Biochem. J. (1969) **113**, 765
Printed in Great Britain

Studies on the Polyphenol Metabolism of Tissue Cultures Derived from the Tea Plant (*Camellia sinensis* L.)

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(*Received* 17 *January* 1969)

1. The growth characteristics on various media of solid and liquid suspension cultures derived from the stem of the tea plant are described; chlorophyll and anthocyanin synthesis occurred in the light. 2. Only the simplest catechins and leucoanthocyanins were present in callus tissue, although oligomeric and polymeric leucoanthocyanin fractions were also represented. Light caused an increase in all monomeric components analysed, but inhibited polymerization of the leucoanthocyanins. 3. The polyphenol oxidase activity of cultures was comparable with that of the apical regions of the intact plant, and was inversely correlated with growth rate. 4. Growth was stimulated by hormonal variation, and inhibited by high concentrations of sucrose and by high light-intensity; polyphenol concentrations were generally inversely correlated with growth rate. 5. From the inability of callus tissue and of cultured root apices to synthesize complex catechins, it is inferred that complex catechin formation in intact plants is associated with the process of cell vacuolation.

Previous work has shown the main classes of polyphenols present in the tea plant to have characteristic distributions within the plant (Forrest & Bendall, 1969*a*,*b*). To gain some insight into the factors controlling polyphenol synthesis and perhaps leading to such distributions, it is desirable in the first instance to study the effects of simple environmental modifications on the metabolism of the polyphenols. However, it is difficult to control rigidly the environment of an intact plant, whereas tissue cultured *in vitro* as a callus provides a relatively simple system of predominantly undifferentiated cells which, on suitable media, may be maintained indefinitely by periodic subculturing. Such callus cultures may be grown on media of known composition and subjected to a range of controlled environmental conditions.

Previous work on the metabolism of polyphenols in tissue cultures has tended to be concentrated on anthocyanin synthesis, which is generally claimed to be light-dependent (Gautheret, 1941; Slabecka-Szweykowska, 1955; Ardenne, 1965). de Capite (1955*a*,*b*) was able to induce stable anthocyanin synthesis in the phloem of carrot root explants by a short initial cold-treatment, which gave a purple proliferating callus after a few weeks when the medium contained sugar and the system was aerobic. Typical results obtained with intact plants were repeated with tissue cultures when Heller (1948) demonstrated anthocyanin synthesis in cultures of virginia creeper by suppression of nitrogen in the medium, and Szweykowska, Gierczak & Luszczak (1959) and Szweykowska (1959) showed that increased anthocyanin synthesis in cultured red-cabbage embryos was correlated with increased sugar and with decreased nitrogen. Leucoanthocyanin synthesis has received little attention, although it has been shown to be dependent on an adequate air supply in sycamore cultures (Goldstein, Swain & Tjhio, 1962; Wickremasinghe, Swain & Goldstein, 1963). Finally, tannin synthesis in cultures derived from juniper was depressed by illumination, and the tannin content was inversely related to the growth rate or to the dry weight of the callus (Constabel, 1963).

The object of the present work was, initially, to describe the growth characteristics of callus tissue from tea and to compare its polyphenol composition with that of the mature plant from which it was derived, and then to investigate the effects of environmental and nutritional modifications on growth and polyphenol metabolism.

MATERIALS AND METHODS

Analytical methods

Chromatography and polyphenol determination. Cultured tissue was extracted with aq. 50% (v/v) methanol, as described for tissues from the intact plant by Forrest & Bendall (1969*a*), and t.l.c. and colour reactions were used for

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the separation and identification of polyphenols (Forrest & Bendall, 1969*a*). Flavylogens were determined with the acid–butanol reagent (Swain & Hillis, 1959), catechins with the benzidine reagent and total phenols by the potassium titanium oxalate method (Forrest & Bendall, 1969*a*). Flavylogens were fractionated into different molecular sizes by gel filtration on a Sephadex column as described by Forrest & Bendall (1969*b*). A column of length 12·7cm. and volume 2·82ml./cm., packed with Sephadex G-25 (fine grade), was used, the flow rate being about 58ml./hr.

