DIFFERENTIATION IN *PINUS RADIATA* CALLUS CULTURE: THE EFFECT OF NUTRIENTS

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ABSTRACT

Pinus radiata D. Don callus maintained for 2.5 years by subculturing was observed to differentiate, producing nodules containing xylem, cambium, and phloem. It has been shown that there is a pronounced optimum in the concentration of sucrose and major nutrients for this differentiation. Reduced levels of either sucrose or major nutrients alone were not sufficient to induce formation of this vascular tissue, although simple-pitted parenchyma cells with some secondary walls were produced.

INTRODUCTION

Plant tissue cultures have been used to study cellular differentiation for a number of years (e.g., Camus, 1949; Wetmore and Sorokin, 1955; Jeffs and Northcote, 1966, 1967; Haddon and Northcote, 1975). Much of this work centred around experiments to investigate the chemical factors controlling vascular tissue differentiation in angiosperm callus cultures. No one factor has been shown to be responsible for the varying degrees of differentiation which have been obtained, although an auxin seemed to be essential to initiate xylary elements (Roberts, 1969). Other factors cited as critical were the level of carbohydrate (Wetmore and Rier, 1963), the presence of cytokinins (Torrey and Fosket, 1970), and the physical-chemical environment to which the tissues are subjected (Torrey, 1975).

Callus from many conifer species has been successfully grown in culture (Brown and Sommer, 1975): individual tracheid-like structures have been induced in callus from *Sequoia sempervirens* (D. Don) Endl. (Ball, 1950), *Pinus strobus* L. (Gautheret, 1956), *P. banksiana* Lamb. (Geissbuhler and Skoog, 1957), *Picea glauca* (Moench) Voss (White, 1967; Durzan, 1973), *Pinus pinaster* Ait. (Lavaud, 1970), *P. palustris* Mill. (Perry, 1972), *Picea abies* (L.) Karst. (Chalupa and Durzan, 1973), and *Pinus cembra* L. (Salmia, 1975). Organised nodules of vascular-type tissue within callus have, however, been reported only once (Schnurbusch, 1973). *Abies concolor* (Gord.) Engelm. callus, when transferred to a medium containing coconut milk, casein hydrolysate, and 2 mg/litre of kinetin, developed hemispherical nodules containing cambium- and xylem-type cells.

It was recently observed that callus of *Pinus radiata* D. Don, maintained on one medium on filter-paper bridges for 4 months, became hard and compact and contained vascular nodules. Depletion of nutrients was suspected to be a main factor inducing differentiation and an attempt was made to verify this suggestion.

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MATERIALS AND METHODS

Callus tissue, initiated from excised 18-day-old hypocotyl segments, was maintained on a modified Linsmaier and Skoog (1965) basal medium (Table 1) supplemented with 5 mg/litre of indolylbutyric acid (IBA) and adjusted to pH 5.6-5.8. "Difco" purified agar was added (concentration of 0.425% by volume) and the mixture was autoclaved for 15-20 min. at 103 kPa. Aliquots (25 ml) were poured into 90-mm petri dishes. To determine the influence of total nutrient-medium concentration on differentiation, plates were made up containing half-, quarter-, and eighth-strength medium, the concentration of IBA being kept constant. To investigate the effect on differentiation of varying the concentration of three classes of nutrient, viz major nutrients, micro-nutrients, and sucrose, plates were made up as in Table 2, again maintaining IBA concentration at 5 mg/litre.

TABLE 1—Composition of medium (based on Linsmaier and Skoog, 1965*)

SOLUTION A (Major elements)	mg/litre	SOLUTION C (micro-nutrients)	mg/litre
NH_4NO_3	1 650.0	H_3BO_3	6.2
KNO_3	1 900.0	$MnSO_4.4H_2O$	22.3
$MgSO_4.7H_2O$.	370.0	$ZnSO_4.4H_2O$	8.6
KH_2PO_4	170.0	KI	0.83
Na_2EDTA	37.3	$Na_2MoO_42H_2O$	0.25
$FeSO_4.7H_2O$	27.8	$CuSO_4.5H_2O$	0.025
		$CoCl_2.6H_2O$	0.025
		Thiamine. HCl	0.4
		Myo-inositol	100.0
SOLUTION B (sucrose component plus calcium chloride)			
Sucrose	30 000.0		
$CaCl_2.2H_2O$	440.0		

* 3-Indoleacetic acid (IAA), kinetin, and the optional constituents used by Linsmaier and Skoog were omitted.

