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Experimental Induction of Vascular Tissue in an Undifferentiated Plant Callus

By R. A. JEFFS and D. H. NORTHCOTE
Department of Biochemistry, University of Cambridge

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1. By the implantation of wedges containing indol-3-ylacetic acid and sucrose into blocks of undifferentiated bean-callus tissue it has been possible to induce the formation of xylem and phloem cells. 2. The differentiation has been investigated cytologically and measured chemically. 3. The optimum concentrations of the nutrients in the wedge, which gave differentiation closely resembling the vascular development found in the stem of the intact plant, was 0·1mg. of indol-3-ylacetic acid/l. and 2% sucrose. 4. The ratios of the xylose/arabinose concentrations of the tissues increased in the differentiated callus tissue compared with those of the undifferentiated tissue. A similar increase has been found for the ratios determined for xylem tissue compared with those for cambium. 5. The lignin content of the differentiated tissue compared with the undifferentiated tissue was greater in both the callus and stem tissue. 6. Chemical analysis of lignin showed that in the differentiated callus tissue it consisted of sub-units based on *p*-hydroxybenzaldehyde and vanillin. This was compared with the lignin obtained from undifferentiated callus tissue and that obtained from the tissues of the intact stem. 7. The results of the investigation have been discussed with reference to the problems of cell growth and differentiation and related to the changing patterns of the ultrastructure of the cell during its development.

The differentiation of plant cells is accompanied by characteristic changes in the cell wall. These have been studied extensively by optical microscopy (Cruger, 1855; Barkley, 1927; Sinnott & Bloch, 1945; Esau, 1965) and more recently by an electron-microscopic investigation of xylem (Porter, 1961; Hepler & Newcomb, 1963; Buvat, 1964; Wooding & Northcote, 1964; Cronshaw & Bouck, 1965; Pickett-Heaps & Northcote, 1966*a*) and of phloem (Esau & Cheadle, 1961, 1962; Buvat, 1963; Wooding & Northcote, 1965; Northcote & Wooding, 1966).

As the plant cell differentiates the wall material changes in composition and character. Thornber & Northcote (1961*a*,*b*, 1962) have measured the change in polysaccharide composition of the cambial cells of sycamore, ash, birch and pine as they develop into mature xylem and phloem in the intact tree. Jensen & Ashton (1960) has compared the changes in the primary walls of the meristematic cells of pea-roots during the development of the cells in the first 2mm. behind the tip.

If the polysaccharide contribution to the wall material is considered as being derived from two series of monosaccharides, (*a*) glucose, (mannose), glucuronic acid and xylose and (*b*) galactose, galacturonic acid, arabinose and rhamnose, then during secondary growth the polysaccharides laid down are almost all derived from the glucose series and little or none of the polysaccharides containing the monosaccharides of the galactose series are formed (Northcote, 1963*a*). In the xylem vessel extensive lignin deposition also occurs. Thus the ratio of xylose to arabinose in the cell wall will give a measure of the degree of secondary thickening and, together with measurements on the lignin content of the wall, a biochemical index of differentiation can be obtained. Xylose and arabinose are chosen for the experimental measurements since glucose occurs in the starch of the cell in addition to the wall and the pentoses are simpler to estimate than the uronic acids.

Isolated tissue cultures have been used to study the control of differentiation. Several workers have reported the induction of organized tissues in plant-callus tissue (Wetmore & Sorokin, 1955; Skoog & Miller, 1957; Clutter, 1961; Wetmore & Rier, 1963; Bergmann, 1964; Haccius & Lakshmann, 1965) and others have succeeded in obtaining intact plants from a clone of callus cells (Steward, Mapes & Mears, 1958; Earle & Torrey, 1965; Vasil & Hildebrandt, 1965). How-

ever, most of these studies have been cytological, morphological changes alone being used as criteria of differentiation.

Bergmann (1964) reported an increase in lignin synthesis in tobacco-callus tissue after the application of kinetin. Barnoud (1965), Lamport & Northcote (1960*a*,*b*) and Stoddart, Barrett & Northcote (1966) have investigated the polysaccharide composition of various callus tissues, but no previous attempt has been made to follow chemical changes during induced differentiation of the tissue.

In this paper we describe the cytological and biochemical changes which occur in callus tissue of *Phaseolus vulgaris* during induced differentiation.

METHODS AND MATERIALS

Reagents. These were of A.R. grade where possible. IAA* was obtained from Roche Products Ltd. (Welwyn Garden City, Herts.) and special Agar-Noble from Difco Laboratories (Detroit, Mich., U.S.A.). For the culture media glass-distilled water was used.

Tissue culture of Phaseolus vulgaris. Seeds of *P. vulgaris* (Sanders Ltd., Cambridge) were soaked and grown in vermiculite for 2–3 weeks until they had acquired a flourishing root and stem growth. Pieces of stem about 2–3 cm. in length were cut with a sterile scalpel and the surface was sterilized in 70% ethanol followed by 5% (w/v) calcium hypochlorite soln. (Gautheret, 1959).