A number of unidentified compounds with similar u.v. fluorescence, apparently characteristic of callus tissue, were determined by their u.v. absorption. For this purpose, redistilled ethanol was used as a solvent to decrease blank extinction values as much as possible. Cellulose spots were removed from various points on blank gridded thin-layer cellulose plates, having only aq. 50% methanol applied as a load, with a microspatula into glass centrifuge tubes and weighed, and 5ml. of redistilled ethanol was added to each tube. The cellulose was homogenized in the ethanol in a small glass homogenizing tube (Wesley Coe Ltd., Cambridge; internal volume 5·5ml.) by using an 8mm. mechanical stirrer [Baird & Tatlock (London) Ltd., Chadwell Heath, Essex] fitted with a small hard-plastic homogenizer with a metal stem; the solution was then shaken rapidly on a neoprene-cup Vortex mixer for 1min. The tubes were spun at maximum speed in a bench centrifuge, the supernatants were removed with a Pasteur pipette, and their extinctions were read at 260, 275 and 280nm. in a Unicam SP.500 spectrophotometer. It had been found that the u.v. extinction of the eluate from the cellulose was directly proportional to the weight of cellulose taken for elution, so that it was now possible to determine the cellulose blank extinction value for any position on the plate, from the known weight of each spot. A standard solution of (+)-catechin was applied in various loads to cellulose layers, and the extinctions at 280nm. of the spot eluates were measured. After correction for ethanol and cellulose extinctions, there was a linear relationship between the weight of catechin applied and the extinction up to loads of at least 0·05mg.; a molar extinction coefficient of 3700 was obtained, which compares well with the value of 3585 given by Vuataz, Brandenberger & Egli (1959); the elution was therefore efficient. The foregoing elution procedure and extinction measurements were adopted for the determination of the unknown compounds.

Measurement of polyphenol oxidase activity. A Clark oxygen electrode (Yellow Springs Instrument Co., Yellow Springs, Ohio, U.S.A.) coupled to a Honeywell–Brown Electronik recorder was used to follow the uptake of oxygen when pyrogallol was supplied as substrate, as described by Gregory & Bendall (1966). The reaction solution (3ml.) contained (final concn.) 0·1M-pyrogallol in 0·05M-sodium citrate buffer, pH5·6; blank tubes were assayed with glass-distilled water replacing the enzyme solution.

Culturing methods

Media. The standard medium used (Table 1) had a mineral composition based on that of the H2 medium described by Heller (1953). The seven solutions *A*–*E*, *S* and *O* (Table 1), were stored separately (solutions *S* and *O* at −15°, the rest at 4°), and mixed together when required. Coconut milk, obtained from fresh coconuts, was filtered through muslin, autoclaved at 15lb./in.^{2} for 30min. to precipitate most of the protein, and then filtered through Whatman no. 1 filter paper. The milk was stored at −20° and added to the medium to give a final concentration of 20% (v/v). The pH of the complete medium was about 4·7.

Table 1. *Composition of standard medium (H2) used for culturing tissue*

The medium H2 (Heller, 1953) was made up by mixing these solutions.

Inorganic solutions	Concn. (mM)
Soln. *A*	
KCl	10·1
$NaNO_3$	7·05
$MgSO_4,7H_2O$	1·00
Soln. *B*	
$NaH_2PO_4,2H_2O$	0·91
Soln. *C*	
$CaCl_2,6H_2O$	0·51
	Concn. (μM)
Soln. *D*	
$FeCl_3,6H_2O$	3·66
Soln. *E*	
$ZnSO_4,7H_2O$	3·48
H_3BO_3	16·2
$MnSO_4,4H_2O$	0·448
$AlCl_3$	0·225
$NiCl_2,6H_2O$	0·126
KI	0·601
$CuSO_4,5H_2O$	0·120
Organic solutions	**Concn. (p.p.m.)**
Soln. *S*	
Sucrose	20000
Soln. *O*	
Thiamin	0·1
Calcium pantothenate	0·1
2,4-Dichlorophenoxyacetic acid	6·0

Callus tissues were grown in boiling tubes; each tube contained 15ml. of the medium gelled by heating it with 0·8% (w/w) of Special Agar–Noble (Difco Laboratories, Detroit, Mich., U.S.A.). The tubes were plugged with non-absorbent cotton wool and sterilized by autoclaving them at 15lb./in.^{2} for 30min.

