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The Relationship between Lignin and Morphological Characteristics of the Tracheary Elements from Cacao (*Theobroma cacao* L.) Hulls

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Tracheary elements (TEs) were physically separated from the hulls of cacao pods (*Theobroma cacao* L.). Their morphological features were extensively investigated with scanning electron microscopy and chemical characterization. Spiral TEs were covered with a thin layer of primary wall that had a web-like structure on its outer surface. These TEs had a spiral circularity diameter of 8.2 ± 0.6 μm and an estimated secondary wall thickness of about 2.1 ± 0.2 μm. Polarized microscopy analysis revealed that the cellulose microfibrils were aligned parallel to that thickening. Lignin content was 36.1%, with a 0.13:1.00 molar ratio of syringyl to guaiacyl units and a 1.09:1.00 molar ratio of erythronic acid and threonic acid. Total yields of the alkaline nitrobenzene oxidation and ozonation products were 324.5 and 148.8 μmol g^{-1} of extract-free TEs, respectively. Based on these morphological and lignin characteristics, we conclude that fully ripened cacao hulls exhibit the same features of secondary wall thickening as those seen at an earlier stage.

Keywords: lignin, nitrobenzene oxidation, ozonation, tracheary element, *Theobroma cacao* L.

Tracheary elements (TEs), components of the xylem vessels, are equipped with elaborately patterned secondary cell walls under their primary cell walls, which present a characteristic appearance. Compared with the primary cell walls, the secondary walls are relatively thicker and stout due to high cellulose content and lignin and hemicellulose deposits, which provide the TEs with sufficient strength to withstand high negative pressure within the vessels (Ye, 2002; Oda et al., 2005; Jung and Park, 2007). This secondary wall system consists of a first-order framework (the helical system) and a second-order framework (secondary walls deposited between the gyres). The latter type occurs in diverse forms, appearing at various times throughout TE development. This two-phase wall deposition accounts for a wide range of pit and reticulum patterns, as well as several types of perforation plates (Bierhorst and Zamora, 1965). Although distinctive vessels from various ferns and other species have been well-described in terms of their morphological features, these have not been fully explored in plants such as cacao (*Theobroma cacao* L.).

Tracheary elements are characterized by the structural features of secondary wall thickening. This is accompanied by lignification, which can, therefore, serve as a marker of TE differentiation (Fukuda and Komamine, 1982; Fukuda, 1997). Lignin is a complex polymer of hydroxylated and methoxylated phenylpropane units, linked via oxidative coupling. It is a common chemical and morphological component of the tissues in higher plants, including pterodophytes and spermatophytes (gymnosperms and angiosperms), where it typically occurs in vascular tissues that are specialized for liquid transport and mechanical strength (Fengel and Wegener, 1984). The amount of lignin present among species and within individual plant parts is quite variable. Moreover, secondary cell walls show a significant degree of fluctuation in their lignin homogeneity over a life cycle. Three kinds of monolignol units, *p*-coumaryl alcohol, coniferyl alcohol, and sinapyl alcohol, are incorporated at different stages of cell wall formation, and are deposited into various morphological regions of that developing cell wall. That is, the structural features of lignin from early and late secondary walls can be quite variable because of an increase in the structural heterogeneity within a growing lignin polymer. At the immature stage, lignins exist as highly formed condensed types, e.g., β-5 (phenylcoumaran), β-1 (diarylpropane), β-β (pinoresinol), and 5-5 (biphenyl); in comparison, an arylglycerol-β-aryl ether (β-O-4) linkage is the main form at maturity (Terashima et al., 1989; Jin et al., 2003; Kim et al., 2004).