TABLE 2—Effects of concentration of medium components on differentiation

Mixture	Concentration (relative to full strength) of solution			Appearance of callus		Degree of diffn.	
	A	B	C	12 days	29 days	20 days	33 days
1	½	½	½	Nodules	Nodules	SPPC*	XCP†
2	1	1	1	Normal, no nodules	Normal, no nodules	None	None
3	½	1	1	Brown, no nodules	Brown, compacted areas	None	SPPC
4	1	½	1	Normal, no nodules	Compacted areas	None	SPPC
5	1	1	½	Yellow, no nodules	Compacted areas	None	None
6	1	½	½	Normal, no nodules	Compacted areas	None	SPPC
7	½	1	½	Yellow, no nodules	Compacted areas	None	SPPC
8	½	½	1	Nodules	Nodules	SPPC	XCP

* SPPC — Simple-pitted parenchyma cells

† XCP — Differentiated into xylem,cambium and phloem

Subculturing of Callus, Growth Conditions, and Assessment

Five pieces of fresh green callus (approx. 0.1-0.15 g) were subcultured aseptically on to each of four plates for each nutrient concentration. The cultures were illuminated continuously using "cool-white" fluorescent tubes 60 cm from the plates, while temperature was maintained at 22° ± 2°C. The radiant energy at the plate surfaces did not exceed 2.0, 1.4, and 0.5 $\mu W/cm^2$ in the blue, red, and far-red respectively.

Callus diameter was used as a non-destructive growth indicator and was measured using a Wild stereomicroscope and a 1-mm grid. Two diameter measurements at right angles were made for each callus, and the average of the 20 calluses in each set of nutrients was calculated.

At intervals during the experiment, squashes and hand-cut sections of callus were prepared and examined. Where differentiation was found, tissue was prepared for electron microscopy by fixation in 4% glutaraldehyde in Sorensen's phosphate buffer (pH 7.2) for 4 h, followed by an overnight wash in buffer and then post-fixation in 2% osmic acid for 2 h. After dehydration through an ethanol series the tissue was embedded in Epon 812. The schedule was similar to that employed in a study of *P. radiata* cambium, which has been fully described elsewhere (Barnett, 1971).

Sections for light and electron microscopy were prepared using an LKB Ultrotome III. Aqueous toluidine blue was used to stain sections for cellulose before bright-field microscopy. These sections were also examined using Nomarski interference contrast and polarisation optics.

RESULTS

Effect of Concentration of Complete Medium

Differences in growth rate between cultures grown on different concentrations of complete medium are illustrated in Figs. 1 and 2a. Growth rate was related to nutrient

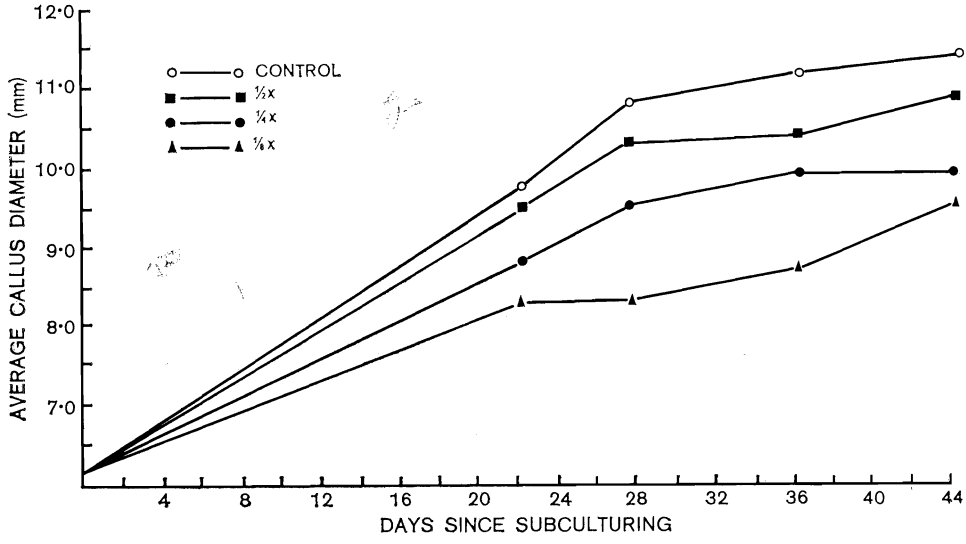

FIG. 1—Difference in callus growth on various dilutions of Linsmaier and Skoog (L + S) medium, all containing 5 mg/litre IBA. Average of 20 calluses.

