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Differences in chemical constituents between vascular bundles and nonvascular bundles of cacao (*Theobroma cacao* L.) hull

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Abstract Cacao (*Theobroma cacao* L.) hulls were physically separated into vascular bundles (VBs) and nonvascular bundles (NVBs) to investigate their chemical compositions and the structural features of abundant polyphenolic compounds. Glucose content was determined to be 21.4% for VBs and 17.5% for NVBs, together with xylose content as 13.1% for VBs and 2.8% for NVBs. In addition, uronic acid in NVBs (12.9%) was much higher than that in VBs (5.2%). The content of total (poly)phenolic compounds (35.9%–39.1%) quantified as Klason residues (KRs) and acid-soluble phenolic compounds (ASPs) were similar in both cell types, although there were great differences in the structural characteristics of polyphenolic compounds. The pyrogram of VBs clearly showed high intensities of guaiacol and 4-vinylguaiacol together with low intensities of catechol and 4-methylcatechol. On the other hand, that of the NVBs showed opposite trends. These results were confirmed by alkaline nitrobenzene oxidation based on total yields of vanillin and syringaldehyde. Therefore, the accumulation of various polyphenolic compounds in cacao hulls relies strongly on the cell type and is correlated with the development of a secondary wall.

Key words *Theobroma cacao* L. · Cacao hull · Vascular bundles · Polyphenolic compounds · Lignin

Introduction

The effective utilization of cacao hull as biomass is hampered by a lack of knowledge concerning its major polyphenolic compounds. Only a few studies[1,2] have investigated polyphenolic compounds, including lignin in cacao hull, owing to the fact that there are no proper methods to determine them quantitatively. There are also no reports so far about the structural characteristics of polyphenolic compounds including lignin in vascular (VB) and nonvascular (NVB) bundles of cacao hull. The various cell types have walls that differ in morphological structure, stage of differentiation, and rate and degree of digestion by microorganisms.[3] Hence, it is desirable to reduce this complexity by studying individual cell types isolated from tissues; however, few cell types have been isolated to refine the botanical samples of the walls being analyzed (*Sorghum bicolor* cv. Atlas,[3,4] *Dactylis glomerata* L. and *Panicum virgatum* L.,[5] *Lolium perenne* cv. Perma and *Lolium multiflorum* cv. RvP[6]).

The isolation procedures used for leaves and stems of grasses (*Dactylis glomerata* L.,[5] *Sorghastrum nutans* L. Nash[7]) and those for the leaves[8] and stems[9] of kale (*Brassica oleracea* L.) involved maceration and repeated sieving of tissues in liquid media. Kawamura et al.[10] also applied a pectinase pretreatment. Wilson et al.[3] pointed out that these procedures lead to loss of cell solubles and fine wall particles and to modification of wall characteristics with an enzyme pretreatment. Therefore, physical separation of fresh material without liquid media to give two cell types such as VBs and NVBs has the advantage of minimizing the risk of changes in cell wall composition and chemical characteristics during isolation. In this study, a mechanical separation of cell types was applied to obtain valuable information not only on the differences in chemical constituents but also in the structural features of the major polyphenolic

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compounds in various cell types from cacao (*Theobroma cacao* L.) hulls.

Materials and methods

Separation of cells

Cacao hulls were collected from the Rajamandala Cacao Plantation at Rajamandala in West Java (Indonesia). The mucilage was removed by scraping with a razor blade, and the VB zone was collected by cutting under magnification. Outer layers (epidermis and sclerenchyma) were stripped off using fine tweezers, and the VBs, which were easily separated from the outermost layers, were scraped off. The NVBs were cut and trimmed off in slivers. Isolated cells were dried over P_2O_5 in a vacuum oven overnight at 40°C and ground finely for 30 min using an MM 200 Vibratory Ball Mill (Retsch, Germany). The sample was embedded in Technovit 7100 resin (Kulzer, Germany) and was sliced at a thickness of 10–20 μm with a hand microtome. Observations were carried out with an optical microscope (model BM-50, Olympus, Tokyo, Japan) and a stereo-microscope (model SZH-10; Olympus, Tokyo, Japan). The isolated VBs were observed after staining with safranin.

