Distribution of Esterified Phenolic Acids in Cell Walls of Immature Bamboo

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幼竹細胞壁におけるフェノール酸エステルの分布

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Résumé

Variation of distribution of esterified ferulic acid and *p*-coumaric acid during growth of moso-bamboo (*Phyllostachys pubescence* Mazel) was investigated by ultraviolet and fluorescence microscopic techniques. The results indicate that the esterified phenolic acids was widely distributed in the tissue of immature bamboo having 6 m in height, but some distinctive differences in distribution of both acids were detected. Metaxylem vessel cell walls contained feruloyl groups at young stage of growth, and accummulated *p*-coumaric acid with progress of lignification. Fiber cell walls were rich in esterified ferulic acid and their accummulation progressed from inner to outer layers of vascular bundle. Degree of esterification of parenchyma cell walls was lower than these two cells, and the esterification progressed slowly with growth.

要　　旨

モウソウチク（*Phyllostachys pubescence* Mazel）の幼竹（高さ6 m）を用いて竹の成長に伴うエステル結合したフェルラ酸及び*p*-クマール酸の分布を紫外線・蛍光顕微鏡法により調べた。エステル結合した両フェノール酸は幼竹の組織中に広く分布しており，両者の分布には明確な相違が認められた。後生道管細胞壁中には成長の初期にエステル結合したフェルラ酸が存在するが，木化が進行するにしたがい*p*-クマール酸が堆積した。また，繊維細胞壁中にはエステル結合したフェルラ酸が多く含まれており，維管束鞘の内部から外側に向かって堆積した。柔細胞壁中のフェルラ酸のエステルは上記の組織に比較して少なく，その堆積が遅いことがわかった。

1. Introduction

Bamboo has been attracted considerable interest in studying the growth mechanism of higher plant because of its rapid growth rate[1-3]. Bamboo belongs to *Bambusoideae*, a subfamily of *Gramineae*, and the cell walls contained a large amount of phenolic acid in addition to polysac-

charides and lignin[4]. The most commonly observed and predominant phenolic acids were ferulic acid and *p*-coumaric acid which could be released by saponification[5].

Previously, we analyzed a detailed chemical composition change from the top to the bottom of an immatured moso-bamboo, and reported that, while *p*-coumaric acid content increased in good accordance with increase of lignin content, ferulic acid content was inversely proportional to the lignin content[6]. This indicates that the location and function of these two phenolic acids in bamboo are different. It has been suggested that *p*-coumaric acid was esterified at both γ and α positions of bamboo lignins in a ratio of 4:1[7]. Recently it was reported that several oligosaccharides esterified with *p*-coumaric and ferulic acids were isolated when a moso-bamboo shoot was enzymatically digested[8,9]. However, distribution of these phenolic acids in bamboo tissue has not been clarified yet.

Histochemical research on the phenolic acids indicates that the esterified ferulic acid could be visualized specifically in the cell wall by its characteristic blue autofluorescence when it was irradiated with ultraviolet (UV) light and a bathochromic shift to green was noticed after exposure to ammonia or dilute alkali[10-12]. UV microscopy has also been effectively used for determination of lignin in wood tissue[13] and the phenolic acids in rice cell wall[14].

In the present study, we intend to investigate variation of distribution of the phenolic acids during growth of moso-bamboo by UV microscopy and fluorescence microscopy.

2. Experimental

2. 1 Samples

An immature moso-bamboo (*Phyllostachys pubescens* Mazel) having 620 cm in height was harvested on May 16, 1989, at the Botanical Garden of Kyoto Universty. Immediately after the harvest, the immature bamboo was cut from the bottom into 6 portions (No. I-VI) having 100 cm in length except the top portion (No. VI) which had 120 cm in height. After bamboo-sheaths were peeled off, a small block was cut out from the lower part of the internode at about center of each portion: No. 1 from 7th , No. 2 from 13th, No. 3 from 18th, No. 4 from 22nd, No. 5 from 26th, No. 6 from 32nd internode, respectively as shown in Fig. 1. Each sample was fixed with glutaraldehyde by soaking in cold 3% glutaraldehyde-0.1M potassium phosphate buffer (pH 7.2). The remaining portions were cut into small pieces, lyophilized and milled to pass 24 mesh screen by a Wiley mill.

