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CHARACTERIZATION OF POLYPHENOLS IN CELL WALLS OF CULTURED *POPULUS TRICHOCARPA* TISSUES

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Key Word Index—*Populus trichocarpa*; Salicaceae; black cottonwood; tissue culture; plant growth regulators; polyphenol; lignin; cell wall; auxin; cytokinin.

Abstract—The amount and composition of cell wall-bound polyphenol (lignin) in cultured *Populus trichocarpa* tissues which formed numerous xylem elements (xylogenic) or no xylem (non-xylogenic) were compared. Polyphenol accounted for *ca* 15% of the dry wt of the cell wall and did not differ significantly in amount in xylogenic and non-xylogenic tissues. The syringic acid derivative, 3,4,5-trimethoxybenzoic acid, was identified as one of the oxidation products of methylated cell walls and was recovered in similar amounts irrespective of xylem formation. In contrast, lignin from xylogenic cultures contained more *p*-coumaryl alcohol derivatives and less coniferyl alcohol derivatives than lignin from non-xylogenic cultures. In this respect the lignin composition of xylogenic tissues more closely resembled that from stems.

INTRODUCTION

Lignin is a heterogeneous, random and optically inactive polymer synthesized during secondary cell wall formation in vascular plants. Guaiacyl lignin, normally found in gymnosperms, is derived primarily from coniferyl alcohol and yields vanillic acid with permanganate oxidation [1]. Syringyl lignin, on the other hand, is normally found in angiosperms and is derived from both coniferyl and sinapyl alcohol. It yields syringic acid on permanganate oxidation [1]. Fergus and Goring [2] provided microspectrophotometric evidence that syringyl residues are compartmentalized in birch wood, with guaiacyl lignin occurring primarily in xylem cells and middle lamellae with syringyl lignin restricted to the secondary walls of fibre and ray cells. Support for this hypothesis was obtained by Wolter *et al.* [3] who failed to detect syringyl residues in lignin isolated from xylogenic callus cultures of *Populus tremuloides*. Venverloo [4], on the other hand, did find syringyl residues in the lignin of xylogenic cultures of *P. nigra*, although tracheids gave a weak Mäule reaction when compared with other regions of the callus.

To further study the regulation of lignin synthesis, we have examined lignin from callus cultures of *P. trichocarpa* which, by varying the growth regulators in the culture medium, produced either numerous xylem elements or none. Our results show that the amount of syringyl lignin in callus cell walls does not vary significantly in the two tissue types.

RESULTS AND DISCUSSION

Xylem formation in callus cultures of *P. trichocarpa* varied markedly depending on the amount and type of growth regulator supplied in the growth medium (Table 1). Tissues maintained on growth medium containing 10 μM α-naphthaleneacetic acid (NAA) and 1 μM benzylaminopurine (BAP) produced large numbers of xylem elements. When the NAA concentration was increased to 100 μM, xylem elements still formed, but only in the absence of BAP supplements. No xylogenesis occurred in tissues grown with 5 μM 2,4-dichlorophenoxyacetic acid (2,4-D) as the sole growth regulator in the medium. Xylem formation was not correlated with growth, and tracheary elements were found in the most rapidly growing as well as in the most slowly growing tissues (Table 1).

Table 1. Effect of plant growth regulators on growth and xylem formation in *P. trichocarpa* callus grown for two weeks in culture.

Growth regulator treatment*	Growth ($[W-W_0]/W_0$)†	Xylem cells (cells/g fr. wt)
2,4-D (5 μM)	4.95 ± 0.67 (9)‡	0
NAA (100 μM)	9.61 ± 1.25 (9)	$1.35 \times 10^5 \pm 1.26 \times 10^4$ (5)
NAA (100 μM) + BAP (1 μM)	6.43 ± 1.49 (9)	0
NAA (10 μM) + BAP (1 μM)	1.85 ± 0.20 (9)	$6.49 \times 10^5 \pm 3.9 \times 10^5$ (5)

*Growth media were supplemented as indicated with 2,4-dichlorophenoxyacetic acid (2,4-D), α-naphthaleneacetic acid (NAA) and 6-benzylaminopurine (BAP).

