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POLYPHENOLS OF *QUERCUS ROBUR*: ADULT TREE AND *IN VITRO* GROWN CALLI AND SHOOTS

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Abstract—Bark, wood, leaves and *in vitro* grown calli and shoots from pedunculate oak (*Quercus robur*) were extracted by aqueous methanol. The polyphenols, analysed by paper chromatography and HPLC, were principally hexahydroxydiphenoylesters–vescalagin, castalagin and pedunculagin–proanthocyanidins and pentagalloylglucose. In the adult tree, inner bark, heartwood and leaves were found to contain high amounts of hexahydroxydiphenoylesters (3.5 to 8.7% DM), mainly castalagin and vescalagin. Leaves were also rich in pedunculagin. Proanthocyanidins were characteristic of bark and leaves. Calli synthesized both hexahydroxydiphenoylesters and proanthocyanidins; the former were largely oxidized and poorly resolved by chromatography. In *in vitro* grown shoots phenolic compounds in leaves were similar to those of adult tree leaves, whilst stems did not contain pedunculagin but were rich in pentagalloylglucose.

INTRODUCTION

Wood, bark and leaves of pedunculate oak (*Quercus robur* L.) are known to accumulate high quantities of polyphenols, which are mainly hexahydroxydiphenoylesters (ellagitannins) and proanthocyanidins (condensed tannins). Both play a role in chemical defence; proanthocyanidins in leaves may be involved in herbivory deterrence [1] whereas hexahydroxydiphenoyl esters (HHDP esters) are responsible for the high durability of oak wood [2]. Hexahydroxydiphenoyl esters of oak wood are also of industrial interest. Apart from tanning, they might be used as chelating agents in the formulation of anticorrosion mixtures for the steel industry [3], or in the recovery of metal ions from waste waters [4]; they might also be used as protein-precipitating agents in the brewery industry [5] or as organoleptic agents in the aging of brandies [6] They contribute to formation of taste and colour of brandies [7, 8] and wines [9, 10] kept in oak barrels.

The main polyphenols of pedunculate oak have been characterized. In leaves [1], several HHDP esters were identified as main components: pedunculagin, castalagin, vescalagin, casuarictin, other components, including ellagic acid, (+)-catechin, (+)-gallocatechin, procyanidin B-1, procyanidin B-3 and flavonol glycosides, were also identified In wood, the main polyphenols are castalagin, vescalagin and to a lesser extent castalin and vescalin [11]. A lignan, (+)-lyoniresinol has also been identified [12]. (+)-Catechin, (+)-gallocatechin [13] and various procyanidin dimers [14] were isolated from bark

The structures of the main HHDP esters are now well established and some biosynthetic pathways have been proposed by Haslam [15, 16] and Okuda [17, 18]. Very probably, they all derive from a common precursor, the β-penta-*O*-galloyl-D-glucose which is further oxidized and hydrolysed. The biosynthesis of β-penta-*O*-galloyl-D-glucose is currently studied by Gross *et al* [19], but none of the pathways leading to HHDP esters has been definitely proved. *In vitro* cultures of calli and shoots would be particularly useful for investigating such pathways as they provide a stable, homogenous and aseptic medium whose nutritional and hormonal conditions can be easily controlled.

In this work, a general survey of pedunculate oak polyphenols is presented. The polyphenols were extracted from leaves, bark and wood of an adult tree and from *in vitro* cultures of calli and shoots. They were compared by HPLC and paper chromatography. The suitability of *in vitro* systems for biosynthesis studies is evaluated.

RESULTS AND DISCUSSION

Polyphenols of adult pedunculate oak

The majority of the extracted polyphenols are water soluble and consist of HHDP esters and proanthocyanidins (Table 1) Drastic differences of nature and amounts are observed between outer and inner bark, sapwood, heartwood and leaves Outer bark and sapwood had a relatively low polyphenol content. Relative amounts of HHDP esters and proanthocyanidins vary greatly: woods contain mainly HHDP esters, outer bark only proanthocyanidins and inner bark and leaves as much of both.