These lengths were explanted into a salt medium (Heller, 1953) supplemented with coconut milk (20%, v/v), sodium 2,4-dichlorophenoxyacetate (6 p.p.m.), sucrose (2%) and solidified by addition of 1% of agar. Callus growth resulting from the cut upper surface was transferred to fresh medium for subculturing.

The sterile operations were performed in a special cabinet fitted with rubber gloves and ultraviolet lamp. Solutions and apparatus were autoclaved at 15 lb./in.^{2} for 30 min. The tissue was grown either in liquid or on solid culture with the medium described above and it has been maintained in the Laboratory during the last 3 years.

Sycamore cambium and xylem tissue. This was prepared from freshly cut young trees obtained from Madingley Wood (Cambridge) (Thornber & Northcote, 1961*a*).

Histochemical detection of differentiation. The differentiated callus blocks were fixed in a chrome fixative (Johansen, 1951) for 4 hr., and dehydrated through an ethanol series. After clearing in 2-methylpropan-2-ol, the blocks were embedded in paraffin wax (m.p. 49°). These blocks were then sectioned (8–10μ) with a sledge microtome. The sections were floated on to gelatine-coated slides and stained with Heidenhain's haematoxylin (20–30 min.), or with safranin (20 min.) and counterstained with picric aniline blue (30 sec.), or with phloroglucinol (Jensen, 1962). The stained sections were mounted in D.P.X. mountant (E. Gurr and Co. Ltd., London, S.W. 14) and examined with an optical microscope (Zeiss Ultraphot model II). Polarized optics were used to detect the birefringent bands of thickening in xylem cells and u.v.-fluorescence microscopy of aniline-blue stained sections was used to detect phloem (Currier & Strugger, 1956; Currier, 1957). During all these manipulations the orientation of the callus was carefully noted and related to its position in the incubation tubes used for the induction experiments.

Analytical procedures

Preparation of tissue. The callus blocks used in the induction experiments were carefully taken from the agar medium and the wedges (Fig. 1) gently removed from them by means of a blunt needle. Any small amount of fresh growth which had occurred at the edges of the tissue block during the incubation period were removed. The top 3–5 mm. of the callus immediately below the wedge (Fig. 1) was dissected away from the block and this portion was freeze-dried and then refluxed with 80% ethanol for 30 min., further refluxed with ethanol–benzene (2:1, v/v) for 30 min. and dried over P_2O_5 *in vacuo*. The extraction removed fats, waxes, phenolic compounds and free sugars.

Determination of monosaccharides in polysaccharide hydrolysates. The extracted tissue (70–100 mg.) was dissolved in 1 ml. of 72% (w/w) H_2SO_4 for 2–3 hr., diluted to 28 ml. with water and autoclaved for 1 hr. at 15 lb./in.^{2}. The residue was centrifuged and the supernatant solution and washings brought to within pH 3·6–5·1 with $BaCO_3$. The Ba^{2+} ions were removed from the solution after filtration by passage through a short column of Amberlite IR-120 ion-exchange resin (H^+ form) (British Drug Houses Ltd., Poole, Dorset), and the solution was evaporated to dryness under reduced pressure below 60°. The neutral sugars were redissolved in saturated benzoic acid and stored at 4°.

The neutral monosaccharides present in the polysaccharide hydrolysates were separated by descending paper chromatography for 16 hr. on Whatman no. 1 paper with ethyl acetate–pyridine–water (8:2:1, by vol.) as the solvent system.

The sugars were detected and estimated by the aniline hydrogen phthalate method of Wilson (1959) and the results expressed as μg. of sugar/mg. of starting material. Standard solutions of monosaccharides were prepared in saturated benzoic acid and stored at 4°. Percentage recoveries of standard sugars after the hydrolysis procedure and chromatographic estimation by the Wilson method

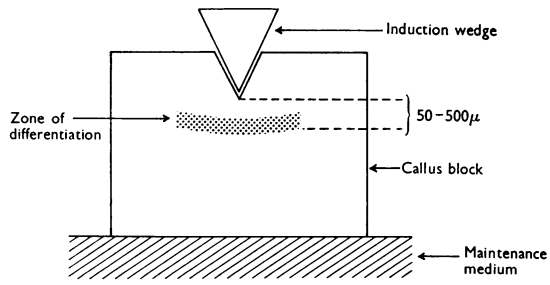


Fig. 1. Diagram to illustrate the insertion and position of the induction wedge containing sucrose and indol-3-ylacetic acid in the callus block.

* Abbreviation: IAA, indol-3-ylacetic acid.

were 93±5% (six determinations) for the sugar standards used. No epimerization of the standard sugars was detected.