The following modifications to the standard H2 medium were made.

(*a*) Ammonia (AM) medium. Ammonium ions replaced nitrate as the nitrogen source; $NaNO_3$ was replaced by 7·05mM-Na_2SO_4 + 3·00mM-$(NH_4)_2SO_4$.

(*b*) Low-sucrose (LS) medium. No sucrose was added, the only source of sucrose being the coconut milk, in which its concentration is about 5% (Tulecke, Weinstein, Rutner & Laurencot, 1961). Thus the final concentration of sucrose was about 1%.

(*c*) High-sucrose (HS) medium. This contained 5% of sucrose.

(*d*) Naphthoxyacetic acid (NA) medium. Naphthoxyacetic acid at a concentration of 0·5p.p.m. (w/w) replaced 2,4-dichlorophenoxyacetic acid.

(*e*) Naphthoxyacetic acid/low sucrose (NA/LS) medium. This was a combination of the NA and LS media, containing about 1% of sucrose, 0·5p.p.m. (w/w) of naphthoxyacetic acid, and no 2,4-dichlorophenoxyacetic acid.

(*f*) Naphthoxyacetic acid–2,4-dichlorophenoxyacetic acid (NAAD) medium. Both growth hormones were present, the concentrations being 0·5p.p.m. (w/w) of naphthoxyacetic acid and 6·0p.p.m. (w/w) of 2,4-dichlorophenoxyacetic acid.

(*g*) Synthetic (SM) medium. An attempt was made to replace the growth-promoting activity of coconut milk by an amino acid–vitamin mixture. The medium differed from the H2 medium in the omission of coconut milk and in the addition of the following components: L-asparagine, 150mg./l.; L-glutamine, 150mg./l.; L-alanine, 40mg./l.; L-glutamic acid, 20mg./l.; inositol, 150mg./l.; naphthoxyacetic acid, 0·5p.p.m. (w/w).

Technique. Explants were obtained from tea bushes growing in Cambridge Botanic Gardens. Stem explants were taken from 1-year-old branches, about 3–4mm. diameter, which were defoliated, scrubbed in water, submerged in 5% (w/w) calcium hypochlorite for 10min., and then in 75% (v/v) ethanol for 15min. Callus tissue was grown from these in boiling tubes (see above).

Liquid suspension cultures were set up by sterilizing 150ml. samples of the standard H2 medium (without agar) in narrow-necked 1l. glass bottles, and inoculating each bottle with the partially broken-up callus tissue from one culture tube.

Growth conditions. All cultures were grown in a constant-temperature room at 25°. The lighting array for illuminated cultures consisted of two parallel Ekco 20w white 24in. fluorescent tubes, 9in. apart and, placed centrally between them, one 100w Mazda tungsten-filament bulb to supply the red wavelengths in which the fluorescent light was deficient. The cultures were arranged 2·5ft. below the lights, the incident-light-intensity being 1000 lux. The lights were controlled by a Vennerette mark II time switch (Venner Ltd., New Malden, Surrey), initially set to give 8hr. of darkness alternating with 16hr. of light; the light period was later decreased. Liquid suspension cultures were maintained in darkness, the bottles lying on their sides and continuously rotated about the horizontal axis by rollers driven by an electric motor; the bottles rotated at 45rev./min.

RESULTS

Growth characteristics

Compared with the tissues of several other plant species, the growth rate of the tea callus on the H2 medium was slow, and the consistency was hard and compact. Cultures had a tendency to become brown and unhealthy on aging or due to bruising sustained during subculturing.