Chemical analysis

The neutral sugars in a sulfuric acid hydrolysate were analyzed as their alditol acetates[11] using myoinositol as an internal standard on the Shimadzu GC-18 gas chromatograph (TC-17 capillary column 30 m × 0.25 mm i.d.; column temperature 200°C, injection and detector temperature 250°C, detector FID). Uronic acids in the sulfuric acid hydrolysate used for alditol acetate analysis were determined spectrophotometrically using the method of Scott.[12] Nitrogen content was determined using a CHN microanalyzer (Perkin Elemer 240). Klason residue was determined gravimetrically by TAPPI standard T-222om-88; and acid-soluble phenolic components were determined by the Shimadzu UV-200 spectrometer at 205 nm using 110 $g^{-1}1cm^{-1}$ extinction coefficient.[13] The sample (30 mg) was subjected to alkaline nitrobenzene oxidation,[14] and the products were quantified using ethylvanillin as an internal standard by the Shimadzu GC-17 gas chromatograph (NB-1 capillary column 30 m × 0.25 mm i.d.; injection and detector temperature 300°C; detector FID). Column temperature was maintained for 10 min at 180°C and then programmed to 280°C in 5°C min^{-1} increments.

Pyrolysis-GC/MS

Samples (20–50 μg) were pyrolyzed for 4 s at 500°C using a Microfurnace Pyrolyzer (PYR-4A; Shimadzu). The pyrolyzer was interfaced (interface temperature 270°C) with a gas chromatography-mass spectrometry (GC-MS) system consisting of a Shimadzu GC-17A gas chromatograph coupled to a Shimadzu QP-5000 mass spectrometer. The products were separated using an NB-1 capillary column. The column temperature was maintained at 50°C for 1 min and then programmed at 5°C min^{-1} to 270°C. The peaks of the pyrolysis products were identified by comparing the retention times and mass spectra with those of authentic compounds and those in previous reports.[15,16]

Results and discussion

The VB and NVB zones are shown in Fig. 1. Tracheary elements (TEs) composed of secondary walls with spiral thickening were observed in the VBs (Fig. 1c). The TEs had split down from top to bottom (Fig. 1a), and they were clustered and embedded (Fig. 1b).

The neutral sugar compositions of VBs and NVBs are given in Table 1. It can be inferred that most of the glucose was derived from cellulose. Xylose and mannose would be components of noncellulosic wall polysaccharides, such as *O*-acetyl-4-*O*-methylglucuronoxylans and glucomannans, respectively. Rhamnose, arabinose, and galactose probably originated from pectic substances, such as arabinogalactans, galactans, and rhamnogalacturonans.[17] Glucose was determined as 21.4% for VBs and 17.5% for NVBs. In addition, VBs were rich in xylose (13.1%) but less so in rhamnose (0.5%), arabinose (1.5%), and galactose (1.7%), whereas NVBs had slightly higher concentrations of rhamnose (0.9%), arabinose (2.5%), and galactose (1.9%) and lower levels of xylose (2.8%) than VBs. Uronic acid was much higher in NVBs (12.9%) than in VBs (5.2%) (Table 2). The sugar composition results suggest that NVBs contain higher amounts of pectic substances than VBs. In addition, VBs would have more secondary wall development than NVBs, based on the results in previous reports[6,18–21] that secondary wall thickening is characterized by high contents of glucose and xylose but a low content of uronic acid. The results were similar to those reported by Hatfield et al.[4] They separated sorghum (*Sorghum bicolor* cv Atlas) into sclerenchyma, a VB zone, and pith parenchymal cell walls and found that the latter contained higher proportions of arabinose, galactose, rhamnose, and uronic acid than was found in sclerenchyma or the VB zone walls. It is striking that the xylose contents among cell types in sorghum were little different, ranging from 28% to 33%[4]; however, in cacao hulls the xylose contents were significantly different for VBs (13.1%) and NVBs (2.8%). The large amount of xylose in VBs suggests that the content of noncellulosic wall polysaccharides in VBs is much higher than that in NVBs. The noncellulosic wall polysaccharides would be composed mainly of *O*-acetyl-4-*O*-methylglucuronoxylans, not glucomannans, owing to low levels of mannose in VBs. Furthermore, the arabinose/xylose ratio was approximately 1:9 for VBs and 1:1 for NVBs. A similar result was reported in previous reports[6,18–21] that stated that the arabinose level was low in fiber cells (with secondary walls) but was present in much higher proportions in mesophyll cells (only with primary walls) and that the arabinose/xylose