Determination of lignin by alkaline nitrobenzene oxidation. The extracted material (30mg.), nitrobenzene (0·06ml.) and 1ml. of 8% (w/v) NaOH were heated under pressure to 160±5° for 2·5hr. in a stainless-steel bomb (Stone & Blundell, 1951). Samples processed without callus material were used as reagent blanks.

The vanillin, syringaldehyde and *p*-hydroxybenzaldehyde produced by the oxidation were separated by descending paper chromatography for 22hr. on Whatman no. 1 paper with butan-1-ol–aq. ammonia (sp.gr. 0·88)–water (4:1:5, by vol.) as the solvent system. The spots on marker strips cut from the chromatograms were coloured with a 0·4% solution of 2,4-dinitrophenylhydrazine in 2N-HCl as a spray reagent (Bland, 1949) and used to locate the position of the compounds produced by the oxidation. These compounds were also separated by thin-layer chromatography on 20cm.×20cm. plates coated with a layer (0·25mm.) of MN-cellulose powder 300 [Machery, Nagel and Co., obtained from Camlab (Glass) Ltd., Cambridge] with a two-dimensional solvent system: benzene–acetic acid–water (10:7:3, by vol.) and butan-1-ol–aq. ammonia (sp.gr. 0·88)–water (4:1:5, by vol.) (Said el Basouni, Neish & Towers, 1964).

The oxidation products were eluted from the chromatograms with 0·2% KOH in ethanol and the extinctions of the eluates were measured on a Unicam SP.500 spectrophotometer at 352mμ for vanillin, 368mμ for syringaldehyde and 335mμ for *p*-hydroxybenzaldehyde. Standard curves of these derivatives were prepared so that the concentrations could be read off directly. Percentage recoveries of each compound after nitrobenzene oxidation and chromatography were 77±4% (eight determinations).

Determination of lignin by extraction in sodium hydroxide solution. The method of Stafford (1960) devised from the extraction procedures of Bondi & Meyer (1948) and the spectrophotometric investigations of Goldschmidt (1954) was used. Extracted material (50mg.) was ground thoroughly with ether in a mortar to remove any chlorophyll present. The tissue was then extracted three times with water followed by an extraction with 50% methanol. The residue was centrifuged and extracted for 16hr. with 0·5N-NaOH (2–3ml.) at 70°, centrifuged again and the residue washed with 0·5N-NaOH (1ml.), and the alkaline solutions were combined. The alkaline extract was adjusted to pH8·5–9·0 with N-HCl and samples (1ml.) of this solution were diluted with either 5mN-NaOH (2ml.) or 0·05M-phosphate buffer, pH7·0 (2ml.). The difference spectrum of these two solutions was determined on Unicam SP.500 and SP.800 spectrophotometers.

Phenol analyses of the alkaline extract were performed by using a modification (Stafford, 1960) of the method of Gierer (1954) for native lignin preparations. Samples (0·5–1·0ml.) of the extract, 0·4ml. of 0·5M-tris buffer, pH9·0, and 1·0ml. of freshly prepared ethanolic solution (26μg./ml.) of 2,6-dichloroquinonechloroimide (Koch–Light Laboratories Ltd., Colnbrook, Bucks.) were incubated for 1hr. at room temperature. The extinctions at 610mμ of these solutions were then measured on a Unicam SP.500 spectrophotometer. The quantities of phenols present in the solutions were expressed in terms of μg. of guaiacol by comparison with a standard curve.