Even though the formation of secondary wall thickenings is saliently affected by lignification (Nakashima et al., 1997), TE lignin characteristics have not yet been analyzed in detail. Previous studies have focused primarily on determining only lignin content or observing lignified cells by microscopy (Fukuda and Komamine, 1982; Nakashima et al., 1997; Kaliamoorthy and Krishnamurthy, 1998). Nevertheless, an analysis of the structural features of lignin would allow deeper insight into the relationship between secondary wall formation and lignification. Here, we performed alkaline nitrobenzene oxidation (NBO) and ozonation analyses because these are the most comprehensive among various chemical degradation methods for qualitatively and quantitatively determining the building units of lignins. Indeed, it is generally difficult to obtain or isolate the

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amount of TEs necessary for chemical evaluation. However, because the vascular bundles of cacao hulls comprise only the spiral form, these were selected for the current research, which was undertaken to improve our understanding of the extensive morphological features and unique lignin characteristics of spiral TEs.

MATERIALS AND METHODS

Sample Preparation

Hulls of cacao (*Theobroma cacao* L.) pods were obtained from the Rajamandala Cacao Plantation at Rajamandala in West Java (Indonesia). They were soaked in water for 3 d with constant stirring to remove mucilages. After the excess water was removed, the mucilages were scraped off with a razor blade. Tracheary elements (TEs) that could be easily separated with forceps from the most outer layers, the epidermis and sclerenchym, were collected by cutting the tissue under magnification. These isolated TEs were finely ground for 30 min in an MM 200 Vibratory Ball Mill (Retsch, Germany), then extracted twice with 70% acetone (v/v) at ambient temperature for 12 h. The extract-free TEs were dried over P_2O_5 in a vacuum oven at 40°C overnight.

Observations by Stereo Microscope and Scanning Electron Microscope (SEM)

Samples were viewed horizontally with a stereo microscope (Olympus Model SZH-10; Tokyo, Japan). Following the general steps of fixation and dehydration (Robards and Wilson, 1993), we attached the separated TEs with double-sided tape to stubs, which were then dried over P_2O_5 in a vacuum oven at 40°C overnight. Afterward, the stubs were sputter-coated with Pt and observed with an Hitachi S-4000 SEM (Tokyo, Japan), using an accelerating voltage of 10 kV. To observe their web-like primary walls, we broke down most of the phenolic compounds by soaking several hulls in 150 mL of distilled water before treating them four times with 1 g of sodium chlorite ($NaClO_2$) for 1 h at 70 to 80°C, followed by 0.2 mL of acetic acid (Chung et al., 2003). Data for the diameters and thicknesses of these isolated TEs were analyzed for mean values ± SD from 100 measurements (n = 10 hulls).

Lignin Determination

Extract-free samples (1 g each) were treated with 72% (w/w) sulfuric acid (10 mL), then homogenized by stirring with a glass rod. The mixtures were kept for 3 h at ambient temperature, with frequent stirring. Each reaction mixture was diluted with distilled water to achieve 3% sulfuric acid, then autoclaved for 30 min at 121°C. After hydrolysis, the samples were filtrated through a fritted glass filtering crucible (1G4) and dried overnight at 105°C. Klason lignin was gravimetrically determined while acid-soluble lignin was analyzed on a Shimadzu UV-200 Spectrometer (Tokyo, Japan) at 205 nm, using 110 g^{-1} cm^{-1} L for the extinction coefficient (Schöning and Johansson, 1965).

Alkaline Nitrobenzene Oxidation (NBO) Analysis

Aromatic features were examined via NBO according to a procedure modified from that of Iiyama and Lam (1990). Samples (40 mg each) were placed in a stainless steel reactor with 4 mL of 2 M NaOH and 0.25 mL of nitrobenzene, then kept in a heating block for 2 h at 170°C. The reactor was cooled in iced water and 0.1 mL of ethylvanillin (250 mg per 50 mL) was added as an internal standard. The reaction mixture was extracted three times with dichloromethane, and the aqueous phase was acidified with 4 M HCl to pH 1. This acidified solution was extracted twice with dichloromethane and ether. The organic phase was then evaporated to dryness and trimethylsilylated with N,O-bis(trimethylsilyl)acetamide (BSA) at 105°C for 10 min. The products were analyzed on a Shimadzu GC-17A gas chromatograph (Tokyo, Japan) (conditions: NB-1 capillary column, 30 m × 0.25 mm i.d.; injector and detector temperature, 300°C; detector, FID). The column temperature was raised from 180 to 280°C at 5°C min^{-1} after holding the initial temperature for 10 min.