The nature of the HHDP esters varies according to the organs considered (Figs 1 and 2). Apart from outer bark, they all accumulate castalagin and vescalagin, together with some unidentified HHDP esters; the proportions of the two isomers, castalagin and vescalagin, vary according to the samples, the former being predominant in

Table 1 Polyphenol contents in barks, woods, leaves and *in vitro* grown calli and shoots of pedunculate oak (% DM)

	Total phenols	Hexahydroxydiphenoyl esters		Proanthocyanidins	
		HNO_2	Ellagic acid	Vanillin	Anthocyanidins‡
Outer bark	2 6	1 8†	tr	0 56	tr
Inner bark	8 3	3 5	0 60	4 2	+ +
Sapwood	0 74	0 40	0 07	0 16	—
Heartwood	6 7	8 7	1 63	0 03	—
Leaves	7 2	3 8	0 74	2 9	+
Calli from seedlings					
Obscurity	7 1	5 2	1 41	0 7	+
light	5 9	3 5	1 18	0 8	+
Calli from young tree					
obscurity	6 9	4 6	1 29	1 0	+
light	7 7	5 1	1 40	1 1	+
Calli from adult tree					
obscurity	10 1	6 1	1 72	2 5	+ + +
light	9 2	4 9	1 47	3 1	+ + +
Shoots from seedlings					
leaves	3 4	2 6	0 33	0 2	—
stems	4 1	2 6	0 72	0 3	—
Shoots from young tree					
leaves	3 8	3 7	0 77	0.4	tr
stems*	6 4	3 8	1 25	1 8	+ +
Shoots from adult tree					
leaves	5 9	2 7	0 78	0 3	-
stems*	10 6	3 8	0 67	1 3	+

*These stem samples are largely contaminated by calli

†The absorption maximum appears as a shoulder, the value is probably largely overestimated

‡(—) no red colour, (+) weak red colour, (+ +) medium red colour, (+ + +) strong red colour

wood and bark and the latter in leaves It was also observed that leaves differ from wood and bark by their ability to accumulate pedunculagin

Not all HHDP esters were resolved by HPLC Paper chromatograms showed in all samples analysed, a streak (A in Fig 2) which gives the characteristic coloration of HHDP esters with nitrous acid Relative to castalagin, vescalagin and pedunculagin spots, this streak may be more or less important It is particularly pronounced for inner bark, heartwood and to a lesser extent, leaves This streak corresponds probably to HHDP esters which have been oxidized and polymerized [20]

In bark and leaf samples, rich in proanthocyanidins (Table 1), the extracts contain compounds reacting with vanillin which overlap by paper chromatography the streak of HHDP esters These compounds are proanthocyanidins oligomers

Other polyphenols, diethyl ether and ethyl acetate soluble, were also identified ellagic acid is the main ether soluble phenol of sapwood, heartwood and outer bark Its amount (never over 1 mg/g DM) is always less than a tenth of that obtained after acid hydrolysis of HHDP esters, except in the case of outer bark. Hexahydroxydiphenoyl units are thus mainly present in a combined form in the ellagitannins

Gallic acid was identified in heartwood extract (0.15 mg/g) and as traces in the other samples (+)-Catechin is the main phenol in ether or ethyl acetate fractions obtained from proanthocyanidin rich samples, i e inner bark and leaves Vanillin was identified as traces in sapwood and heartwood. The presence of pentagalloylglucose could not be detected either by HPLC or by paper chromatography (Fig 2)

Polyphenols of calli and shoots grown in vitro

All the calli and shoots grown *in vitro*, whatever their origin, produce HHDP esters and proanthocyanidins (Table 1). In most samples, these tannins correspond to the majority of phenols

The polyphenols detected in calli by HPLC or paper chromatography included few HHDP esters, the main ones being vescalagin and castalagin (Fig 1d) No pedunculagin could be identified unambiguously It appears that most HHDP esters in calli are those eluted in the form of a streak by paper chromatography (Spot A on Fig 2) which probably results from oxidation of simpler molecules such as vescalagin or castalagin The HHDP esters of calli would thus be more largely prone to oxidation than those of adult tree