RESULTS

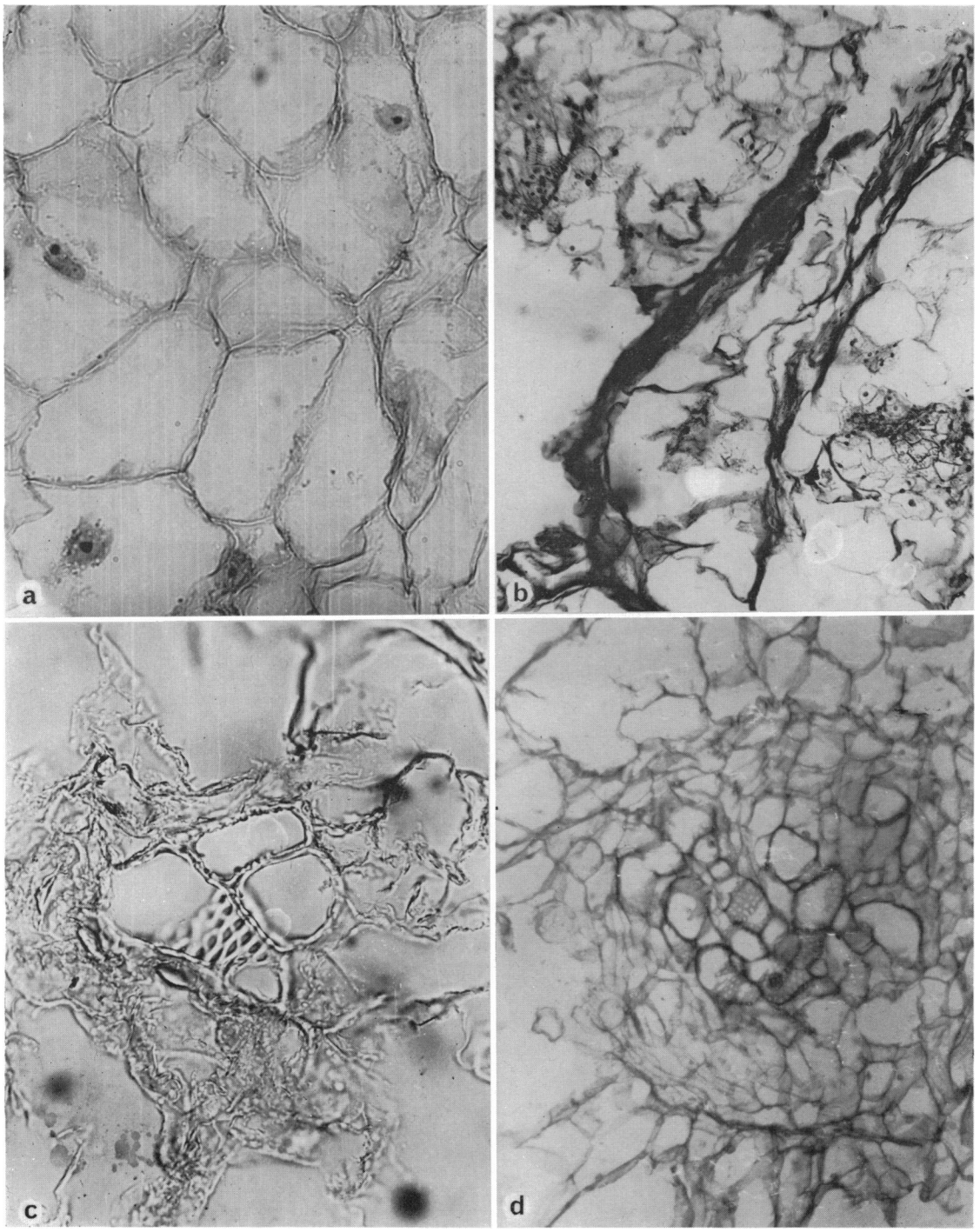
Callus tissue. This consisted of creamy, extremely friable tissue masses which grew quite rapidly and required subculturing every 5–6 weeks. The typical appearance of the tissue consisted of loosely packed parenchymatous cells which contained large vacuoles and prominent nuclei (Plate 1*a*). There was no organization of the cells within the tissue and no differentiation.

Experimental induction of differentiation. The techniques used were those employed by Wetmore & Rier (1963). Pieces of callus from agar slopes were cut into portions of approx. 1cm. cube; a wedge-shaped groove was cut across the upper surface of the callus block with a sterile scalpel. An equivalent close-fitting wedge, cut from an agar gel (1%) containing the experimental solution, was carefully laid in the groove so that it was in intimate contact with the callus tissue. The whole block was transferred to a boiling-tube containing 15ml. of a solid maintenance medium and left for 1–2 months at 25° (Fig. 1). This medium contained the basic salts, coconut milk (20%, v/v), naphthoxyacetic acid (50μg./l.), sucrose (1%) and agar (1%); it maintained the callus yet prevented profuse growth. The solutions used in the induction agar wedges were sucrose and IAA. Sucrose at each of the concentrations 0·0%, 1·0%, 2·0%, 3·0% and 4·0% was combined in every combination with IAA at concentrations 0·05, 0·10, 0·25, 0·50 and 1·00mg./l.

Callus blocks which carried agar wedges containing neither sucrose nor IAA (control callus) when examined microscopically contained nothing having the appearance of secondary thickened cells in the region 50–500μ below the wedge. The callus always consisted of the typical parenchymatous cells.

In all the blocks carrying wedges containing 2% sucrose plus varying concentrations of IAA patches or nodules of mitotically active and differentiating cells were found. A section of the tissue which had carried a wedge containing 50μg. of IAA/l. and 2% sucrose is shown in Plate 1(b). The position of the wedge can be seen running across the section and two areas of differentiation are located nearby. These do not occur exactly in contact with the wedge, but some way into the callus mass.

In all the blocks carrying wedges containing IAA the localization of the differentiation to a region between 50 and 500μ from the lower edge of the wedge was demonstrated. A patch of differentiating xylem cells, stained red with safranin, is shown in Plate 1(c). The lignified bands associated with this tissue can be clearly seen. The blocks carrying the wedges containing 50μg. of IAA/l. and those with the other low IAA concentrations



Plates 1 and 2. All photographs are of callus tissue obtained from the stems of *Phaseolus vulgaris*.

