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POLYPHENOLS OF MATURE PLANT, SEEDLING AND TISSUE CULTURES OF *THEOBROMA CACAO*

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Key Word Index—*Theobroma cacao*; Sterculiaceae; cocoa; plant and tissue cultures; composition; polyphenols; phenolic acids and flavonoids.

Abstract—The major flavone in mature cocoa leaves is isovitexin, with smaller amounts of vitexin and 7-*O*-glucosides of apigenin, luteolin and chrysoeriol. Flavones are absent in the cotyledons but two quercetin glycosides are present, the 3-*O*-glucoside and 3-*O*-galactoside. The stem contains few polyphenols and none of the newly identified flavone and flavonol glycosides. High concentrations of total phenols, particularly leucocyanidins and (−)-epicatechin occur in the cotyledons, intermediate amounts in the leaves and low levels in the stem. No flavones were detected in the seedling and the flavonol glycosides found in the seedling stem and hypocotyl probably originate from the cotyledon. This reduced capacity to synthesize polyphenols in meristematic tissue is even more marked in the callus which contains none of the flavonol glycosides and phenolic complexes found in the parent tissue. Instead the callus contains two new flavonoid glycosides, phenolic acid complexes and *p*-coumaric and caffeic acids. This pattern was constant for each callus clone despite the clones being initiated from different parts of the seedling, each with its characteristic pattern. Epicatechin and leucocyanidins are the only compounds common to all tissues.

INTRODUCTION

The polyphenols of the cocoa bean have long been regarded as an important component of flavour in the fermented and roasted cotyledon. As a result there has been considerable investigation of the polyphenol content of the bean [1–4] and also of the vegetative parts of the plant [5]. Some comparison of the major polyphenols within the genus *Theobroma* has also been made [6]. Only anthocyanins, leucocyanidins, catechins and some phenolic acids were positively identified previously. Since we have been able to develop tissue cultures of cocoa [7], we decided to compare the polyphenol composition of various parts of the mature plant and seedling, with tissue cultures derived from the same parts to see, firstly, the effect of dedifferentiation from plant to tissue culture on the polyphenol composition and, secondly, the potential value of the tissue culture for studies on the biosynthesis of the polyphenols in cocoa.

RESULTS

Major components of all the cocoa extracts are anthocyanins, leucocyanidins, (−)-epicatechin, catechin, *p*-coumarylquinic acid and chlorogenic acid. The presence and identity of these in mature tissue had already been established from earlier investigations [1–5]. The identity of the same compounds in the present work was confirmed by chromatographic data and the response to specific spray reagents. Additional caffeylquinic esters were identified by R_f and UV data. The plant material also contained a variety of previously unidentified flavonoid compounds (A–G) which were isolated and identified as described.

The TLC data shows the distribution and relative amounts of the polyphenols in the different sources (Fig. 1). A number of compounds are common to each source of the mature plant. These are the leucocyanidins (L_1 and L_2), (−)-epicatechin (EP), *p*-coumarylquinic acid (H) and chlorogenic acid (I). The stem extract contained only one additional compound which was a caffeylquinic ester (J). The cotyledon extract was more varied since there were further leucocyanidin spots (L_5, L_6, L_7 and L_8), two anthocyanins (AN_1 and AN_2), catechin (CA), a further caffeylquinic ester (K) and two previously unidentified flavonols, quercetin 3-*O*-glucoside (F), and quercetin 3-*O*-galactoside (G). A large number of polyphenols were present in the leaf. Besides the compounds common to each source, there were the leucocyanidins (L_3 and L_4) and the newly identified compounds, apigenin 7-*O*-glucoside (A), luteolin 7-*O*-glucoside (B), chrysoeriol 7-*O*-glucoside (C), vitexin (D), isovitexin (E) and two caffeylquinic esters (J and K). Isovitexin was the flavonoid present in the largest amount in the leaf. In the seedling some of the polyphenols such as *p*-coumarylquinic (H) and chlorogenic acids (I), a caffeyl ester (J) and (−)-epicatechin (EP) were found in all parts. The leucocyanidins (L_1–L_3) were also universally distributed but no species of leucocyanidin was common to all of the seedling parts. Of more restricted distribution were the anthocyanins ($AN_{1,2}$) and a caffeylquinic ester (K), both of which were found only in the cotyledons (Fig. 1). Quercetin 3-*O*-glucoside and quercetin 3-*O*-galactoside on the other hand were present in the cotyledon, hypocotyl and stem but not the root. The most common of the unidentified compounds was the one which turned yellow-green (Yg) in ammonia and this was detected in extracts of the root, hypocotyl and stem. The root and to a much lesser extent the stem contained large amounts of a yellow compound which turned red in ammonia (Rb). All the other unknowns, i.e. colourless spots which either turned bright yellow (By), yellow-brown (Yb) or

