glauca (white spruce)	(WS5B)8	Yes	4960 ± 861	119 ± 14	434 ± 21	252 ± 87
	(WS5B)8	No	2067 ± 874	210 ± 78	524 ± 116	606 ± 301
Larix decidua (European larch)	(L1-18)5	Yes	1859	85 ± 37	N.D.	N.D.
	(L253-2)12	No	53	848 ± 206	N.D.	N.D.

N.D. = Not determined.

[a]Means ± S.D.; N = 3.

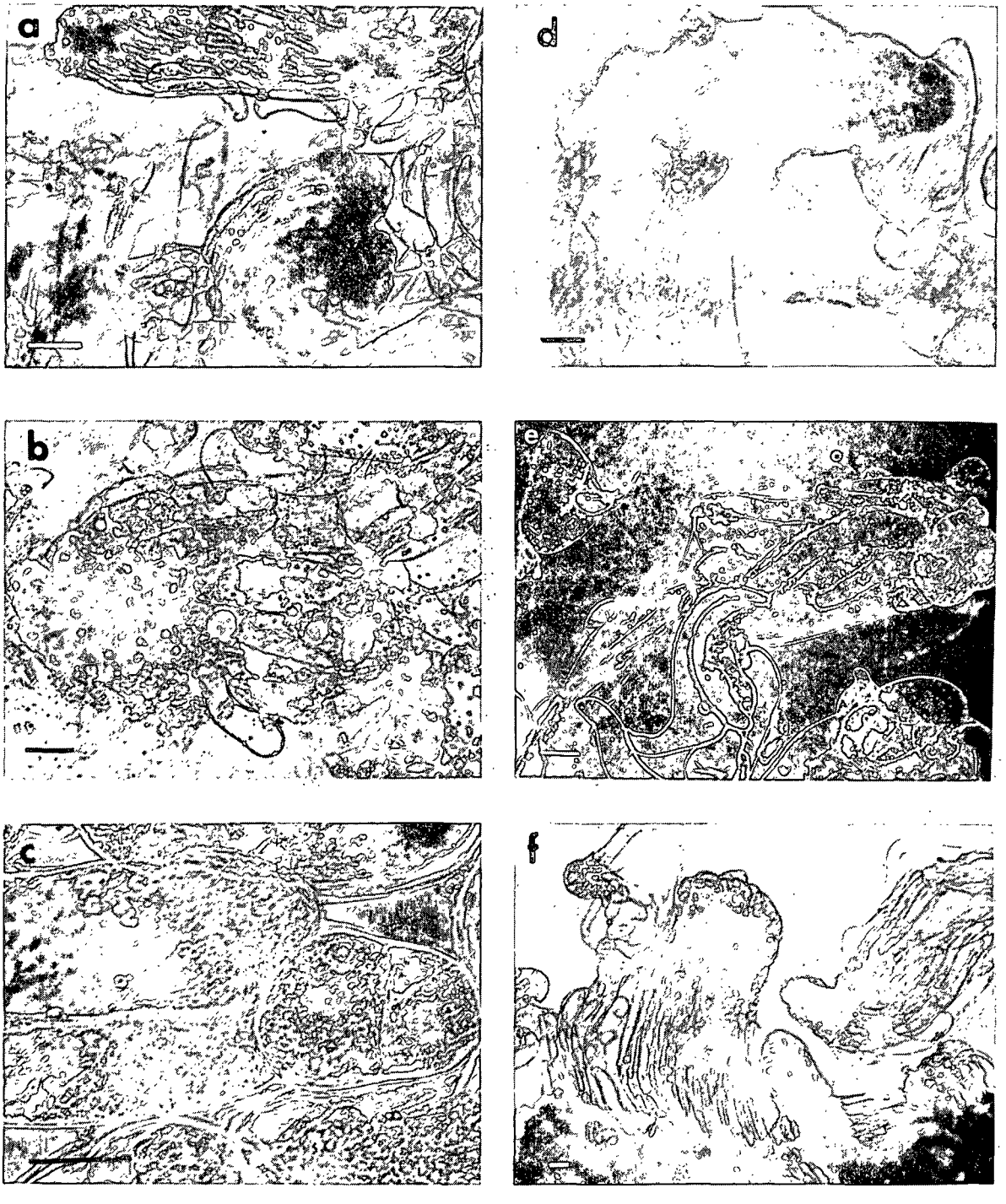

Figure 1. Typical level of somatic embryo development attained in embryogenic callus without transfer from proliferation medium: (a) Norway spruce, (b) white spruce, (c) loblolly pine, (d) white pine, (e) pond pine, (f) European larch. Scale bar = 100 μm.