Plate 1. (a) Callus tissue grown on solid medium. Cells completely undifferentiated. Stained with safranin; picric aniline blue (×440). (b) Area of differentiation produced by a wedge containing 50μg. of IAA/l. and 2% sucrose. The position of the wedge can be seen across the section between the nodules containing xylem and mitotically active cells. Stained with Heidenhain's haematoxylin (×270). (c) Secondary thickening in differentiating xylem cells in the callus tissue induced by a wedge containing 50μg. of IAA/l. and 2% sucrose. Stained with safranin; picric aniline blue (×1100). (d) A nodule containing 10–12 differentiating xylem cells surrounded by dividing cells. The nodule was induced by a wedge containing 0·5mg. of IAA/l. and 2% sucrose. Stained with safranin; picric aniline blue (×160).

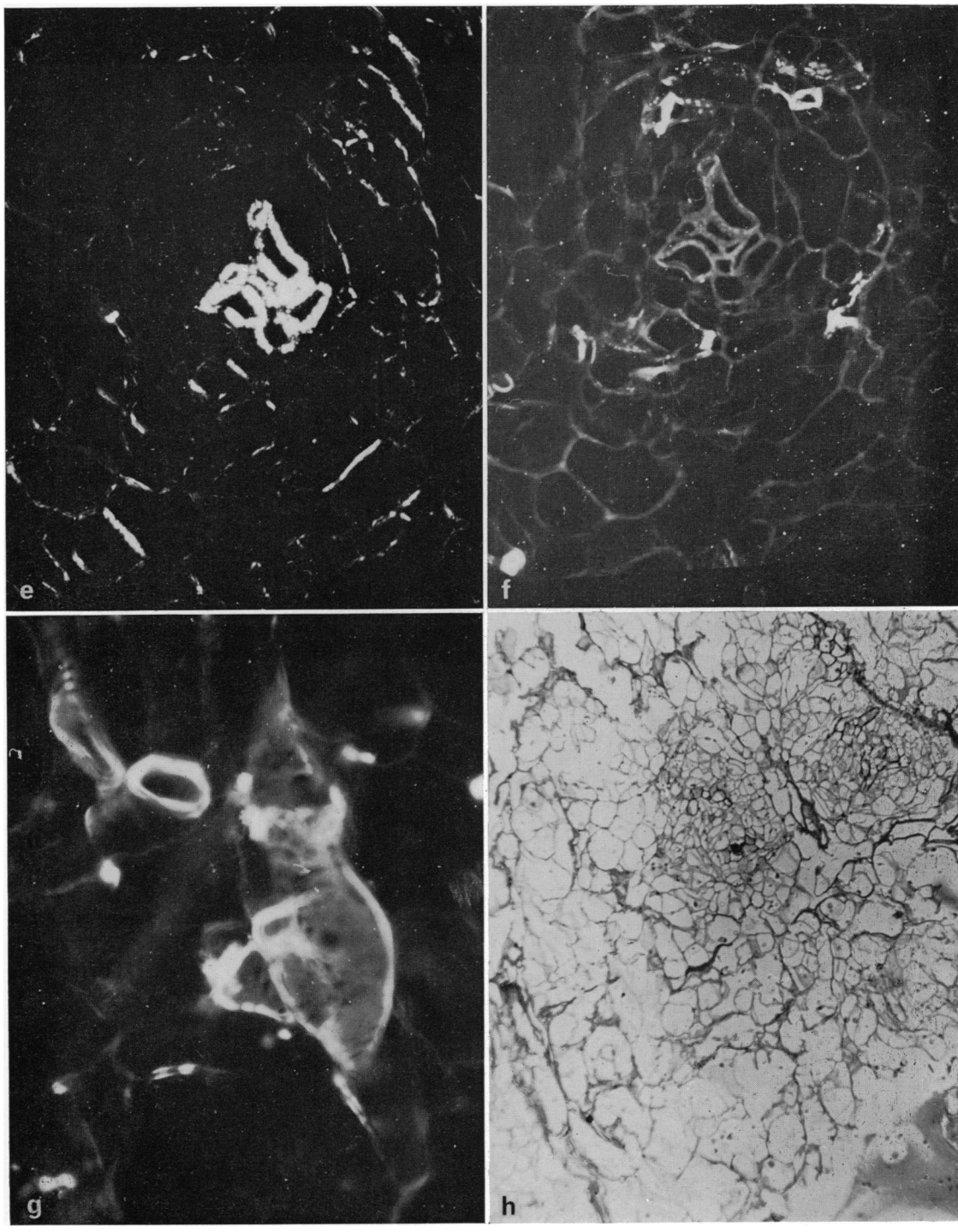


Plate 2. (e) A nodule of differentiated tissue induced by a wedge containing 0·1 mg. of IAA/l. and 2% sucrose. The section is photographed with polarized light and the birefringence of the secondary thickening of the xylem cells is apparent. These cells are at the centre of the nodule (× 180). (f) The same nodule as that shown in (e). The section is stained with aniline blue and photographed with u.v. light. The callose of the sieve plates of the phloem cells is apparent. These cells are at the perimeter of the nodule (× 180). (g) A nodule containing phloem cells induced by a wedge containing 0·1 mg. of IAA/l. and 3% sucrose. The section is stained with aniline blue and photographed with u.v. light. The sieve plates are very apparent (× 450). (h) Region of differentiation induced by a wedge containing 0·1 mg. of IAA/l. and 2% sucrose (optimum concentrations). Stained with safranin; picric aniline blue. Four areas of xylem differentiation are apparent together with cambial activity between them (× 40).

had three to four xylem cells within these areas (Plate 1c) whereas those with the higher IAA concentrations, 0·5 mg. of IAA/l. and 1·0 mg. of IAA/l., had 10–12 cells within each region of differentiation (Plate 1d).

All the blocks carrying wedges containing 0·5 mg. of IAA/l. plus varying concentrations of sucrose showed areas of differentiating tissues and in some of these regions the presence of phloem cells was demonstrated by u.v.-fluorescence microscopy. The callose produced in the sieve-plates of phloem cells gave a characteristic yellow fluorescence. Pictures of a nodule from a block carrying a wedge containing 0·1 mg. of IAA/l. and 2% sucrose are shown in (e) and (f) of Plate 2. The nodule photographed with polarized light to detect the birefringent walls of the xylem elements is shown in (e), and (f) is a photograph of the u.v.-fluorescence of the nodule. In the fluorescent specimen the phloem cells stand out as light areas against a dark background at the perimeter of the nodule while the xylem is present at the centre. A cambial layer is formed between these differentiating cells after 6–8 weeks' incubation (Plate 2h).