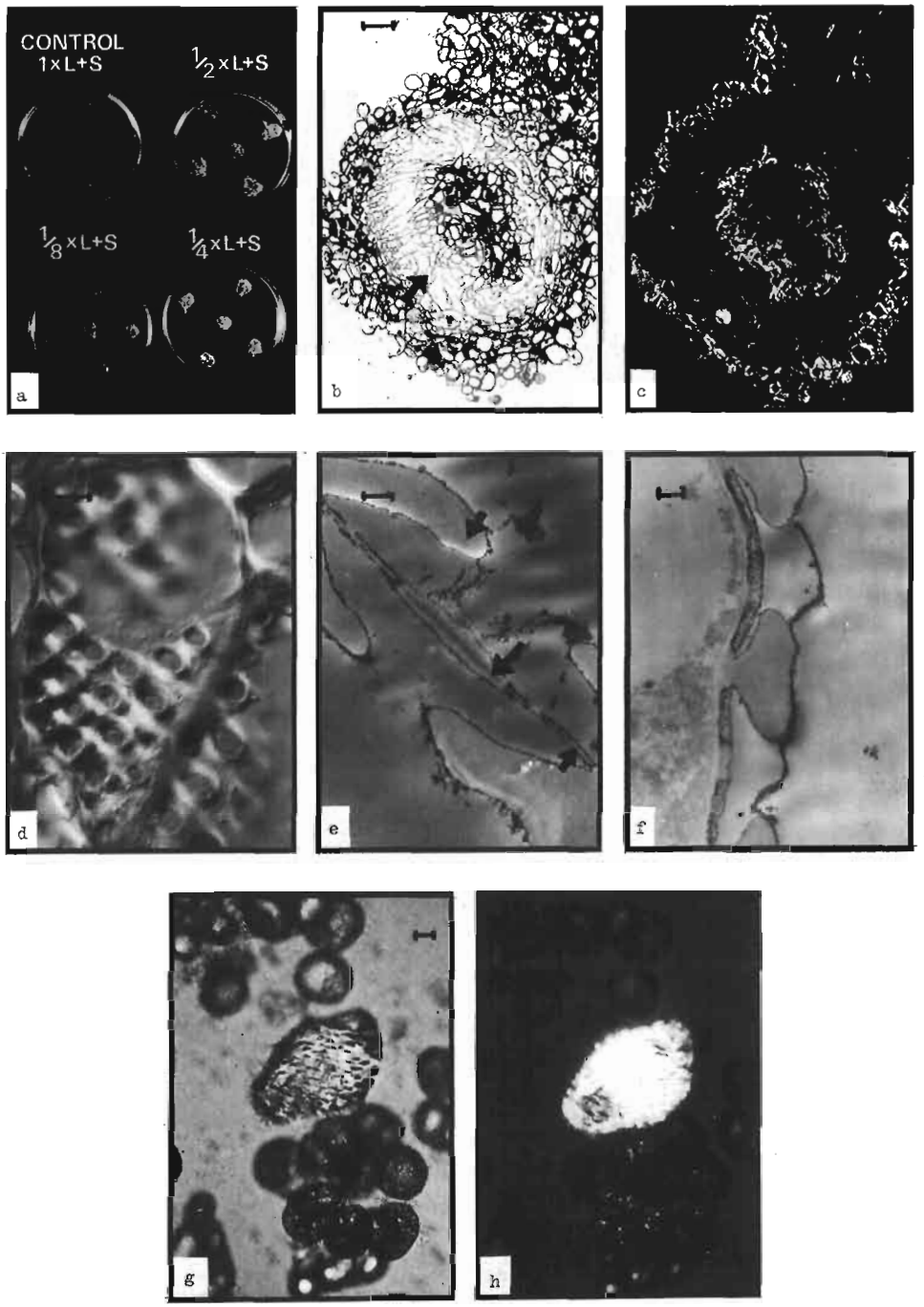

FIG. 2—(a) Growth of callus cultures on different dilutions of Linsmaier and Skoog (L + S) medium. (b) Section through callus nodule. Around the central parenchymatous region, tracheids are present, which are in turn surrounded by a cambium and a