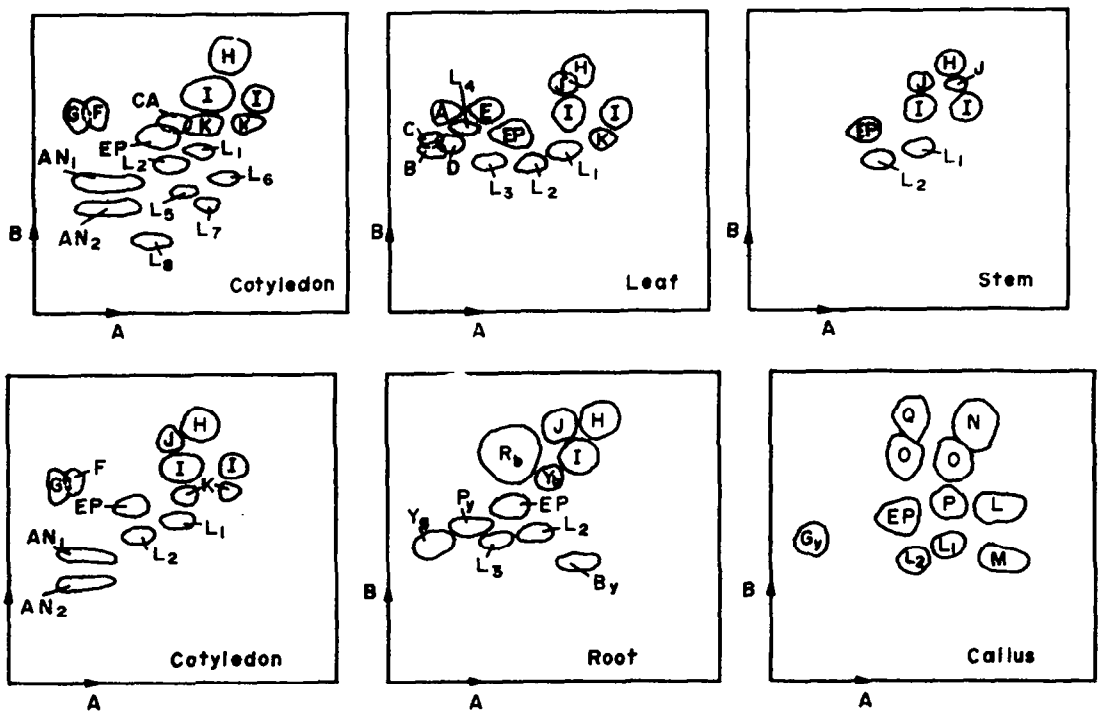

Fig. 1. The TLC pattern of the major polyphenols of cocoa in the cotyledon, leaf and stem (upper) and seedling cotyledon, root and callus derived from the cotyledon (lower). (Solvent A is 5% HOAc and solvent B is *n*-BuOH–HOAc–H_2O, 4:1:5).

Spot	Polyphenols	Spot	Polyphenols
AN_1	cyanidin 3-α-L-arabinoside	I	chlorogenic acid
AN_2	cyanidin 3-β-D-galactoside	J, K	caffeylquinic esters
EP	(−)-epicatechin	L	flavonoid glycoside
CA	(+)-catechin	M	flavonoid glycoside
L_1–L_8	leucocyanidins	N	*p*-coumaric acid
A	apigenin 7-*O*-glucoside	O	caffeic acid
B	luteolin 7-*O*-glucoside	P, Q	caffeic acid complexes
C	chryseriol 7-*O*-glucoside	Rb	red–brown } unidentified
D	vitexin	Yb	yellow–brown } compounds which
E	isovitexin	Yg	yellow–green } developed
F	quercetin 3-*O*-glucoside	Py	pale yellow } fluorescent
G	quercetin 3-*O*-galactoside	By	bright yellow } colours in
H	*p*-coumarylquinic acid	Gy	golden yellow } ammonia

pale yellow (Py) in ammonia, were located only in the root extract (Fig. 1).