A relatively high concentration of sucrose (3–4%) as well as physiological concentrations of indol-3-ylacetic acid in the wedges favoured the induction of phloem, Plate 2(g) (Wetmore, DeMaggio & Rier, 1964). The most advanced stage of differentiation reached in these experiments was obtained with wedges containing 0·1 mg. of IAA/l. and 2% sucrose when all the elements of a vascular strand were found in the callus block as compact nodules (Plate 2e and f).

Sugar ratios. Table 1 shows the results of repeated determinations of the concentrations of xylose and arabinose in the hydrolysates of callus blocks that have been subjected to the optimum induction stimulus (0·1 mg. of IAA/l.; 2% sucrose) for 1 and 2 months, and blocks which have carried wedges of 1% agar only for similar periods. There is an increase in the xylose/arabinose ratio of the treated tissue compared with the initial concentrations or with that in the control callus.

Lignin. Qualitative chromatograms indicated that control callus blocks had traces of *p*-hydroxybenzaldehyde, as did the cambium of sycamore after alkaline nitrobenzene oxidation. The differentiating callus had quite marked concentrations of *p*-hydroxybenzaldehyde and vanillin, but no syringaldehyde was detected.

Quantitative measurements revealed that the ratios of *p*-hydroxybenzaldehyde to vanillin (Table 2) produced in the lignin of the differentiating xylem elements was different from 1:2 which Bondi & Meyer (1948) had found for the mature lignin of

Table 1. *Concentrations of xylose, arabinose and rhamnose in polysaccharide hydrolysates of extracted bean callus and sycamore tissues*

Values are given ± S.D. with number of experiments in parentheses.

Tissue	Xylose (μg./mg. dry wt.)	Arabinose (μg./mg. dry wt.)	Rhamnose (μg./mg. dry wt.)	Xylose/arabinose ratio
Control callus	31·6	77·8	2·5 ± 0·5 (12)	0·409 ± 0·053 (12)
Differentiated callus (1 month induction)	34·0	64·8	5·5 ± 0·5 (12)	0·527 ± 0·045 (12)
Differentiated callus (2 months' induction)	46·5	68·0	5·5 ± 0·5 (12)	0·682 ± 0·038 (12)
Sycamore cambium	29	63	4·6	0·46
Sycamore xylem	133	6·5	Trace	20·5

Table 2. *Concentration of phenolic aldehydes derived from oxidation of lignin by nitrobenzene*

Tissue	*p*-Hydroxybenzaldehyde (μg./mg. of dry tissue)	Vanillin (μg./mg. of dry tissue)	Syringaldehyde (μg./mg. of dry tissue)
Control callus	85	27	0
Differentiated callus (1 month induction)	95	69	0
Differentiated callus (2 months' induction)	187	118	0
Sycamore cambium	20	50	30
Sycamore xylem	70	125	195

Table 3. *Spectrophotometric analysis of the alkaline extract of lignin from bean callus and sycamore tissue*