phloem. Files of tracheids derived from neighbouring cambial cells are arrowed. (c) The same area seen between crossed nicols, showing strong birefringence of older tracheids near the centre of the nodule. (d) Part of a tracheid wall seen with Normaski optics, showing closely packed pits. (e) Electron micrograph showing a bordered pit in a callus nodule tracheid. Arrows show, from top, a typical border, margo, and torus. (f) Electron micrograph showing half-bordered pits in the wall between a parenchyma cell (left) and a tracheid (right). (g) Light micrograph of a squash preparation showing a simple-pitted parenchyma cell. (h) The same cell viewed between crossed nicols, showing strong birefringence of the cell wall. Scale marks: in (b) and (c), 100 μm; in (d), 10 μm; in (e) and (f), 1 μm; in (g) and (h), 1000 μm.

concentration, differences becoming more pronounced with time. After 44 days, callus grown on full-strength medium remained bright-green and friable and showed no sign of vascularisation (Fig. 3). Callus grown on half-strength medium, however, had yellow nodules of varying size on the surface furthest from the medium (Fig. 3), and that grown on quarter-strength medium was similar in appearance, although the yellow nodules were smaller. Callus grown on eight-strength medium carried only a few clumps of colourless translucent cells and no nodules.

Examination of squashes and sections revealed that all the calluses except those on full-strength medium had differentiated to some extent. Callus cells grown on full-

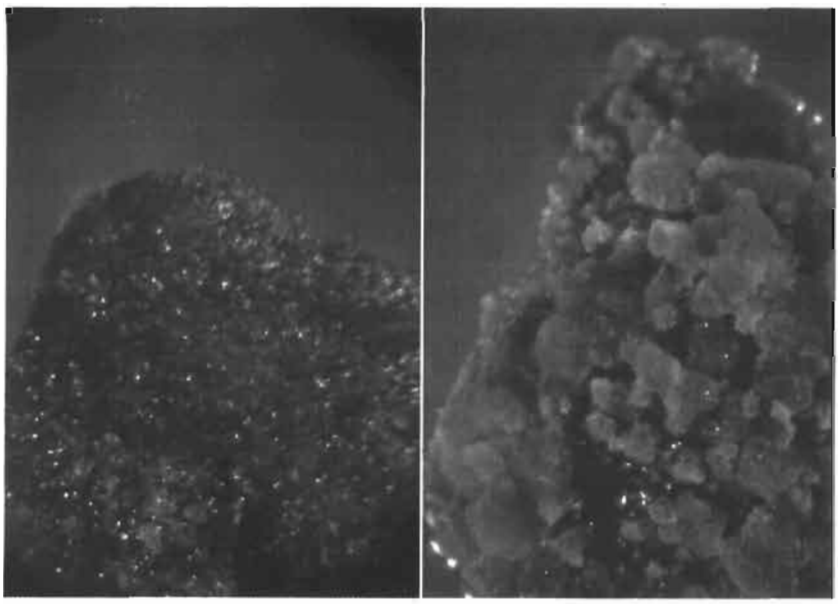

FIG. 3—Undifferentiated green callus grown on full-strength L + S medium (left) and (right) callus grown on half-strength medium, showing proliferation of yellow nodules at the callus surface. Magnification: left, 5×; right 7×.

strength medium were highly vacuolated parenchymatous cells with many plastids and without cell wall specialisation (cf. background cells in Fig. 2g). Differentiation was most pronounced in the half-strength medium, where the nodules displayed a stem-like anatomy (Fig. 2b). Cambium cells formed a ring enclosing tracheids, which in turn enclosed a central region of tannin-containing parenchymatous cells. To the outside of the cambium was a ring of phloem cells. The phloem, cambium, and tracheids were only locally oriented, resembling the situation in the differentiated callus nodules observed in resin pockets of *Pinus ponderosa* Laws. by Harris and Barnett (1975).

The presence of an organised secondary wall in the sieve cells of the phloem and in the tracheids was confirmed by viewing the sections between crossed nicols; the tracheids were found to be strongly birefringent and the sieve cells slightly birefringent (Fig. 2c). The walls of these cells exhibited a strong staining reaction with toluidine blue, confirming their cellulosic nature.

With the aid of Nomarski interference contrast optics, numerous bordered pits were observed in the walls of the tracheids (Fig. 2d). The structure of these pits resembled that found in normal tracheids as revealed by electron microscopy (Fig. 2e). Typical half-bordered pits were found where tracheids butted aaginst parenchymatous cells (Fig. 2f).