Polyphenols differ according to origin of the calli Calli obtained from seedlings only gave HHDP esters in form of a streak (Spot A on Fig 2) but no other spots were observed, whereas in calli obtained from young trees or adult trees many other spots could be seen (Figs 1d and 2) The agar culture media of calli obtained from seedlings were also the only ones that were deeply coloured Their extraction with successively methanol and water revealed the presence of low amounts of ellagic acid (methanol) and of the same streak of HHDP esters (water) The agar

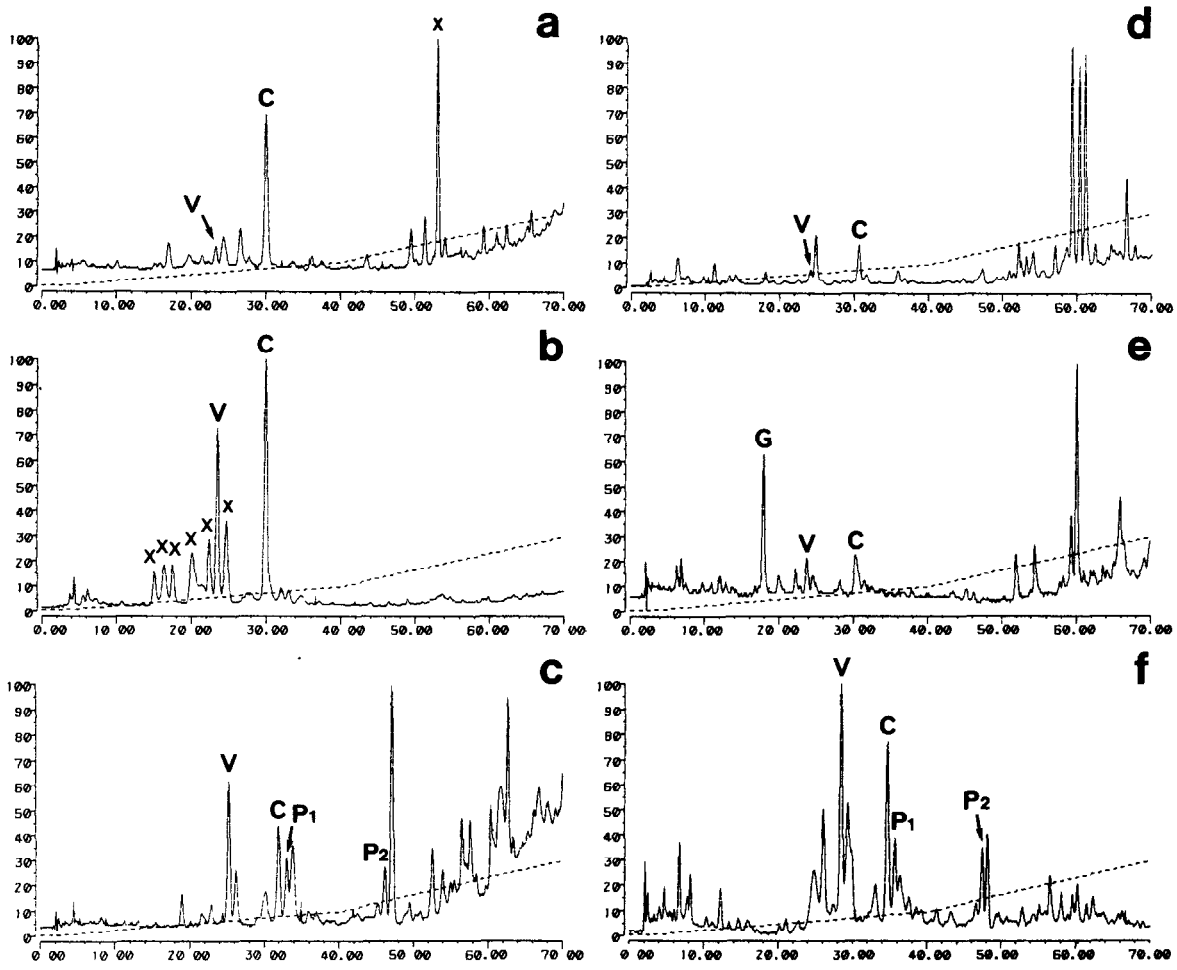


Fig 1 High-performance liquid chromatography of water-soluble polyphenols extracted from (a) inner bark, (b) heartwood, (c) leaves, (d) *in vitro* grown calli, (e) stems of *in vitro* grown shoots and (f) leaves of *invitro* grown shoots V-vescalagin; C-castalagin; P_1 and P_2-pedunculagin (two isomers); X-unknown HHDP esters, G-gallic acid

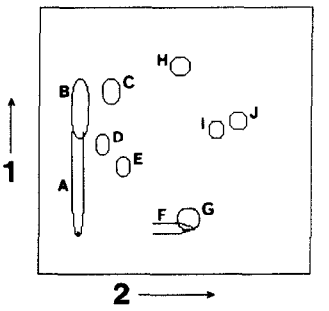
culture media of other calli or in *in vitro* grown shoots were colourless and devoid of any polyphenols. Dark brown agars have often been associated with difficulties in the *in vitro* micropropagation of woody plants [21]. The brown colour is probably due to the oxidation of polyphenols by phenoloxidases In no case can it be related to a high amount of polyphenols (Table 1). These oxidized polyphenols would result in the streak observed by paper chromatography, although oxidation during extraction cannot be excluded.

Calli obtained from adult trees produce about three times more proanthocyanidins than other calli (Table 1) whereas concentration of HHDP esters remains more or less the same whatever the sample. We can suppose that in pedunculate oak, age has a larger influence on the biosynthesis of proanthocyanidin than on that of HHDP esters As was reported for leaves harvested throughout the season [1], the proanthocyanidin concentration increases with the age of the tissue.