Cultures derived from different clones showed characteristic modes of growth: callus from the clones Namabinzi 4/D2 and S.61 had a friable appearance and grew comparatively rapidly on the H2 medium, whereas that from clones C/7, IN/13 and IN/15 had a much slower growth rate, with densely packed and very hard callus. Reactions to illumination were also different, although the general effect was to lower the growth rate (see Table 4). Initial growth of explants was almost completely inhibited by light; however, after a dark period of a few weeks, light caused little retardation of growth of the young cultures, and intense greening sometimes occurred. The slow-growing callus from clones C/7, IN/13 and IN/15 showed a very marked tendency for anthocyanin synthesis when cultured in the light, becoming intensely red–purple; synthesis was initiated on the part of the callus next to the surface of the medium, where supplies of nutrients and of air were maximal. Cultures of the other clones could also be induced to synthesize anthocyanin in the light on certain media (see Table 4), but the production was relatively poor and localized. Callus sections showed anthocyanin to be confined to the vacuoles of the outer layers of cells. Chlorophyll synthesis was initiated by light in cultures of all clones, but occurred less readily and much less intensely in those that were synthesizing anthocyanin.

Attempts to culture other tissues from mature plants *in vitro*, including parts of leaves, intact and dissected apical buds, and flower buds, were thwarted by bacterial and fungal contamination.

Liquid suspension cultures showed a lag period of several weeks before growth occurred, but thereafter growth was fairly rapid and of a characteristic type: the shearing forces generated by the constant rolling motion smoothed the callus fragments to yellow–brown spheres, and growth apparently occurred by production of protuberances, which became dislodged and which in turn increased in size and then fragmented. Most of the tissue present was thus in the form of spherical cell clumps, so that the system was not a true cell suspension culture.

Polyphenol content

T.l.c. (Fig. 1) showed the callus polyphenol composition to differ from that of the secondary phloem whence it was derived, and indeed from that of any part of the intact plant (Forrest & Bendall, 1969*a*,*b*). The only catechins present were the simple epicatechin and catechin, and the only flavonol glycoside was quercetin 3-rhamnoglucoside, present in low concentration. By far the most important components were the leucoanthocyanins, both monomeric and polymeric. The leucoanthocyanin composition was similar to that found in the bulkier tissues of the intact plant, the main spots being the most highly mobile compounds LA-13, -49 and -50, which are thought to be the simplest in structure (Forrest & Bendall, 1969*b*). The characteristic feature, however, was the presence of a series of spots appearing dull-green or blue in u.v. light, and generally highly mobile in aqueous solvents. They reacted with none of the spray

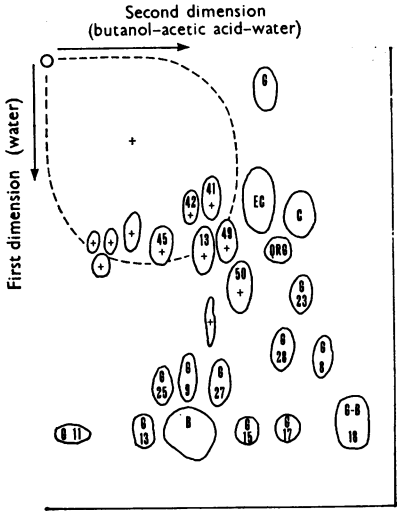

Fig. 1.* Thin-layer cellulose chromatogram of extract of callus tissue derived from clone S.61, cultured in the light. Colours of fluorescence under u.v. light with ammonia vapour are shown.

reagents employed, and their identity remains obscure.

Table 2 gives the concentrations of the catechins and of the main leucoanthocyanins in callus cultured in darkness and in light. Light caused no qualitative change in catechins, but increased the concentrations of all the components severalfold. The catechin concentrations were low compared with those in actively growing parts of the intact plant, and in this respect callus tissue was similar to relatively inactive tissues such as stem wood and root, where there is little catechin but a high proportion of flavylogens (Forrest & Bendall, 1969*a*). Flavylogen separations on a column of Sephadex G-25 (fine grade) showed the dark-grown callus to have approximately the same polymer content, on a dry-weight basis, as mature-green-stem tissue (Fig. 2 and Table 2); higher concentrations of oligomers and monomers were present. The effect of light was to eliminate polymer synthesis, decrease the oligomer concentration and increase greatly the monomer fractions; the ratio of complex to simple monomers was increased.