Tissue	$10^5 \times$ Values of difference spectra (Δ) at various wavelengths (λ)/mg. dry wt. of extracted tissue: $\Delta\lambda$270/mg. dry wt.	$\Delta\lambda$300/mg. dry wt.	$\Delta\lambda$350/mg. dry wt.	Phenol (μg./mg. dry wt. estimated as guaiacol)
Control callus	0·434	0·306	0·047	18
Differentiated callus (1 month induction)	1·720	1·240	1·240	84
Differentiated callus (2 months' induction)	2·260	3·040	1·954	142
Sycamore cambium	10·0	0·500	0·500	34
Sycamore xylem	40·0	30·0	70·0	288

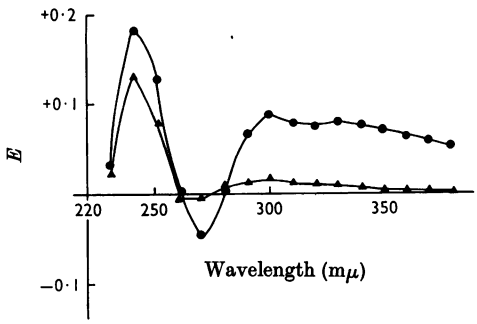


Fig. 2. Ultraviolet-absorption difference spectra of lignin extract at pH 7 and pH 12·3. ▲, Control callus; ●, differentiated callus (0·1 mg. of IAA/l. and 2% sucrose, 2 months).

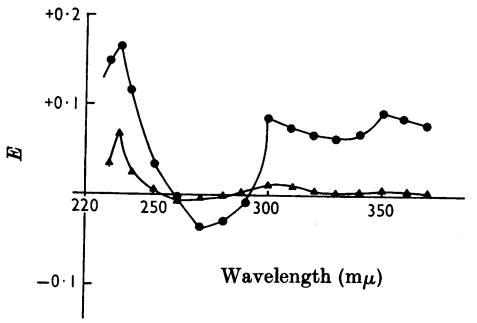


Fig. 3. Ultraviolet-absorption difference spectra of lignin extract at pH 7 and pH 12·3. ▲, Sycamore cambium; ●, sycamore xylem (original alkaline extract diluted 1:1 compared with the cambial extract).

Leguminosae. This agrees with the finding of Barnoud (1965) who showed discrepancies between naturally occurring and induced lignins.

The ultraviolet-absorption difference spectra of the lignin extracts at pH 7 and pH 12·3 are shown in Figs. 2 and 3. Extracts from mature shoots are characterized by the minimum in the region 270–280mμ (Stafford, 1960). Non-conjugated aromatic hydroxyl radicals give the peaks in the 250mμ and 300mμ region whereas phenols with large conjugated side chains, e.g. *p*-hydroxycinnamic acid, absorb beyond 350mμ (Goldschmidt, 1954). As the water-soluble and methanol-soluble polyphenols lying free in the tissue had been previously extracted, these polyphenolic compounds must have been produced by the alkaline degradation of young lignins in the cell walls. Thin-layer chromatography of these extracts showed the presence of vanillin, *p*-hydroxybenzaldehyde and *p*-hydroxycinnamic acid, whereas no flavanoids, catechins or leucoanthocyanins could be detected by the benzidine spray reagent (Lindstedt, 1950).

The results obtained by performing these estimations on samples of sycamore cambium and xylem are shown in Fig. 3. The similarity between the callus control blocks and the cambium, and also between the differentiated callus and the sycamore xylem, is apparent. The cambium and the callus tissue contain smaller absolute values of lignin and also show a small peak at 250–270mμ, which indicates the presence of some non-conjugated polyphenols. The mature xylem and differentiated callus contained more polyphenolic substances in their walls, and a substantially larger percentage of these exist as conjugated polyphenols.

Table 3 shows the differences of lignin content between several observations on control and induced blocks. The induced callus contains significantly more lignin-like substances than the controls and there is slightly more lignin in the tissue carrying the wedges for 2 months than in those carrying the wedges for 1 month.

DISCUSSION

The results of this work have fully confirmed those of Wetmore & Rier (1963), who used tissue cultures of *Syringia*. In addition to the *Phaseolus*

tissue described above, we have also obtained similar results with *Camellia* tissue blocks. It would appear therefore that the diffusion-gradient technique might provide a general method for the introduction of organized growth within a plant cell culture. Both xylem and phloem cells were formed during the induction period. When the induction periods were prolonged for 6–8 weeks the distribution of the xylem and phloem became comparable with that of the primary vascular strands seen in intact stems and included an area of cambial activity between the xylem and phloem cells (Plate 1d; Plate 2h) (Wetmore *et al.* 1964).