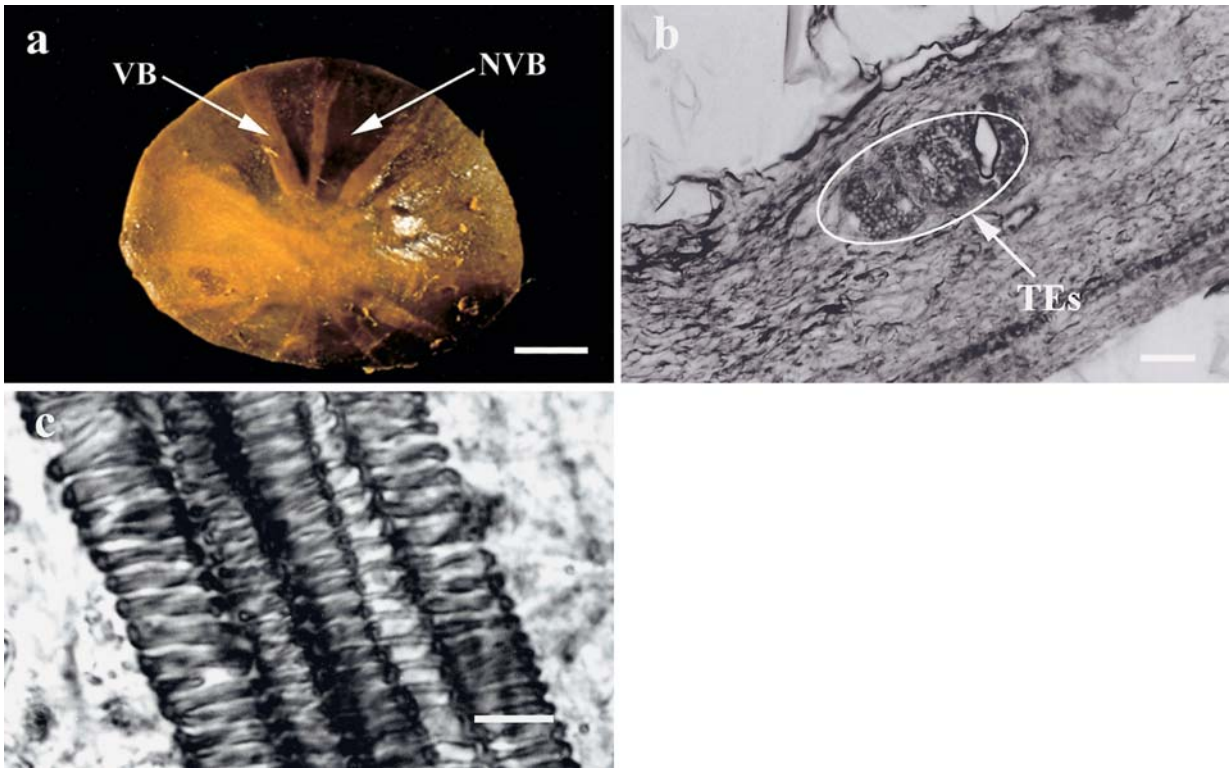

Fig. 1. Vascular bundles (*VB*) and nonvascular bundles (*NVB*). **a** Top-projection of cacao hull. bar 2 mm. **b** Cross section of cacao hull. Bar 100 μm. *TEs*, tracheary elements. **c** Tracheary elements of VB. *Bars* **a** 2 mm; **b** 100 μm; **c** 5 μm