Extracts from the various callus sources also showed each had a number of compounds in common (Fig. 1). The major polyphenols were *p*-coumaric (N) and caffeic acids (O), (−)-epicatechin and two leucocyanidin species. Gentisic, sinapic, *p*-coumaric and caffeic acid were also present in the acid-hydrolysed callus extract. Some of the callus clones contained one or two phenolic acid complexes (P and Q), each of which consisted of caffeic acid and an unidentified component. Two new flavonoids (L and M) were also isolated from most clones but were not fully identified. Their UV data suggested that they were derivatives of the same compound with different glycosylation. The UV spectrum for both was typical of a 5-deoxy dihydroflavonol with a free 7-hydroxyl, with possibly a 3-hydroxyl group substituted by a sugar molecule. Acid hydrolysis of compound (L) released glucose and in compound (M) glucose and xylose were produced.

The distribution of the caffeic acid esters and compound (L) and (M) in the callus was variable and may be a result of clonal differences. For instance only two of the new clones contained a caffeic ester but the limited variation in composition of the callus clones was in no way related to the differences in the original parental tissue.

The concentration of total phenols, leucocyanidins and (−)-epicatechin in each of the source is shown in Table 1. In the mature plant, the stem is low in total phenols whereas the cotyledon, as expected, shows a very high value. The cotyledons appear to contain

Table 1. Total phenols, epicatechin and leucocyanidin content of parts of the mature plant, seedling and callus of *Theobroma cacao*

Source of material	% Dry weight	(−)-Epicatechin mg/g	Leucocyanidin (E550/g)	Total phenols (mg equiv. pyrogallol/g)
Mature plant				
cotyledon	52.08	14.64	8.68	19.53
leaf	26.02	1.58	2.56	1.44
stem	30.87	0.30	0.91	0.37
Seedling				
stem	16.67	0.95	1.06	0.65
hypocotyl	12.30	0.63	2.09	1.04
cotyledon	47.93	5.45	8.04	7.08
root	7.88	0.36	0.49	0.66
Callus				
stem	6.89	0.28	2.55	0.87
hypocotyl	6.33	0.18	2.48	1.09
cotyledon	6.32	0.24	2.69	0.95
root	7.19	0.47	2.39	1.49

proportionately more (−)-epicatechin than leucocyanidin while the leaf does show some synthesis and/or accumulation of these compounds since they are present in much larger amounts than in the stem. In the seedling the cotyledons again had the highest level of (−)-epicatechin, leucocyanidin and total phenols (Table 1). Estimates of the same polyphenols in the callus showed some variation between callus of different sources, but these differences were not as large as the differences between the cotyledon and any other part of the plant or seedling.

DISCUSSION

The polyphenols in the mature plant parts were numerous and varied with wide differences between the leaf, stem and cotyledon. There were a number of polyphenols common to each source, most of which had been identified in previous investigations [1–6]. The compounds referred to earlier as anthoxanthins [5] were identified here as flavones or flavonols. It was in these compounds that the stem, leaf and cotyledon extracts differed so markedly. The stem contained none of these compounds, whereas the leaf contained five and the cotyledon two. The mature cotyledon contained a number of polyphenols which, even after three weeks germination, remained relatively unchanged. There was only a loss in some of the leucocyanidins and in catechin, which were presumably metabolised or translocated to other parts of the seedling during germination. The two flavonols found in the cotyledon were also present in the seedling hypocotyl and stem, probably as a result of translocation from the seedling cotyledon. The other polyphenols in the seedling stem which also included the young first leaves were less numerous than in the mature tissue of the stem and leaf. This situation was even more marked in the callus which contained a smaller number of polyphenols, with only leucocyanidins and (−)-epicatechin in common with the seedling and mature tissues. The altered pattern of polyphenols was found to be the same in all the callus clones irrespective of origin. There appeared to be no inheritance of the characteristic polyphenol pattern of each seedling part. Although there were some compounds common to callus and the intact plant, most of the polyphenols found in the callus were not detected in the plant at all; this is a common situation in tissue cultures [8–10].

EXPERIMENTAL

Plant material. The youngest part of a mature tree (variety Amelonado), grown under greenhouse conditions at Calderstones Park, Liverpool 18, provided the leaf and stem material. The leaves were at the final stage of the flush cycle and had developed to maximum size and chlorophyl content. The stem was the internode between these leaves and this was still green when used for analysis. Amelonado seeds were imported in the fresh pod from Ghana Cocoa Research Station, Tafo, Ghana. The seeds were either used directly for analysis or, after the sterilization procedure of ref. [7], provided surface sterile seedlings for a tissue culture source. At 3 weeks the seedlings were sectioned into root, cotyledon, hypocotyl and stem, and portions of these incubated onto nutrient agar medium [7] and subcultured every 6 weeks. Samples for analysis were taken at a time when the callus had been subcultured for one year.