Ozonation Analysis

The side-chain structure of lignin was characterized by ozonation, with the method modified from that of Akiyama (2002). Samples (100 mg each) were dissolved in an ozonation solvent (30 mL) that consisted of acetic acid:water:methanol (16:3:1, v:v:v); oxygen containing 3% ozone was added to the solution for 2 h at 0°C. The reaction mixture was reduced with sodium thiosulfate ($Na_2S_2O_3$) to remove excess ozone and peroxides, and was evaporated with 1 to 2 mL of water until no smell of acetic acid could be detected. Each reduced sample was saponified overnight with 30 mL of 0.1 M NaOH; 1 mL of erythritol solution (1 mM, 122.12 mg L^{-1} in water) was added as an internal standard. The mixture was passed through a cation exchange column (Dowex-50W-X4 resin, ammonium form) to convert organic acids into their ammonium salts, washing with water until a pH of 7 to 8 was achieved. After concentrating the ammonium salt solution to 2 to 3 mL, we dried the products under vacuum at 40°C overnight. These products were then trimethylsilylated with a 3:2:1 (v:v:v) mixture of DMSO (dimethyl sulfoxide), hexamethyldisilazane (HMDS), and trimethylchlorosilane (TMCS). Analysis was performed on a GC-17A Shimadzu gas chromatograph. Conditions included an NB-1 capillary column (25 m × 0.25 mm i.d.), flame-ionization detector (injector temperature: 250°C, detector temperature: 280°C), and a column temperature initially held at 120°C for 5 min, then programmed at 4°C min^{-1} to 170°C and 10°C min^{-1} to 250°C. All chemical analyses were conducted in triplicate.

RESULTS AND DISCUSSION

Morphological Features of Tracheary Elements

From our samples of cacao hulls, numerous parallel rays composed of clustered TEs became visible after we removed the cover material of white mucilage, which can swell in excess water (Figs. 1a, b, 2a). Although TEs can occur in var-

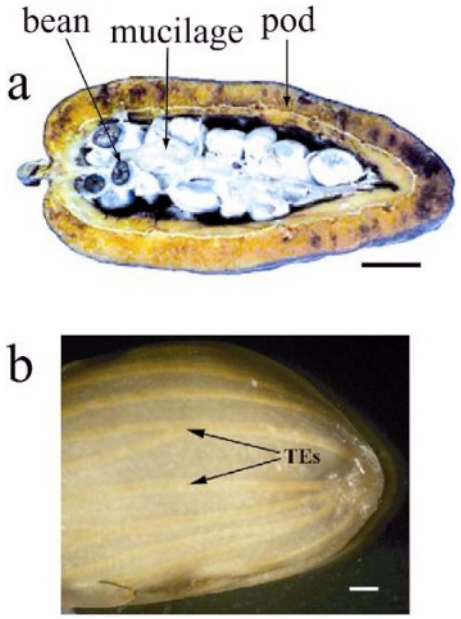

Figure 1. Section through cacao pod (**a**) and horizontal view of cacao hull (**b**). Bars: a = 2 cm; b = 1 mm.

ious forms, e.g., annular, spiral, reticulate, and/or pitted wall thickenings (Fukuda, 1997), only spiral thickenings were observed here. These were arranged parallel to the long axis of the cell, forming connected tubes (Fig. 2a-h).

Figure 2b shows the tip of an elongated and tapered TE; those with spiral wall sculpturing had imperforate ends. The thin layer of the primary wall has general rheological properties and protects against water leakage after lignification (Romberger et al., 1993); here, it covered the outside of the TEs, producing a disordered web-like (Fig. 2d) and lamellate structure (Fig. 2e). The ability of TEs to withstand stretching is demonstrated in Figure 2g and h, where primary walls were retained despite being distorted. These results suggest that TEs with spiral thickenings have some degree of extension for preserving their conformation. Patterns of secondary thickening can also affect mechanical strength when combined with certain flexibility (Burgess, 1985).