Table 1. Anhydrous neutral sugar composition of vascular bundles and nonvascular bundles

Monosaccharides (%)	VBs	NVBs
Rhamnose	0.5	0.9
Arabinose	1.5	2.5
Xylose	13.1	2.8
Mannose	1.0	1.6
Glucose	21.4	17.5
Galactose	1.7	1.9
Total neutral sugars	39.2	27.2

VBs, vascular bundles; NVBs, nonvascular bundles

Table 2. Chemical composition of vascular bundles and nonvascular bundles

Chemical composition (%)	VBs	NVBs
Uronic acid	5.2	12.9
Total neutral sugar	39.2	27.2
Nitrogen content	1.7	1.8
Acid-soluble phenolics (ASP)	2.5	3.9
Klason residues (KR)	36.6	32.0
Total (poly)phenolics (ASP + KR)	39.1	35.9

ratio was around 1.0:1.5 for mesophyll cells and 1.0:7.0 for fiber cells. These data suggest that the higher xylose/arabinose ratio in VBs than in NVBs is correlated with the increase in the portion of secondary wall.

The quantity of Klason residue (KR) was not significantly different between VBs (36.6%) and NVBs (32.0%) (Table 2), together with 2.5% and 3.9% of acid-soluble phenolics (ASPs) in VBs and NVBs, respectively. However, there were significant differences in the analytical pyrograms for VBs and NVBs (Figs. 2, 3). High intensities of guaiacol and 4-vinylguaicol, which are particularly characterized by guaiacyl units of lignin,[15,16] appeared, whereas catechol and 4-methylcatechol were of low intensity, which might be derived from the B-ring of condensed tannins[22] in VBs (Fig. 2). On the other hand, the pyrogram of the NVBs showed that high intensities of catechol and 4-methylcatechol were detected, together with low intensities of guaiacol and 4-vinylguaicol (Fig. 3). In addition, appreciable intensities of phenol, *m*-cresol, and *p*-cresol appeared in both VBs and NVBs. The intensities of 2,6-dimethoxyphenol and 4-vinyl-2,6-dimethoxyphenol, which are assumed to be the main products of syringyl units of lignin,[15,16] were negligible in both VBs and NVBs. These results suggest that differences in accumulated polyphenolic compounds are strongly dependent on the cell type. Based on the pyrolysis results, it can be deduced that the lignin content in VBs is higher than in NVBs. This was confirmed by alkaline nitrobenzene oxidation (Table 3).

A relatively high yield of vanillin (0.24 mmol/g) was obtained, together with a low yield of syringaldehyde (0.03 mmol/g) from VBs. However, these compounds in the NVBs were not detected in alkaline nitrobenzene oxidation products. Although it was difficult to determine the lignin

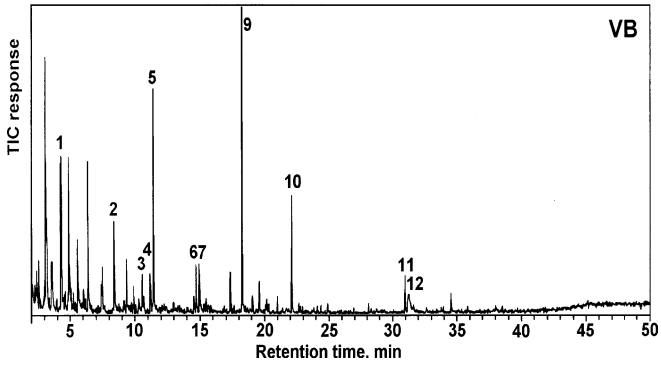

Fig. 2. Pyrogram of vascular bundles (*VB*). *1*, furfural; *2*, phenol; *3*, *m*-cresol; *4*, *p*-cresol; *5*, guaiacol; *6*, 4-methylguaiacol; *7*, catechol; *9*, 4-vinylguaiacol; *10*, eugenol; *11*, caffeine; *12*, theobromine or theophylline