2. 2 Phenolic acid analysis

Phenolic acid analysis was carried out as previously described[6,15]. Briefly, 100 mg of the dried native sample was saponified with 10 ml of 2 N NaOH for 24 hr at room temperature. The suspension was adjusted to pH 2.1 with HCl and extracted with ether (10 ml × 5). The extracts were evaporated to dryness. Phenolic acids were converted into TMS derivatives and analyzed by g.l.c. (Shimadzu GC-15A) on a column of CBP-1 (25 m × 0.25 mm) at 200°C using methyl heptadecanoate as an internal standard.

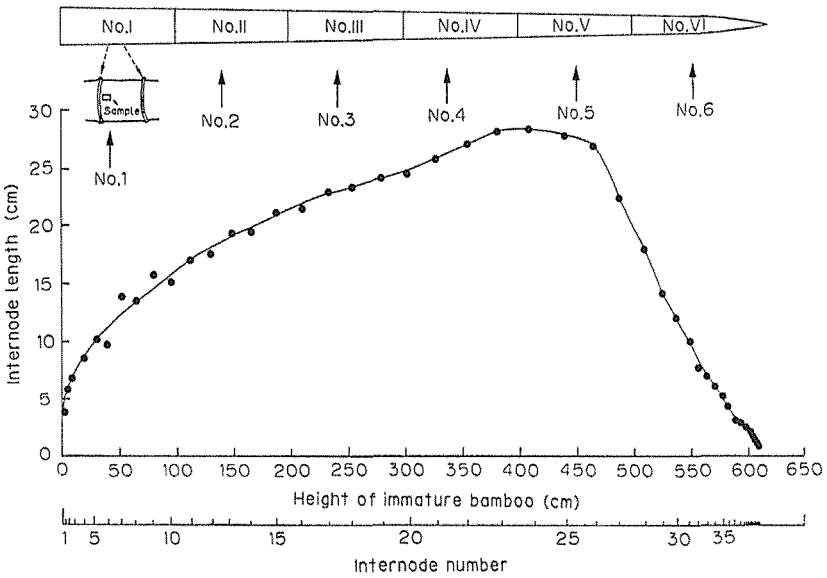

Fig. 1 Relation of internode length with its serial number and height of immature moso-bamboo, and positions of samples.

An immature culm of moso-bamboo 620cm in height cut into 6 portions (No. I-VI) and small block was cut out from the lower part of the internode at about center of each portion as indicated by an inserted schematic illustration.

Arrows indicate the position of samples (No. 1-6) used for ultraviolet and fluorescence microspectroscopic analyses.

2. 3 UV microscopic spectrometry

A small block was cut into 2 mm long pieces which were dehydrated in a graded ethanol series and embedded in Luveak 812 resin. Transverse section of 1 μm thickness was cut on a Sorvall JB-4 microtome equipped with a glass or a sapphire knife. Ultraviolet absorption spectra were measured by a Zeiss UMSP80 microscope. Measurements were made at 5 nm band width and 1.5 μm spot diameter. Photographs were taken at 280 nm.

2. 4 Fluorescence microscopic spectrometry

Transverse section of 20 μm thickness was cut from the fixed block on a sliding microtome with a frozen specimen stage. Since the blocks of No. 4, 5, 6 were very fragile, 40 μm thick sections were cut. The sections were observed with an epifluorescence microscope (BH-RFL, Olympus Kougaku Co.) with UV-excitaion (filter: UG1, DM400, L410, L420). Microfluorescence spectra were measured by a Zeiss UMSP80 microscope. Illumination was carried out with three filters (BP 365, FT 395 and LP 397). Measurements were made at 20 nm band width and 5 μm spot diameter. Effects of alkali on fluorescence spectrometry were determined by treatment with 0.1 N NH_4OH on the sections.

2. 5 UV and fluorescence emission spectroscopic analyses

For comparison, both UV and fluorescence emission spectra were measured for 7 compounds: L-tyrosine, *p*-coumaric acid, ethyl coumarate, *trans*-ferulic acid, methyl ferulate, *O*-[5-*O*-(*trans*-feruloyl)-α-L-arabinofuranosyl]-(1→3)-*O*-β-D-xylopyranosyl-(1→4)-D-xylopyranose (FAX_2) and milled bamboo lignin (MBL) containing 1.03% *p*-coumaric acid and 0.13% ferulic acid (w/w). The latter two were isolated from mature bamboo following the procedure as previouly described[16,17]. Esters of *p*-coumaric and ferulic acids were synthesized according to the method of Gubler *et al.*[18], and the other reagents were purchased from Nakarai Tesque Co., Ltd. Measurements were made for neutral solutions in 50% aqueous methanol or 1,4-dioxane with a Shimadzu UV365 spectrometer and a Hitachi 850 spectrofluorometer at 25°C. Effects of alkali on fluorescence spectra were determined by addition of ammonia to the above neutral solution to make 0.1 N NH_4OH.