The diameters and thicknesses of our spiral TEs are presented in Figure 2(c-j). Tracheary elements were aligned in parallel rows in which circular or elliptical TEs showed a spiral circularity diameter of about 8.2 ± 0.6 μm (Fig. 2f, i). Their size is small when compared with general wood spe-

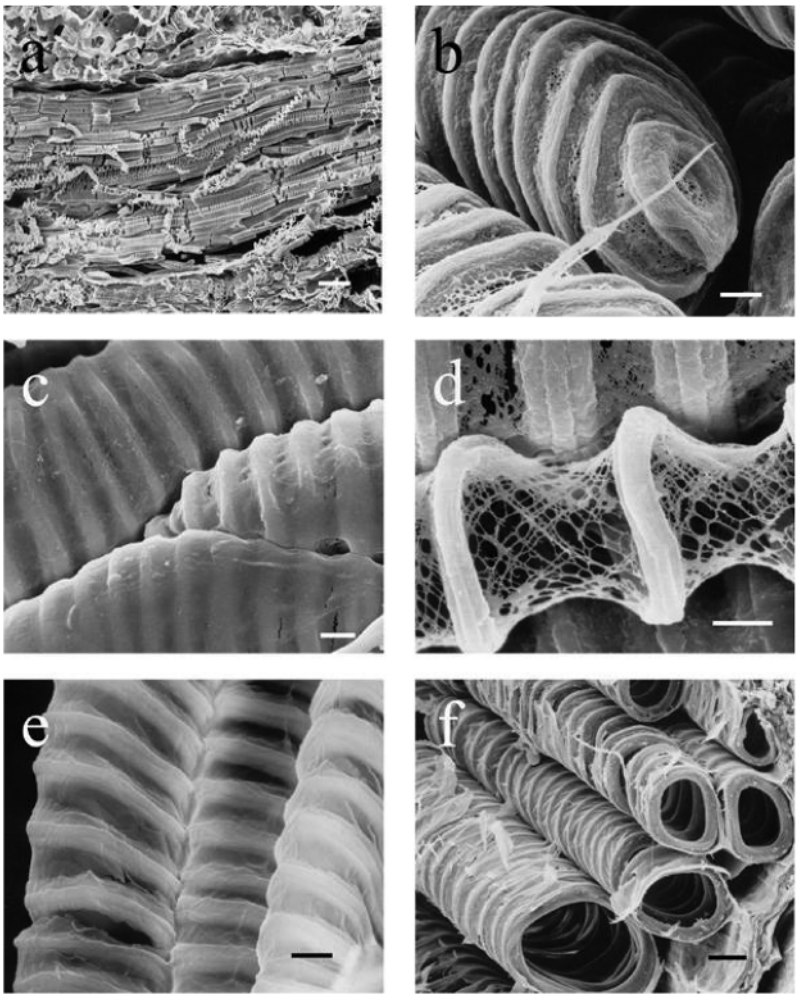

Figure 2. Tracheary elements in cacao hulls: (**a**) longitudinal section of hull, (**b**) tip of elongated TE with tapered end, (**c**) TEs with imperforate ends, (**d**) TE with web-like primary wall, (**e**) TE with lamellate of primary wall, (**f**) different sizes of spiral rings of TEs, (**g**) TE stretched out, (**h**) TE bent with primary wall, (**i**) example of spiral circularity of TE, (**j**) example of TE thickness. Bars: a = 50 μm; b, c, d, and e = 2 μm; f = 3 μm; g = 5 μm; h = 7 μm; i = 2 μm; j = 0.5 μm.