The comparison between the induced vascular tissue and the intact stem was reinforced by the chemical investigation. In the stem it has been shown that the secondary thickening of the xylem is accompanied by an increase in the xylose/arabinose ratio and in the amount of lignification of the cell wall (Thornber & Northcote, 1961*a*,*b*). From the results quoted in Tables 1, 2 and 3, it can be clearly seen that a comparable increase in the xylose/arabinose and the lignin content occurs in the differentiated callus tissue.

The pectic substances which are formed in the primary cell wall consist of polymers of galactose, arabinose, galacturonic acid and small amounts of other sugars including rhamnose (Aspinall & Fanshawe, 1961; Barrett & Northcote, 1965). The rhamnose present in the cell wall occurs mainly in the pectic substances and therefore the rhamnose content of the wall, which is simple to estimate, gives an indication of the amount of primary growth (Thornber & Northcote, 1961*b*). Some primary growth indicated by an increase in the rhamnose content of the tissue did occur and this probably corresponded to the induction of the actively dividing cells which can be seen between the differentiating xylem and phloem.

During the development of sycamore xylem in the stem there is a qualitative change in its lignin composition. This is seen as an increase in the amounts of vanillin and syringaldehyde produced after oxidation with nitrobenzene. The composition of the induced lignin present in the secondary wall of the xylem elements in the callus tissue was also different from that which occurred before induction and gave an increase in the amounts of vanillin and *p*-hydroxybenzaldehyde after oxidation.

The stimulus for the induction of the differentiation within the callus block which had been provided with the wedges of agar containing indol-3-ylacetic acid and sucrose seems to be the formation within the tissue mass of gradients of concentrations of these nutrients. At a particular position, a ratio of concentrations of growth factors may exist which initiates and maintains the differentiation process (Northcote, 1963*b*). Wetmore & Rier (1963) have demonstrated that there is no polarized transport of indol-3-ylacetic acid in callus blocks so that it is probable that this material diffuses as a simple gradient from the wedges. An external environment for the cells at the particular position, 50–500 μ below the wedge, is thus produced by the diffusion of substances down from the wedge, materials produced by the cells themselves and materials derived from the maintenance medium below. Once the nodule is established it is probable that the organization can be maintained since the more regular patterns of the cells within the nodule might control the amounts of nutrients and growth factors which enter it (Torrey & Shigemura, 1957; Steward *et al.* 1958).

Whatever the initial mechanisms which cause a cell to differentiate, one very apparent effect is the change in carbohydrate and lignin metabolism. During the secondary growth, the composition of the polysaccharides comprising the matrix material of the wall changes (Northcote, 1963*b*). It has been shown that the pectic substances representing the galactose series of polysaccharides are formed within the Golgi apparatus and that they are transported to the cell wall via the Golgi vesicles (Northcote & Pickett-Heaps, 1966). Similarly, some of the material which is deposited in the secondary thickening arises from the Golgi apparatus (Wooding & Northcote, 1964; Pickett-Heaps & Northcote, 1966*a*; Northcote & Wooding, 1966) and the matrix material of the wall at this stage is composed of polymers of the glucose series.

The two series of sugars are interconverted by epimerase activity on the corresponding uridine diphosphate sugar compounds (Hassid, Neufeld & Feingold, 1959). Since both the pectic substances (galactose series) and some of the hemicelluloses e.g. xylans (glucose series) are apparently formed and transported by the Golgi body, and since there is a change in the relative amounts of these two groups of polysaccharides produced during the change from primary to secondary growth which is a stage in the differentiation of the cell, a possible site of the differentiation stimulus could be at the epimerase activity associated with the Golgi body.

If the growth substances are influencing the metabolism of the cells by an alteration of enzyme activities brought about by a control of the amount of enzyme synthesized by the cell, it might be that their action is mediated by a mechanism comparable with the action of inducer and repressor systems implicated for bacteria (Jacob & Monod, 1963).

The secondary thickening which is laid down in the xylem is highly organized and is deposited in a spiral or reticulate fashion. Both hemicellose and α-cellulose are deposited in the wall during the thickening, and differentiation involves control

mechanisms for the synthesis and deposition of both of these classes of polysaccharide. It has been shown that the secondary thickening of the xylem cells is closely associated with the distribution of the microtubules and endoplasmic reticulum in the cell at this stage of its development (Hepler & Newcomb, 1963; Wooding & Northcote, 1964; Pickett-Heaps & Northcote, 1966*a*). A similar relationship of these organelles to cell-wall development can be seen at the initial formation of the cell plate at mitosis and during subsequent primary growth (Leech, Mollenhauer & Whaley, 1963; Esau & Gill, 1965; Pickett-Heaps & Northcote, 1966*a*,*b*). The mechanism whereby the cell differentiates must thus affect the whole pattern of the distribution and relationships of the microtubules, Golgi bodies and endoplasmic reticulum which are closely connected not only with the synthesis but with the organization and deposition of the wall material of the developing cell.

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