Polyphenol content of cultured root tips

In view of the simple catechin composition of root tips (Forrest & Bendall, 1969*a*), and of their meristematic growth compared with the division of mature vacuolated cells of callus tissue, it was decided to investigate the effect of light on the root-tip catechins. For this purpose, root tips (1–2 cm. long) from seedlings of clone MT 9 were placed

* Abbreviations used in figures: B, blue; C, (+)-catechin; EC, (−)-epicatechin; G, green; LA and +, leucoanthocyanin; QRG, quercetin 3-rhamnoglucoside.

Table 2. *Concentrations of flavanols and of total phenols in clone S.61 callus*

Catechins and leucoanthocyanins (calculated as catechins) are given as % dry wt., flavylogen fractions are given as peak extinction/g. dry wt. of tissue, total flavylogens are given as E_{550}/g. dry wt. of tissue and total phenols are given as mg. of equivalent pyrogallol/g. dry wt. of tissue. The numbers LA-13 etc. refer to different unidentified leucoanthocyanins (Forrest & Bendall, 1969*b*). The values 0·5 V_t etc. refer to elution positions from Sephadex G-25; V_t = total bed volume of the column.

	Concentrations	
	Dark-grown	Light-grown
(−)-Epicatechin	0·19	1·10
(+)-Catechin	0·05	0·13
Total catechins	0·24	1·23
LA-13	Trace	0·19
LA-45	Trace	0·14
LA-49	0·07	0·64
LA-50	0·07	0·29
Total leucoanthocyanins	0·14	1·28
Flavylogen fractions		
Polymer (0·5 V_t)	0·49	0·13
Oligomer (1·5 V_t)	1·49	0·90
Monomer (2·3 V_t)	1·22	3·43
Monomer (3·3 V_t)	0·74	2·90
Total flavylogens	55	157
Total phenols	1·4	5·7

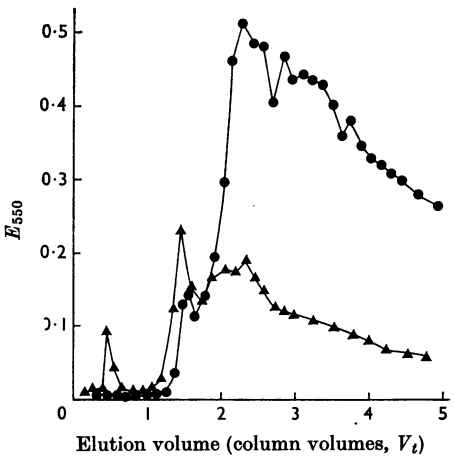

Fig. 2. Fractionation on Sephadex G-25 of flavylogens from extracts of callus tissue grown in darkness (▲) and in light (●). The load in each case was 155 mg. dry wt.

immediately after excision in 10ml. of an aqueous medium containing 20g. of sucrose and 0·01mg. of thiamin/l. (Bonner, 1938) in small conical flasks. The flasks were placed under continuous illumination of 1000 lux from the fluorescent and tungsten lamps (see the Materials and Methods section) for 1 week, after which time the root tips had increased only slightly in length. T.l.c. of an extract showed that the only catechins present were epicatechin and a trace of catechin; it thus appeared that light was incapable of stimulating synthesis of complex catechins in root tips, as in callus tissue.

Polyphenol oxidase activity

Because of its possible involvement in the browning of callus tissue, and also as a point of comparison with the intact plant, it was decided to measure the activity of polyphenol oxidase in the cultures. The activities in solid and in liquid suspension cultures are shown in Table 3. The tissue was homogenized by grinding it in a mortar in the buffer solution, or mechanically in a small homogenizing tube; similar activities were obtained with these two methods, and microscopic examination showed the majority of cells to have been broken. The activity in the clone S.61 callus was similar to that in young leaves; the comparison is complicated by the possibility of enzyme localization in both callus and leaf, and by the different ratios of cytoplasm to total cell volume in the two tissues.