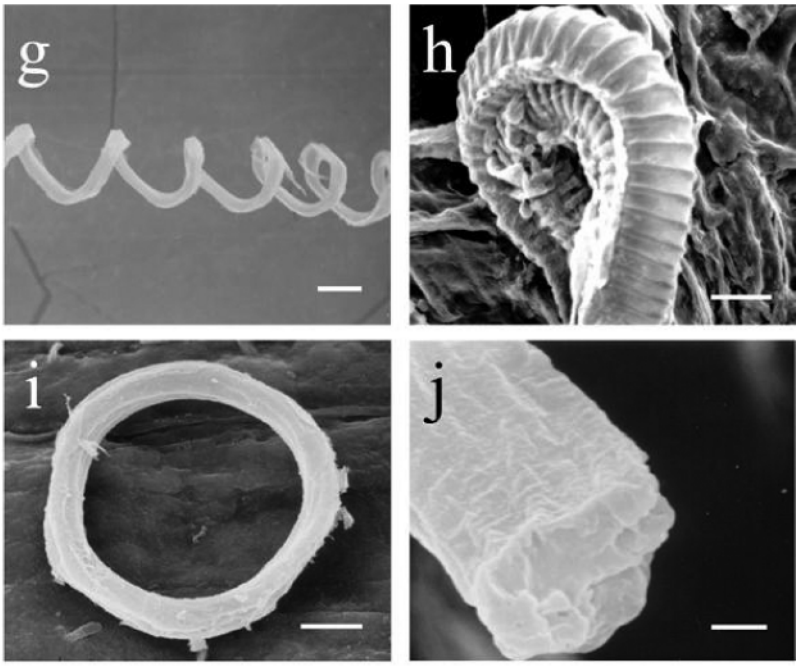

Figure 2. Continued.

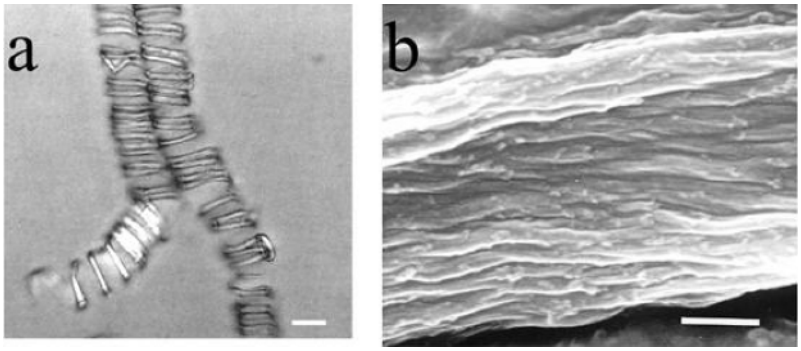

Figure 3. Polarized micrograph of TE (**a**) and high-resolution electron micrograph from inside of TE (**b**). Bars: a = 8 μm; b = 0.5 μm.

cies (Frost, 1930a, b; Carlquist, 1996). Estimated thicknesses here were about 2.1 ± 0.2 μm (Fig. 2j). Based on these morphological observations, we believe that, although the TEs were obtained from fully matured cacao pods, development of their secondary walls was at an incipient stage, as indicated by their spiral shape, imperforate ends, and narrow range in size of circularity.

The orientation of TE cellulose microfibrils was investigated with polarized microscopy and SEM. In the former, these Tracheary elements appeared as bright and dark areas. In the bright area, microfibrils were aligned at a 45° angle to the polarizing axis while those in the dark area were approximately parallel to the axes (Fig. 3a). Our study of these oblique sections provides good evidence that microfibril orientation is along the spiral thickening. This was also confirmed by high resolution of electron micrographs that showed the microfibril bundles lying parallel to those spiral thickenings (Fig. 3b).

Lignin Analyses

Lignins are three-dimensional phenolic heteropolymers covalently associated with polysaccharides in plant cell walls. They are mainly localized in the impermeable water-transport conduits of the xylem and other supporting tissues, such as the phloem fibers (López-Serrano et al., 2004). Their structural heterogeneity varies according to the stage of development during wall formation (Terashima and Fukushima, 1988). Therefore, the particular method, e.g., alkaline nitrobenzene oxidation or ozonation, used for recovering degradative lignin products would depend on the stage that is involved (Faix and Schweers, 1975; Terashima et al., 1989; Kim et al., 2004). Molar ratios of S/V (syringyl to guaiacyl units) from NBO and E/T (erythronic acid to threonic acid) from ozonation are not only important in terms of characterizing lignins, but also for describing the relative proportion of uncondensed lignins. When NBO is conducted in a strong alkali and at elevated temperatures, the side chains of various phenylpropanoids, including those with un-substituted (non-cross-linked) aromatic rings, are cleaved to yield such corresponding aldehydes as *p*-hydroxyphenyl, guaiacyl, and syringyl nuclei (Anterola and Lewis, 2002).