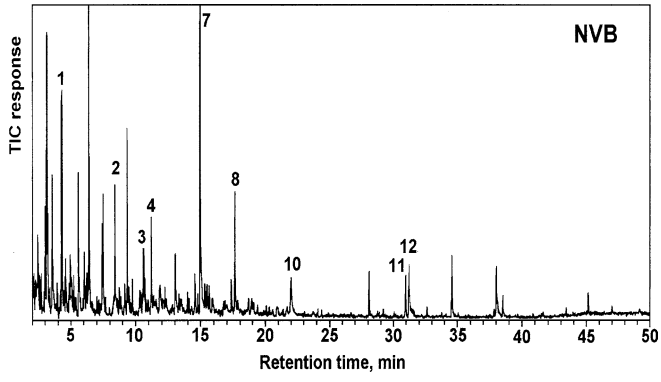

Fig. 3. Pyrogram of nonvascular bundles (*NVB*). *1*, furfural; *2*, phenol; *3*, *m*-cresol; *4*, *p*-cresol; *7*, catechol; *8*, 4-methyl catechol; *10*, eugenol; *11*, caffeine; *12*, theobromine or theophylline

Table 3. Alkaline nitrobenzene oxidation products of vascular bundles and nonvascular bundles

Nitrobenzene oxidation product	Vbs (mmol/g)	NVBs (mmol/g)
4-Hydroxybenzaldehyde	0	0
Vanillin	0.24	0
4-Hydroxybenzoic acid	0.00	0
Syringaldehyde	0.03	0.01
Vanillic acid	0.02	ND
Syringic acid	0	ND
Total yield of S + V	0.27	0.01
S/V molar ratio	0.11	–

ND, not detected; S + V, syringaldehyde + vanillin; S/V, syringaldehyde/vanillin

content quantitatively in polyphenolic compounds, the lignin content in VBs would be higher than in NVBs based on the results of pyrolysis and alkaline nitrobenzene oxidation. Moreover, the total yield of alkaline nitrobenzene oxidation products in both cells was significantly low based on the total amount of (poly)phenolic compounds (KR + ASP). These results suggest that not only lignin but also nonlignin materials such as tannin-like compounds contribute to polyphenolic compounds in both VBs and NVBs.

The syringyl/guaiacyl molar ratio (S/V ratio), which was calculated from the molar yields of vanillin and syringaldehyde, was extremely low (0.11), even in VBs, compared with general wood species. These results suggest that the cacao hull might be at an early stage of development,[23] even if the cacao pod is fully matured, and VBs have much more secondary wall development than NVBs. The nitrogen contents (Table 2) of VBs (1.7%) and NVBs (1.8%) were similar, and the alkaloid compounds (e.g., theobromine, theophylline, caffeine[24]), which have four nitrogen atoms in a molecule, were detected in both VBs and NVBs (Figs. 2, 3). Therefore, it is difficult to use the nitrogen content as a nitrogen-to-protein conversion factor. According to our unpublished data, cacao hull contained 9.9% lipid, 9.3% ash, and so on. It suggests that other compounds as minor components can be compensated for by unidentified parts of VBs and NVBs.

Conclusions

The results reported here provide strong evidence that there are significant differences in the chemical compositions and structural features of polyphenolic compounds for VBs and NVBs. The larger amounts of xylose with low amounts of uronic acid and high intensities of guaiacol and 4-vinylguaiacol in the VB pyrograms, together with opposite trends in NVBs can be explained in terms of different stages of secondary wall development for VBs and NVBs. In addition, the low S/V ratio (0.11) in VBs indicates that even VBs would be at an early stage of development of lignification. Therefore, it can be deduced that all the differences in VBs and NVBs may be related to the less lignified proportion and the less well developed secondary walls.

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