The stimulating effect of light on the accumulation of anthocyanins [22] by tissue cultures has been well documented. In our case, no stimulating or inhibiting effect of light could be obtained (Table 1). Some authors reported a stimulating effect of light on accumulation of proanthocyanidins [22, 23] and HHDP esters [23]; however this effect is less pronounced for HHDP esters and high variability between cultures [23] might explain why we have not observed it.

Shoots grown *in vitro* differ in several respects from calli Phenol and tannin contents are lower in shoots, except in two shoot stem samples which were largely contaminated by calli on which they had grown (Table 1). Among the compounds identified in shoot extracts, vescalagin, castalagin and pedunculagin were the main polyphenols of leaves whereas in stems, only low amounts of HHDP esters (vescalagin and castalagin) could be resolved by HPLC (Fig. 1e and f). Pentagalloylglucose was the main polyphenol of stems, as observed by HPLC and paper chromatography, and was also identified in leaves (Fig. 2). Streak A (Fig. 2) attributed to oxidized HHDP esters has a minor importance except in stem samples contaminated by calli.

Leaves of shoots contained few proanthocyanidins and were similar in that respect to young leaves of an adult



	A	B	C	D	E	F	G	H	I	J
Outer bark	+ +					tr				
Inner bark	+ + *†	+							+†	
Sapwood	+	+								
Heartwood	+ + *	+ + + *				+			tr	
Leaves	+ + *†	+	+ +						+†	
Calli	+ + + *†	tr		+	+	+	+	+	+	
Shoots										
leaves	+	+ + + *	+			+	+		+	+
stems	+			+	+	+	+ + +		+	+