Here, the total yield of NBO products from TEs was 324.5

Table 1. Results from alkaline nitrobenzene oxidation (NBO) analysis of cacao tracheary elements

NBO products* (μmol g^{-1})	TEs
4-Hydroxybenzaldehyde	6.2±0.9
4-Hydroxybenzoic acid	1.3±0.2
Vanillin	259.7±5.8
Vanillic acid	21.5±3.0
Syringaldehyde	30.7±4.3
Syringic acid	5.1±0.7
Total yield	324.5±15.0
Recovery (%)**	18.0
S/V molar ratio	0.13
Lignin (%)	36.1±0.3

*Means ± SD, n = 3.
**% of recovery is based on total lignin content.

μmol g^{-1}, which included a relatively high amount of vanillin (259.7 μmol g^{-1}) and a low amount of syringaldehyde (30.7 μmol g^{-1}) (Table 1). Based on the total lignin content (36.1%, Klason lignin + acid soluble lignin), this meant a recovery rate of 18.0% if one assumed the equivalent molecular weight for one unit of lignin to be 200 (Jin et al., 2003). This value was significantly less than that determined for wood species in general, c.f., 50% for Angiospermae and 34% for Gymnospermae (Chen, 1992). Moreover the molar ratio of S/V, which was calculated from the molar yields of syringaldehyde, syringic acid, vanillin, and vanillic acid, was very low (0.13:1.00) compared with woody plants (Chen, 1992). These NBO results suggest that the lignins in TEs are highly condensed.

To confirm the existence of condensed lignin polymers, we also performed ozonation. Using that method, aromatic moieties of lignin are cleaved while the side chains are mostly left intact and recovered in the form of mono- and dicarboxylic acids. An arylglycerol-β-aryl ether moiety, the most dominant intermonomer linkage in native lignin, gives erythronic and threonic acids as ozonation products depending on its stereo structure (Matsumoto et al., 1986; Habu et al., 1987, 1988; Akiyama et al., 2000). Here, the molar ratios of E/T, which exactly reflected the ratio of erythro to threo forms of the arylglycerol-β-aryl ether moiety, were 1.09:1.00 (Table 2), which is higher than that known for softwood lignins but lower than from hardwood species (Sarkanen et al., 1986). In addition, the total yield of erythronic and threonic acids was 148.8 μmol g^{-1} (Table 2), leading to a recovery rate of 8.2% based on lignin content.

About half of the intermonomer linkages of lignin are of the arylglycerol-β-aryl type (McCarthy and Islam, 2000); yields of erythronic and threonic acids from this structure are estimated to be about 50% (Akiyama et al., 2000, 2002). However, the content of this arylglycerol-β-aryl ether structure might be related to the maturation level of the cell walls, i.e., increasing with growth of the lignin polymer. Therefore, a low recovery value for ozonation products means that it is reasonable to assume that this form of lignin is not normally polymerized but differs structurally from wood lignins. This leads to a diminished arylglycerol-β-aryl ether structure, as well as to a high proportion of condensed types that do not produce erythronic and threonic acids by ozonation. Our total yield of ozonation products (324.5 μmol g^{-1}) was quite reasonable and was similar to that obtained via NBO (148.8 μmol g^{-1}), both of which showed low rates of recovery. Therefore, we can conclude that, although our cacao pods were fully ripened, the structural feature of lignin in their TEs showed characteristics similar to those observed at an earlier stage of secondary wall development because of the low S/V ratios and yields of NBO and ozonation products derived from these condensed types of lignin polymer.

Table 2. Results from ozonation analysis of cacao tracheary elements

Ozonation products* (μmol g^{-1})	TEs
Erythronic acid	77.1±1.9
Threonic acid	71.1±1.0
Total yield	148.8±3.0
Recovery (%)**	8.2
E/T molar ratio	1.09
Klason lignin (%)	36.1±0.3

*Means ± SD, n = 3.
** % of recovery is based on total lignin content.

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