Liquid cultures were harvested by straining them through muslin to separate the large callus clumps; the liquid was spun in a bench centrifuge to collect the smaller cell aggregates and other cell debris, leaving the supernatant medium free of cells. Most of the cell aggregates of the clone C/7 culture, which was slow-growing, contained between ten and 50 cells, but those from the rapidly-growing thick clone S.61 culture were larger, usually consisting of hundreds of cells. The results in Table 3 show that the large callus clumps of clone C/7 had a high activity, four times that of the clone S.61 clumps, which had an activity similar to that of the clone S.61 solid callus. This activity decreased considerably during several days' storage at 4°. The smaller cell aggregates had a much lower activity in both clones; the medium had very low activity, which was destroyed by boiling and therefore real, and presumably due to release of enzyme from broken cells. These results show that enzyme activity is inversely related to growth rate, both between clones and between different fractions within clones. It was possible to render soluble only a small fraction, usually about 10%, of the enzyme by grinding or homogenizing, most of the activity remaining associated with the cell walls and debris.

From the curve of the oxygen uptake of a homogenate of the smaller cell aggregates of the clone S.61 suspension culture, the Michaelis constant for the enzyme was calculated as $1·7 \times 10^{-4}$M; this is comparable with the value of $1·4 \times 10^{-4}$M calculated for the enzyme purified from tea leaves (Bendall & Gregory, 1963).

Table 3. *Polyphenol oxidase activity of cell preparations* in vitro

Activity was measured by the rate of uptake of O_2 in the presence of 0·1M-pyrogallol, expressed as μmoles of O_2/g. fresh wt./min. Homogenates were kept at 4° until used.

	Polyphenol oxidase activity		
Clone	S.61	C/7	
Age of homogenate ...	Fresh	Fresh	6 days
Solid callus homogenate	7·4		
Liquid suspension			
Large cell clumps			
Pellet	7·1	28·6	13·2
Supernatant	0·7		1·8
Smaller cell aggregates			
Pellet	1·8	0·7	
Supernatant	0·7	0·3	
Medium	0·03	0·03	
Medium, boiled	0·00		

Nutritional variations

The three main factors varied were the sugar concentration, the nature of the growth hormone, and the light–darkness regime.

Effects on growth. Cultures of clone S.61 callus were grown on HS, LS and NA media through a period of several subcultures. Their appearances after 1 month and then after a further 6 weeks are tabulated in Table 4. Growth rates were always higher in the dark, and were increased by low sugar concentrations, which also stimulated chlorophyll synthesis. Anthocyanin synthesis was poor in this clone, occurring to the greatest extent on the NA medium. Repeated subcultures on to any one of these media caused an eventual cessation of growth and induced an unhealthy appearance in the cultures, although the callus remained most healthy on the NA medium. If cultures grown on NA medium were subcultured on to the standard H2 medium, there was a burst of fresh growth, the tissue becoming loose, friable and almost transparent. After two or three subcultures on H2 medium the growth rate again slowed down, but it was further stimulated by a reversion to NA medium. In this

Table 4. *Appearance of clone S.61 cultures growing on various media*

Three cultures were subjected to light-treatment and three to dark-treatment in each medium. Growth rate, chlorophyll and anthocyanin were estimated by inspection; the number of × signs shows the relative amounts of growth or synthesis.