*Positive reaction with HNO_2
†Positive reaction with vanillin

Fig. 2 Paper chromatography of methanol/water (4 1) extracts of barks, woods, leaves and *in vitro* grown calli and shoots of pedunculate oak, with relative abundance of the different compounds A-oxidized HHDP esters and oligomeric proanthocyanidins, B-vescalagin and castalagin, C-pedunculagin (two isomers), F-ellagic acid, G-pentagalloyl-glucose, in inner bark and leaves, I-(+)-catechin, in other samples, I-gallic acid D, E, H, J -unknown compounds (tr) traces, (+) minor compound, (+ +) average compound, (+ + +) major compound

tree grown under natural conditions [1]. No clear differences could be demonstrated between shoots obtained *in vitro* from seedlings, young trees or adult trees explants Free ellagic acid and gallic acid were both found in ether extracts of calli and shoots grown *in vitro* but no (+)-catechin could be identified

Comparison of adult tree and calli or shoots grown in vitro

Although calli produce both HHDP esters and proanthcyanidins, they differ from adult tree and *in vitro* grown shoots by their low content of pedunculagin (if any), castalagin and vescalagin, and the high degree of polyphenol oxidation Leaves of *in vitro* grown shoots accumulate vescalagin, castalagin and pedunculagin resembling therefore more the leaves of adult tree than calli (Fig 1f). The degree of polyphenol oxidation is lower than in calli and comparable to that observed in leaves or heartwood of adult tree Stems of *in vitro* grown shoots differ from adult tree in that they do not accumulate HHDP esters, but accumulate pentagalloylglucose, known as a precursor of HHDP esters [16, 17] The same observation was made with some calli of pedunculate oak [24].

The metabolism of polyphenols in pedunculate oak in thus controlled by complex regulatory mechanisms expressed during cell differentiation These mechanisms will lead to accumulation of pentagalloylglucose or to oxidation of pentagalloylglucose to form HHDP esters like vescalagin, castalagin or pendunculagin More extensive oxidation of pentagalloylglucose or of these HHDP esters might result in a complex mixture of HHDP esters of higher molecular weight which are not resolved by HPLC, or even to insoluble HHDP esters which could incrust the cell wall [25] particularly in heartwood [26]. The similarity with proanthocyanidins can be noticed, biosynthesis of proanthocyanidins is also submitted to fine regulation in pedunculate oak (Table 1) They can also be oxidized or polymerized to form insoluble polymers [27]

EXPERIMENTAL

General Castalagin, vescalagin and pedunculagin were extracted from pedunculate oak leaves and purified by chromatography on Sephadex LH 20[1] Other compounds had the following origin gallic acid and ellagic acid, Fluka, (+)-catechin, Serlabo Pentagalloylglucose was a generous gift of Dr Y Cai and Prof E Haslam

Plant material Outer bark, inner bark, sapwood and heartwood were obtained from a same branch (25 cm diameter) of a 100-year-old, freshly felled pedunculate oak The samples were air-dried Leaves were harvested on 21 July 1987 and immediately lyophilized Three types of *in vitro* grown shoots

were used (i) shoots derived from several 3-month-old seedlings, subcultured *in vitro* once, (ii) shoots derived from basal sprouts of a 5-year-old young tree, subcultured *in vitro* 7 times, (iii) shoots derived from a 150-year-old adult tree subcultured *in vitro* 3 times. The cultures were established from stem explants as described in [28] Calli were obtained from internodes taken from the 3 types of *in vitro* grown shoots

Shoots and calli were cultured on an agarized basic medium (BM) composed of half strength Murashige and Skoog [29] macro and micronutrient solutions, Fe EDTA and 88 μM sucrose. For shoot culture BM was supplemented with full strength Gresshof and Doy vitamin solution [30] and 0.44 μM *N*-6-benzyladenine (BA). For callogenesis BM was supplemented with the Morel's vitamin solution [31] concd twice, 4.4 μM BA and 2 7 μM naphthylacetic acid Cultures were kept in a growth chamber at 26 ± 1°, under 16 hr photoperiod for shoots (Sylvania Grolux fluorescent lamps 40 $\mu Em^{-1} sec^{-1}$) Calli were grown under the same photoperiod or in darkness For sampling, shoots were 2-months-old, calli 5-months-old Both were dried at 80° during 48 hr Each sample analysed was composed of 4 calli or 4 shoots grown under the same conditions