†Growth is expressed as $(W-W_0)/W_0$, where W and W_0 are the final and initial fr. wts of the explant, respectively.

‡Mean values expressed ± s.e.m. (n).

The composition of callus cell walls varied with growth regulator treatment. The lignin content of callus, which was always less than that found in two-year-old stem tissue, was correlated with the presence of BAP in the growth medium (Table 2). Cell walls from tissues grown with BAP supplements contained *ca* 15% more lignin

Table 2. Partial composition of cell walls of *P. trichocarpa* stem and callus tissues

Tissue	Growth regulator treatment*	Xylem formation	Polyphenol	Cellulose (mg/g dry wt)	Protein
Stem	—	+	196±3 (6)†	505±23 (3)	37±2 (3)
Callus	2,4-D (5 μM)	−	135±3 (6)	307±12 (3)	323±11 (3)
Callus	NAA (100 μM)	+	134±2 (6)	315±9 (3)	255±7 (3)
Callus	NAA (100 μM)+BAP (1 μM)	−	157±3 (6)	335±27 (3)	309±5 (3)
Callus	NAA (10 μM)+BAP (1 μM)	+	156±3 (6)	297±17 (3)	276±5 (3)

*Growth media were supplemented as indicated.
†Mean values expressed ± s.e.m. (*n*).

than cells grown without BAP, whereas lignin content appeared to be independent of the type and amount of auxin in the growth medium. Callus cell walls also contained less cellulose than stem wood (Table 2), but there was no significant difference in cellulose content with different growth regulator treatments.

The lesser amounts of lignin and cellulose in callus cell walls as compared with stem were due primarily to differences in protein, which accounted for *ca* 30% of callus cell walls but only 4% of stem wood (Table 2). The protein content of cell walls from xylogenic tissues was less than that from non-xylogenic tissues, although some of the protein in callus walls might be attributable to contaminating cytoplasmic protein. Lignin, cellulose and protein together accounted for 70–80% of the cell wall, and the remainder is assumed to consist of hemicellulose and xylosans [5].

The large amount of protein associated with callus cell walls, if solubilized by acetyl bromide treatment, would have led to an overestimate of lignin. However, because of the large difference in the extinction coefficients (280 nm) of protein and polyphenol, protein could have accounted for at most 20% of the lignin values.

Since xylem lignin is believed to lack syringyl residues, it was of interest to compare the phenolic constituents of lignin in xylogenic and non-xylogenic callus tissues. GC and mass spectrometry of silylated permanganate oxidation products of methylated walls revealed *p*-hydroxybenzoic acid (**1**), *p*-methoxybenzoic acid (**2**), protocatechuic acid (**3**), vanillic acid (**4**), veratric acid (**5**) and 3,4,5-trimethoxybenzoic acid (**7**) (Table 3). Since cell walls were methylated prior to oxidation, the presence of free phenols among the oxidation products indicates the occurrence of aromatic ether linkages in the lignin polymer. Although the low yield of free phenols (**1**, **3**, **4** and **6**) relative to the corresponding methoxy derivatives (**2**, **5** and **7**) (Table 3) suggests a relatively low abundance of aryl ether linkages, this result was more likely due to oxidation of free phenols by permanganate. This conclusion is supported by the fact that the phenolic monomers recovered accounted for only 12–19% of the polyphenol solubilized by acetyl bromide (Table 3).