	Growth rate		Chlorophyll synthesis		Anthocyanin synthesis	
Age of culture (weeks) ...	4	10	4	10	4	10
LS medium						
Light 1	××	××	×	××	—	—
2	×	××	×	××	—	—
3	×	×	××	×	—	(×)
Dark 1	××	×××	—	—	—	—
2	×	××	—	—	—	—
3	×××	××	—	—	—	—
HS medium						
Light 1	(×)	(×)	—	(×)	—	—
2	×	—	(×)	—	—	—
3	(×)	—	×	—	(×)	(×)
Dark 1	(×)	×	—	—	—	—
2	×	×	—	—	—	—
3	×	(×)	—	—	—	—
NA medium						
Light 1	×	×	××	×××	×	(×)
2	×	(×)	×	—	—	—
3	××	×	××	××	(×)	(×)
Dark 1	×	×××	—	—	—	—
2	××	××	—	—	—	—
3	××	×××	—	—	—	—

way, alternation between the two media was sufficient to prolong active growth, but transference to the NAAD medium (a combination of the two media) caused growth to slow down very soon, and browning was induced; further subcultures on to NAAD medium caused a complete cessation of growth.

The possibility was considered that the slow growth of the tea callus, compared with that of other species, might be due to a nitrogen deficiency, perhaps caused by lack of the nitrate-reducing enzyme system, since the H2 medium contained no ammonium ions. Growth on the AM medium, containing 3 mM-ammonium sulphate, was poor, for although some cultures grew at a lower rate, most turned brown and stopped growing. After a few months the slowly growing cultures on AM medium were still growing slowly, but young tissue was yellow–brown rather than white; after reversion to the H2 medium they resumed active growth, and chlorophyll synthesis was initiated in the light. It was therefore concluded that nitrogen deficiency was unlikely to be limiting growth, especially as there were a variety of nitrogen sources present in the coconut milk.

Growth on the SM medium, in which coconut milk was replaced by an amino acid mixture, inositol and naphthoxyacetic acid, was moderately fast over the first subculture, but slowed down thereafter. The growth rate was much higher in the dark than in the light, and clone S.61 showed a higher rate than either IN/13 or C/7. It is not possible to say whether growth could be maintained indefinitely on this medium.

Effects on polyphenol concentration. The results shown in Table 5 indicate that the highest flavylogen concentrations occurred in callus grown on standard H2 medium in the light, where growth was slow, and that the most intensely green callus, which was also the slowest growing, had maximal flavylogen concentrations. Naphthoxyacetic acid induced high flavylogen concentrations not only in healthy cultures but also in unhealthy cultures that had been repeatedly subcultured on to the same medium; this is significant since the NA medium was the only one on which any appreciable anthocyanin synthesis was observed (Table 4). High- and low-sugar media both caused a decrease in flavylogen concentration, especially in the dark. Dark-grown cultures consistently had lower flavylogen and total phenol concentrations on all media.

Table 5. *Concentrations of flavylogens and total phenols in callus tissue grown on various media*

Flavylogens are given as E_{550}/g. dry wt.; total phenols are given as mg. of equivalent pyrogallol/g. dry wt.

	Concentrations			
	Flavylogens		Total phenols	
	Light-grown	Dark-grown	Light-grown	Dark-grown
Healthy cultures				
LS medium	25·6	20·0	7·40	5·99
H2 medium	134·0	55·4	23·2	1·36
Intense green	156·9		5·68	
HS medium	29·4	11·8	7·57	3·81
NA medium	62·0	46·5	10·0	7·34
AM medium		33·1		4·94
Unhealthy cultures				
LS medium	1·1		0·20	
H2 medium	4·2		0·28	
NA medium	102·3		3·80	
NA/LS medium	18·7		1·36	

Table 6. *Relative concentrations of leucoanthocyanins and unidentified components of callus tissue grown on various media*

Concentrations are given in arbitrary units and were determined by u.v. absorption. Numbers LA-13 etc. refer to unidentified leucoanthocyanins (Forrest & Bendall 1969*b*), and G-8 etc. to other unidentified components that fluoresce green under u.v. light (see Fig. 1).