Polyphenol extraction Wood and bark were ground in a Retsch mill SM 1 (particle size less than 60 mesh) Leaves, calli and shoots were ground in a Dangoumau vibratory ball mill for 5 min Samples (250 mg) were extracted with MeOH–H_2O (4 1) (5 + 2 5 + 2.5 ml) overnight at room temp MeOH was evapd in a rotary evaporator, the aqueous solutions (made to 5 ml) were acidified to pH 2 with HCl and successively extracted by Et_2O (3 × 2 5 ml) and EtOAc (3 × 2 5 ml) Et_2O and EtOAc solutions were dried with Na_2SO_4. Solvents were evapd *in vacuo* and extracts redissolved in 0 5 ml MeOH.

Determination of polyphenols. Polyphenols were estimated in aq solns, after MeOH evapn and Et_2O extraction, because MeOH interferes in the reaction of vanillin with proanthocyanidin [32] and to eliminate ellagic acid precipitated after MeOH evapn. Total phenols were estimated by the Folin–Ciocalteu method [33], HHDP esters by oxidation with HNO_2 [34] and proanthocyanidins by reaction with vanillin [32], results were respectively expressed in gallic acid, 4,6-hexahydroxydiphenoylglucose [34] and (+)-catechin equivalents.

HHDP esters and poranthocyanidins were also estimated by formation of ellagic acid and anthocyanidins respectively in an aqueous HCl extract (0 09 ml) mixed with MeOH–6N HCl 10 1 (1 ml) in tubes with teflon-lined screw caps were placed 4 hr at 120° Ellagic acid was determined by HPLC as below with the following modification gradient duration 20 min, detection at 370 nm Red colour of anthocyanidins allowed their visual estimation

Paper chromatography. Bidimensional chromatography was run on Whatman paper no 1 (12 5 × 12 5 cm^2) at 20 ± 2°, first dimension aq. AcOH 6%, second dimension *s*-BuOH–AcOH–H_2O 14:1 5 Polyphenols *o*-dihydric phenols) were revealed by spray of $FeCl_3$–$K_3Fe(CN)_6$ [35], HHDP esters with HNO_2 [24], proanthocyanidins and (+)-catechin with vanillin/*p*-toluenesulphonic acid [36], pentagalloylglucose and gallic acid with KIO_3 [37].

HPLC analysis. HPLC analysis of Et_2O and EtOAc extracts were run on a Lichrospher RP 18 E, 10 μm, 25 cm Lichrocart (Merck) with the following elution conditions linear gradient from 0 to 90% solvent B, solvent A H_2O–MeOH–H_3PO_4 (940.50:1), solvent B MeOH/H_3PO_4 990 1, gradient duration: 30 min; flow rate 2 ml/min Detection: UV 280 nm Polyphenols were identified by co-chromatography with authentic references and by spectra (240–400 nm) obtained by on line detection with a diode-array detector Hewlett Packard 1040 A Retention times and maxima of absorption were as follows. Et_2O extracts· gallic acid, 6 6 min, 272 nm; (+)-catechin, 12 6 min, 280 nm; ellagic acid, 21 0 min, 254 nm; EtOAc extracts· pentagalloylglucose, 17 4 min, 282 nm

A Lichrospher RP 18 E, 5 μm, 25 cm Lichrocart (Merck) was necessary to analyse the H_2O extracts Elution conditions were as follows· linear gradient 0–10% solvent B from 0 to 40 min, 10–30% from 40 to 70 min Solvent A H_2O–H_3PO_4 990 1 (v/v); solvent B MeOH–H_3PO_4 990 1, flow rate 1 ml/min Retention times were the following vescalagin, 25.2 min, castalagin, 31.8 min, pedunculagin, 32 9 and 46.0. None of these compounds presented an absorption maximum between 240 and 400 nm, these feature, together with reactivity with HNO_2, was used to detect the presence of other unidentified HHDP esters.

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