Figure 2. Preembryogenic masses in (a) white pine and (b) pond pine embryogenic callus. Scale bar = 100 μm.

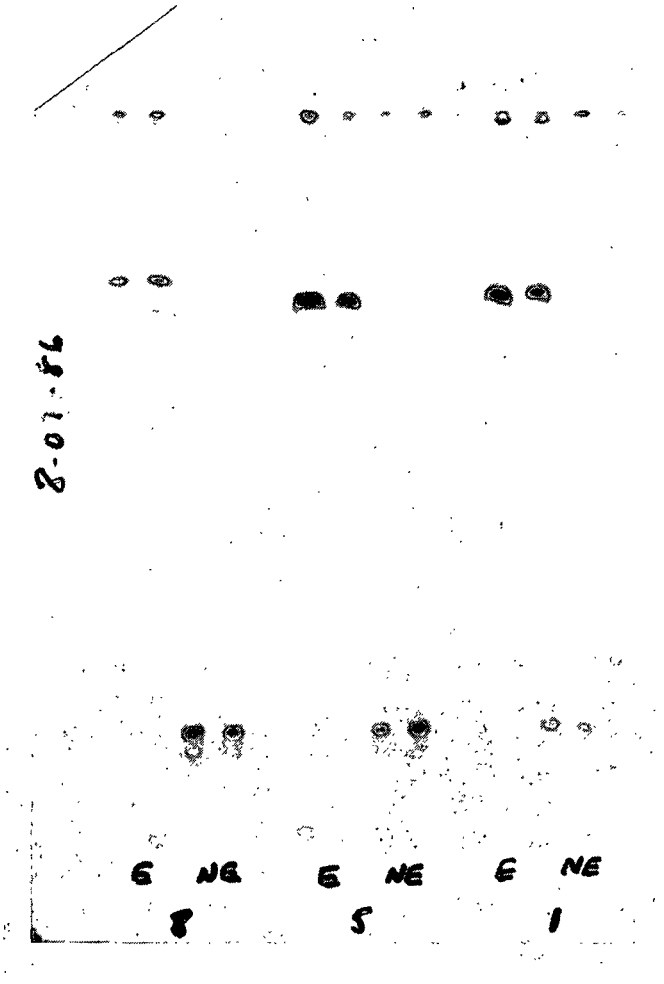

Figure 3. Peroxidase isozyme patterns in duplicate of extracts of embryogenic (E) and nonembryogenic (NE) calli of the three distinct Norway spruce cell lines labeled 1, 5, and 8. The pH gradient runs from 3.5 (bottom) to 9.5 (top).

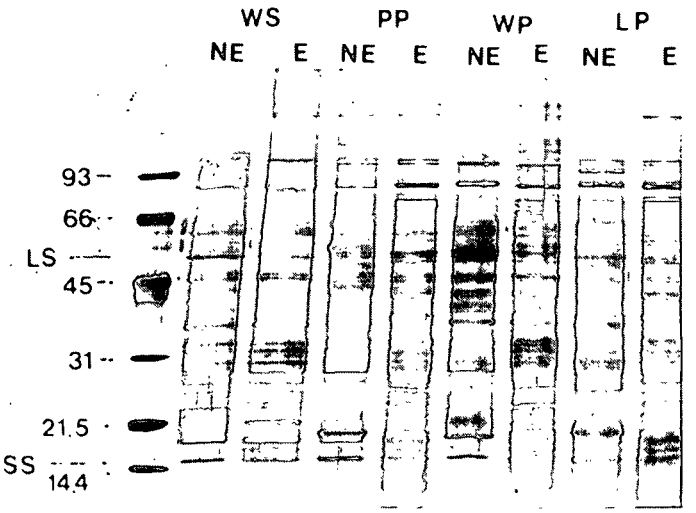

Figure 4. SDS-PAGE of soluble proteins extracted from white spruce (WS), pond pine (PP), white pine (WP) and loblolly pine (LP) embryogenic (E) and nonembryogenic (NE) calli. LS and SS indicate the positions of the large and small subunits of Rubisco.