Table 3. Quantitation by gas chromatography of silylated phenolics obtained from permanganate oxidation of methylated cell walls of *P. trichocarpa* stem and callus tissue

Tissue (treatment)*	Compound† (nmol/mg polyphenol)							Ratio‡ S:V:P	Total§ (mg/mg polyphenol)
	1	**2**	**3**	**4**	**5**	**6**	**7**		
Stem	32±5‖	196±17	23±2	189±12	239±13	nd¶	73±2	0.17:1:0.53	0.12
Callus (5 μM 2,4-D)	48±5	47±3	29±2	76±2	530±15	nd	143±12	0.24:1:0.16	0.15
Callus (100 μM NAA)	82±3	133±12	29±5	307±16	395±23	nd	203±14	0.26:1:0.28	0.19
Callus (100 μM NAA + 1 μM BAP)	19±1	53±2	43±5	163±14	504±16	nd	119±4	0.18:1:0.11	0.15
Callus (10 μM NAA + 1 μM BAP)	34±4	102±4	29±5	175±14	318±7	nd	130±3	0.29:1:0.31	0.13

*Growth media were supplemented as indicated.
†Compounds are as follows: **1**, *p*-hydroxybenzoic acid; **2**, 4-methoxybenzoic acid; **3**, 3,4-dihydroxybenzoic acid (protocatechuic acid); **4**, 4-hydroxy-3-methoxybenzoic acid (vanillic acid); **5**, 3,4-dimethoxybenzoic acid (veratric acid); **6**, 3,5-dimethoxy-4-hydroxybenzoic acid (syringic acid); **7**, 3,4,5-trimethoxybenzoic acid.
‡Ratio of syringyl (s, **6**+**7**); vanillyl (v, **4**+**5**); and *p*-coumaryl (p, **1**+**2**) residues. Protocatechuic acid (**3**) was not included in calculation of ratios.
§M_rs of *p*-coumaryl alcohol (**1** and **2**), caffeoyl alcohol (**3**), coniferyl alcohol (**4** and **5**) and sinapyl alcohol (**7**) were used to calculate total phenolic recovered.
‖Mean values expressed ± s.e.m. ($n = 3$).
¶Not detected.

Moreover, permanganate oxidation of cell walls without prior methylation gave very low yields of phenolic monomers (not shown).

The identification of 3,4,5-trimethoxybenzoic acid (7) among the cell wall oxidation products indicates the presence of syringyl lignin. The yield of 7 was greater in callus than in stem, but there was no relationship between xylem formation and the amount of 7 recovered from callus (Table 3). On the other hand, xylogenic calli showed lower levels of coniferyl residues and higher levels of *p*-coumaryl residues than non-xylogenic calli, and in this respect more closely resembled the phenolic constituents of stem wood (Table 3). These results do not support the work of Wolter *et al.* [3] who failed to detect syringyl lignin in callus of *P. tremuloides*. This discrepancy may be due to species-specific differences or, more likely, to differences in the methods employed. In the aforementioned work, oxidation products were methylated for GC analysis. In our experience, methylation gave low yields owing to solubility problems and syringyl residues were not detected. Much higher yields were obtained with silylation and substantial amounts of 3,4,5-trimethoxybenzoic acid were consistently found.

In all tissues we routinely detected protocatechuic acid (3) which accounted for *ca* 3% of the recovered phenolics (Table 3). This result was unexpected since it indicates that caffeic acid or caffeoyl alcohol is incorporated into lignin. The recovery of 3 may also indicate the presence of other phenolics in cell walls, or may be an artifact of our procedure.

Because tracheids represented a subpopulation of callus cells, our results showing the presence of syringyl lignin in xylogenic callus do not prove that xylem lignin contains sinapyl residues, and hence cannot be used to refute the hypothesis that syringyl lignin is compartmentalized. Xylem accounted for *ca* 20% of the total cell number in the most xylogenic calli. Thus, most of the cell wall polyphenol of callus is not xylem lignin and may also include other phenolic polymers such as suberin [6]. We did find, however, that lignin in xylogenic callus contained elevated levels of *p*-coumaryl alcohol residues, typical of lignin in the Salicaceae [4]. Thus, *p*-coumaryl alcohol may be a preferred substrate for xylem lignin in *P. trichocarpa*.