Differentiation was less pronounced in the nodules of callus grown on quarter-strength medium although tracheid-like cells and parenchymatous cells with simple pitting were present (Fig. 2g). The presence of a secondary wall in these parenchymatous cells was again confirmed using polarisation optics, when strong birefringence was observed (Fig. 2h).

Differentiation in callus grown on eighth-strength medium was limited to white clumps of cells at the callus surface. These clumps had formed only simple-pitted parenchyma cells.

Effect of Varying Medium Component Concentrations

The observations made at intervals during this experiment are summarised in Table 2. The calluses grown on media containing both major nutrients and sucrose at half strength were the only ones to show the degree of differentiation induced in the first part of the experiment where total medium concentration was halved. Examination of hand sections confirmed that the external morphology and internal structure of surface nodules on these calluses were the same as those of the callus nodules in the former experiment.

After 20 days on the medium in which major elements were at full strength and sucrose was at half strength, the callus formed small compacted areas at the surface. These were found to contain simple-pitted parenchyma cells. No further differentiation took place in this tissue. Results for the other treatments were similar in that simple-pitted parenchyma cells were found after 33 days. Only where both major elements and sucrose were at full strength did the callus remain completely undifferentiated. In no treatment did variation in the concentration of micro-nutrients appear to have an effect.

To test the ability of callus with differentiated nodules to differentiate further, pieces of such callus were transferred to fresh half- or full-strength medium. Callus left on its original plate showed no further differentiation after 85 days, when the experiment was terminated. In callus subcultured on to half-strength medium no

further differentiation occurred, while in that on fresh full-strength medium the central, green, undifferentiated material proliferated and eventually obscured the nodules.

DISCUSSION

The results described above clearly show that nutrient concentration can be a factor in inducing differentiation in *P. radiata* callus. Only callus grown on Mixture 8 with major elements and sucrose/calcium at half strength, differentiated in the same way as that on overall half-strength Mixture 1. Concentration of micro-nutrients, and its variation within the limits of these experiments, appeared to have no effect on differentiation. Where only one of the other components was at half strength, differentiation occurred but did not develop beyond the stage of simple-pitted parenchyma cells.

Since the concentration of auxin in the medium was not varied, its role in the differentiation of vascular nodules was not elucidated. However, previous experiments have shown that auxin is essential for continued growth of *P. radiata* callus (New Zealand Forest Service, 1974).

Wetmore and Rier (1963), studying patterns of differentiation in lilac callus, showed that sucrose concentration exerted remarkable control over the type of vascular tissue formed. Xylem was produced at 2% sucrose concentration, phloem at 4%, and a mixture of xylem and phloem separated by a cambium at 3%. This work was later successfully repeated by Jeffs and Northcote (1966; 1967).

The results described here for *P. radiata* are of interest in that higher concentrations of sucrose, in this case 3%, did not lead to nodule formation or differentiation, but reducing the concentration to 1.5% (along with the major nutrients) invariably led to the formation of vascular nodules. This lends support to the hypothesis that the differentiation of *P. radiata* callus originally observed before these experiments were conducted was a consequence of nutrient depletion.

It seems, therefore, that in *P. radiata* callus there is a critical concentration of nutrients which induces differentiation. Ample nutrient supply and extreme deficiency suddenly applied to a callus do not lead to differentiation. Under conditions where ample nutrients are available, callus continues to divide producing only parenchymatous cells. At a critical level, cells remote from the agar, or source of nutrients, begin to be starved as cells nearer the supply take most of what is available. Nodules then form in the remote regions of the callus, furthest from the supply, and differentiate into a stem-like structure. The random orientation of the cells in the differentiated nodule may reflect the lack of auxin-induced polarity such as is found in a normal stem with a single growing point at the tip.

It is suggested that, since vascularisation in plants is primarily an adaptation for conveying nutrients from a source to more remote parts of the plant, vascular differentiation in callus might be a response of the tissue to nutrient shortage — an adaptation to form conduction pathways for the supply of nutrients to remote regions. The lack of a polar auxin-flow, however, makes such a response ineffective, in that the vascular tissue produced does not have the necessary orientation.

That differentiation depends on more than just the effect of a single nutrient concentration or the presence of auxin is well borne out by the variable results produced when different combinations of major nutrients or sucrose were applied. Clearly, many as yet unspecified factors are involved in the process.

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