Extraction and separation. The plant material, seeds (with testas removed) and callus were extracted in cold 70% MeOH [11]. The concd extract was separated on cellulose layers by 2D TLC, using 5% HOAc (solvent A) in one direction then *n*-BuOH–HOAc–H_2O, 4:1:5 (solvent B) in the other direction. To purify the individual compounds, a portion of the concd MeOH extract was diluted with cold H_2O, acidified with 2N HCl then extracted × 5 with equal vols of EtOAc. The combined fractions were evapd to a small vol. then applied as a single streak to Whatman No. 3MM paper and run in solvent A. After locating the bands by UV, they were eluted and rechromatographed in solvent B. The bands were further purified by chromatography in *n*-BuOH–EtOH–H_2O, 4:1:2.2 (solvent C) and finally again in solvent B.

Identification. The compounds on TLC were identified by a number of methods. These included the response to UV light [12], and a variety of spray reagents [13]. After purification the R_f value of each compound relative to rutin was determined in solvents A, B and C, using Whatman No. 1 paper. Authentic markers were used in the chromatography for the hydroxycinnamic acids, *p*-coumaric, caffeic, ferulic and sinapic acids and their derivatives, chlorogenic and isochlorogenic acid, and for (+)catechin, (−)-epicatechin, quercetin and cyanidin. The structure of the purified compounds was established by the UV spectral measurements [14]. The compounds were hydro-

lysed by incubating with 2NHCl at 100° for 30 min. After extracting the aglycone in EtOAc, it was identified using the chromatographic and spectrophotometric methods described. The acid aq. fraction was neutralized with Amberlite IR-45 resin, filtered and reduced in vol. Sugars were detected by PC and quinic acid was identified using TLC in *n*-BuOH–HCO_2H–H_2O, (4:1:5) [15].

Quantitative determinations. The amounts of (−)-epicatechin, leucocyanidins and total phenols were measured in the MeOH extract. Epicatechin was measured by the vanillin method [11] and the leucocyanidins by the method of ref. [16], the amount of leucocyanidins being expressed as total extinction at 550 nm per gram fr. wt of the organ. Total phenols were measured directly on the extract using the K titanium oxalate method [11]. Aglycones formed after hydrolysis were measured by the absorbance at the long wavelength maximum in comparison with the standard molecular extinction coefficient [17]. The sugars were estimated by standard methods [18].

Properties of partly identified polyphenols were as follows: Compound J, colour (UV) blue, (UV/NH_3) blue green. R_fs. A (0.64), B (0.80), C (0.78). λ_{max} (MeOH 241, 287, 320 nm, (NaOMe) 250, 295, 362 nm. Hydrolysis products; caffeic and quinic acid. Compound K, colour; (UV) blue, (UV/NH_3) green. R_fs; A (0.57–0.73), B (0.65), C (0.32). λ_{max} (MeOH) 233, 284, 327 nm, (NaOMe) 234, 250*sh*, 300, 370 nm. Hydrolysis products; caffeic acid and quinic acid. Compound L, colour; (UV) colourless, (UV/NH_3) bright purple. R_fs; A (0.76), B (0.52), C (0.55). λ_{max}(MeOH) 270, 305 *sh* nm, (NaOMe) 244, 287*sh*, 334, (NaOAc) 232, 248, 272, 334 nm, ($AlCl_3$) 271, 300*sh* nm, ($AlCl_3$ + HCl) 271, 300*sh* nm, (NaOAc + H_3BO_3) 232, 270, 303*sh* nm Hydrolysis products; unidentified dihydroflavonol (?) and glucose. Compound M, colour; (UV) colourless, (UV/NH_3) bright purple. R_fs; A (0.70), B (0.44), C (0.37). λ_{max} (MeOH) 271, 303*sh* nm, (NaOMe) 244, 333 nm, (NaOAc) 232, 248, 272, 333 nm, ($AlCl_3$) 271, 300*sh* nm, ($AlCl_3$ + HCl) 271, 300*sh* nm, (NaOAc + H_3BO_3) 232, 271, 302*sh* nm. Hydrolysis products; unidentified dihydroflavonol (?), glucose and xylose. Compound P, colour; (UV) blue, (UV/NH_3) blue–green. R_fs: A (0.39–0.65), B (0.68), C (0.20). λ_{max} (MeOH) 232, 290, 320 nm. (NaOMe) 260, 300, 365* (* spectra deteriorated with time). Hydrolysis products; caffeic acid and an unidentified component. Compound Q, colour; (UV) blue, (UV/NH_3) blue–green. R_fs; A (0.39–0.60), B (0.83), C (0.11). λ_{max} (MeOH 252, 290, 320 nm, (NaOMe) 250, 293, 365 nm. (Hydrolysis products, caffeic acid and an unidentified component.

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