	LS medium		HS medium		NA medium		AM medium
	Light-grown	Dark-grown	Light-grown	Dark-grown	Light-grown	Dark-grown	Dark-grown
LA-13	18	1	8	9	7	11	25
LA-49	8	0	6	0	1	8	37
LA-50	3	0	7	0	0	0	0
G-8	0	0	8	0	8	0	23
G-9	18	13	14	5	6	6	28
G-15	1	15	11	0	5	0	4
G-17	13	10	11	7	5	2	0
G-18	6	0	8	0	0	0	0
G-23	0	3	21	0	8	1	2
G-25	14	6	11	3	17	10	4
G-27	15	6	11	3	14	12	11
G-28	0	0	0	0	10	5	6
Total	96	54	116	27	81	55	140

An analysis of three of the main leucoanthocyanin monomers and of a number of the unidentified compounds fluorescing dull-green in u.v. light is given in Table 6. The concentrations of most of the components were increased by light; with high sugar concentration, values were lower than normal in the dark but higher in light; this phenomenon was also observed for flavylogens and total phenols on this medium. Since high sugar concentration gave poor growth (Table 4), this may have been due to very low polyphenol synthesis in darkness. In the light, synthesis was promoted but, since little growth occurred, the concentrations rose rapidly. Similarly on the AM medium growth was very restricted, so that even in the dark high polyphenol concentrations were observed. Generally, growth rate was inversely correlated with polyphenol concentration.

DISCUSSION

The cultures were capable of synthesizing only the simplest flavonoids in spite of considerable environmental and nutritional variation; this fact is considered to be associated with their mode of growth, which is by division and re-enlargement of

vacuolated cells rather than by meristematic growth, so that there is no true cell development and maturation. In young shoots, both vegetative and floral, of the intact plant, formation of complex catechins began at the stage of cell enlargement by vacuolation (Forrest & Bendall, 1969*a*); the absence of complex catechins from root apices even when grown in the light showed the process to be characteristic of the developing cells of the young shoot. Since the flavonoids occur in the cell vacuoles, and vacuolation is the characteristic of shoot cells that differs in its mechanism from the process in root cells, it was apparent that elaborations on the basic catechin structure were linked with vacuolation in cells derived from aerial meristems.

Synthesis of anthocyanin, of flavonols and of polyphenol oxidase was generally inversely correlated with the growth rate of the cultures; enzyme synthesis was characteristic of large cell clumps showing restricted growth rather than of actively growing, smaller cell aggregates. Similarly, Scott, Daly & Smith (1964) found that the activities of enzymes of the hexose monophosphate pathway of tobacco tissue cultures were decreased when the growth rate was increased by hormonal variation. It is possible that the frequently encountered browning of old cultures may be a consequence of the high concentrations of polyphenol oxidase accumulated.

The cultures were found to share the capacity for leucoanthocyanin polymerization of the stems from which they were derived; the inhibition of polymerization by light was shown also to occur in juniper cultures by Constabel (1963), who similarly showed the tannin content to be inversely related to the growth rate. The increase in synthesis of monomeric leucoanthocyanins in the light has frequently been observed in intact plants (e.g. Hillis & Swain, 1957; Alston, 1958; Bopp & Matthiss, 1962).

High sugar concentrations, which eventually caused growth of the tea callus to stop, have been shown to be inhibitory to a number of species cultured *in vitro*, for example carrot (Hildebrandt, Wilmar, Johns & Riker, 1963), normal and gall tissue of grape stem (Arya, Hildebrandt & Riker, 1962), and red-cabbage embryo cultures (Szweykowska *et al.* 1959). The observation that the NA medium was the most efficient at promoting anthocyanin synthesis in the clone S.61 cultures is similar to that made by Blakely & Steward (1961), who obtained abundant anthocyanin synthesis with the same concentrations of naphthylacetic acid (0·5p.p.m.) in cultures of *Haplopappus gracilis*; higher concentrations of the hormone were inhibitory. In general, it can be said that, as in intact higher plants, a decreased growth rate due to environmental conditions such as intense light or to some nutritional anomaly such as excess of sugar results in the channelling of the excess of accumulated carbohydrate into polyphenol synthesis. Thus there is an inverse correlation between growth rate and polyphenol accumulation.

I thank Dr D. S. Bendall for his interest in this work, and the Science Research Council for financial support.

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