EXPERIMENTAL

Plant tissues. Stem cuttings of *Populus trichocarpa* T. & G. were soaked in H_2O for three days, rooted in soil and grown in large plastic pots in a greenhouse. Stem tissue used for lignin analysis was taken from the main trunk of a two-year-old tree. Callus cultures were initiated from young branches, disinfected with NaOCl, grown on Murashige and Skoog [7] basal medium solidified with 1% (w/v) Bacto-agar (Difco) and supplemented with 5 μM 2,4-D. Established cultures were subsequently maintained on basal medium supplemented with 2,4-D, benzylaminopurine (BAP), and/or NAA as indicated, and subcultured every 6 weeks. Cultured tissues were grown at 27° under constant fluorescent lighting (7–9 $W m^{-2}$ of photosynthetically active radiation). Growth of cultured tissue is expressed as $(W-W_0)/W_0$, where W and W_0 are the final and initial fr. wts of the explant, respectively.

Xylem cell counting. The number of tracheary elements were counted at × 160 magnification after chromic acid maceration [8].

Cell wall purification. Plant tissue (2 g) was ground at high speed in 4 ml of MeOH using a Brinkman Polytron homogenizer. The insoluble residue was collected by centrifugation and extracted for 30 min with mixing using each of the following solvents in succession: MeOH, Me_2CO, EtOAc, MeOH–H_2O (1:1) and (19:1), and H_2O. Cell walls in H_2O were lyophilized and used for all subsequent analyses.

Cell wall analysis. Total polyphenol content was determined by 4 at 280 nm after treatment of cell walls with AcBr [9]. Dehydroconiferyl alcohol polymerizate (DHP) was synthesized from coniferyl alcohol with horseradish peroxidase [10] and used as a standard. Cellulose content was determined as described in ref. [5]. Nitrogen was measured by a microkjeldahl procedure [11]. The protein content was calculated by multiplying the nitrogen content by 6.25.

Lignin analysis. Purified cell walls in dioxane–Me_2CO (1:1) were methylated with CH_2N_2–Et_2O, taken to dryness under an N_2 stream, and then oxidized in alkaline $KMnO_4$ as modified from ref. [12]. To 50 mg of methylated cell walls were added in order 3.75 ml *t*-BuOH–H_2O (3:1), 3.75 ml 0.5 M NaOH, 9.4 ml of 0.06 M $NaIO_4$ and 3.75 ml of 0.03 M $KMnO_4$. The mixture was incubated for 16 hr at 82° in a shaking water bath. The reaction was stopped by adding 1 ml EtOH, cleared by centrifugation and the supernatant extracted twice with 10 ml of Et_2O. The aq. phase was brought to pH 7 with 2 M H_2SO_4, 1 ml of 30% H_2O_2 was added, and the mixture incubated at 50° for 10 min. MnO_2 (20 mg) was then added, the mixture shaken until gas evolution ceased and the ppt removed by centrifugation. The supernatant was brought to pH 2 with conc H_3PO_4 and extracted × 3 with EtOAc. The EtOAc fractions were pooled and dried *in vacuo*. The residue was taken up in EtOAc and silylated with *N,O-bis*-(trimethylsilyl)trifluoroacetamide (BSTFA). GC of silylated oxidation products was accomplished on a 1.7 m × 16 mm i.d. glass column packed with OV-101 using a FID detector. Carrier gas was N_2 (30 ml/min) and the oven temp was 140° for the first two min and then increased at 15°/min. Recoveries of oxidation products were estimated from parallel samples to which known amounts of *p*-coumaric acid, vanillic acid and syringic acid were added prior to cell wall methylation. Conditions for GC-MS were the same except He was used as the carrier gas. MS of lignin oxidation products were obtained at 70 eV and compared with those of synthetic standards.

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