3. Results and Discussion

3. 1 Relation of internode length and height and phenolic acid distribution

Figure 1 shows variation of internode length with its serial number at the bottom, beginning with the lowest. The positions of samples used in this study are also illustrated at the top in this figure. The internode length first increased from the bottom, and was at its maximum at 24th internode about 4 m in height, and decreased to the top. At the lower portion, i.e. No. I and II, the elongation growth period may be over, but the upper portion, especially upper than 24th internode, would continue the growth as reported by Nomura and Yamada[19]. The height of the immature bamboo used in this study (620 cm) was almost equal to that used in the previous study (633 cm), where we studied the chemical composition change accompanied by the growth within one immature culm of moso-bamboo in detail[6]. In this work we report variation of ferulic acid and *p*-coumaric acid contents together with lignin contents. As shown in Table 1, content of the *p*-coumaric acid increased from the top to the bottom in accordance to the lignin content. Ferulic acid content, except No. IV, also increased from the top to the bottom, but it was a max-

Table 1. Variation of phenolic acid and lignin contents in immature moso-bamboo with growth

(% dry weight)

Compound	No. I	No. II	No. III	No. IV	No. V	No. VI
p-Coumaric acid (C)	0.27	0.19	0.16	0.13	0.03	0.01
Ferulic acid (F)	0.18	0.14	0.14	0.24	0.07	0.06
Klason lignin	11.10	9.86	7.91	5.73	1.01	0.45
Ratio of (C) to (F)	1.5	1.4	1.1	0.5	0.4	0.2

Samples No. I-VI represent the six different portions in the same culm of immature moso-bamboo 620 cm in height as shown in Fig. 1

imum at No. IV. The ratio of the *p*-coumaric acid to the ferulic acid increased from the top to the bottom, confirming the results reported previously[5,6]. Based on these results, the present samples are concluded to be suitable for investigation of phenolic acid distribution in relation to growth of moso-bamboo.

3. 2 UV and fluorescence spectroscopic analyses of model compounds

Prior to UV and fluorescence microscopic analysis, UV and fluorescence spectra of 7 model compounds were measured. Results shown in Table 2 and the data reported previously[7,20] indicate that esters of *p*-coumaric and ferulic acids could be differentiated each other by the position of λ_{max}: the former shows an absorption maximum at 312 nm, while the latter at 325 nm. As expected from the phenolic acid composition, the milled bamboo lignin (MBL) showed a typical UV spectrum of the *p*-coumaric acid ester[7,17]. When the fluorescence spectra of the model compounds in neutral solution were compared with those obtained after alkaline treatment, the spectrum of the MBL and L-tyrosine showed only a slight bathochromic shift by 2-3 nm after alkaline treatment. The other compounds showed a larger bathocromic shift. Among them the λ_{max} of the feruloylated trisaccharide (FAX_2) shifted to the longest wavelength, 475 nm.

Table 2. Characteristics of ultraviolet and fluorescence spectra of model compounds

Model compound	λ max (nm)		
	ultraviolet spectral data	fluorescence spectral data	
	neutral condition[a]	neutral condition[a]	alkaline condition[b]
L-Tyrosine	275	310	313
p-Coumaric acid	290	390	436
Ethyl coumarate	312	395	451
Ferulic acid	315	421	458
Methyl ferulate	325	426	455
FAX_2[c]	325	440	475
Milled bamboo lignin	312	456	458

a) In 50% aqueous methanol except milled bamboo lignin which measurement was made in 50% aqueous 1,4-dioxane.

b) In 0.1N NH_4OH.

c) *O*-[5-*O*-(*trans*-feruloyl)-α-L-arabinofuranosyl]-(1→3)-*O*-β-D-xylopyranosyl-(1→4)-D-xylopyranose

3. 3 UV microscopic spectra on some cell walls

Figure 2 is a series of UV micrographs of culm wall from the 6 different portions. In these micrographs, 2 kinds of tissue, metavessel and fiber, were observed. Density of the UV absorptions became weaker from the bottom to the top with ascending height. At the bottom portion of the culm (No. 1, 2), metaxylem vessel and fiber cell walls were thick, and showed UV absorption with dense absorption. At the middle portion of the culm (No. 3, 4), fiber cells of outer bundle sheath showed substantial amount of UV absorption, and density of the UV absorp-

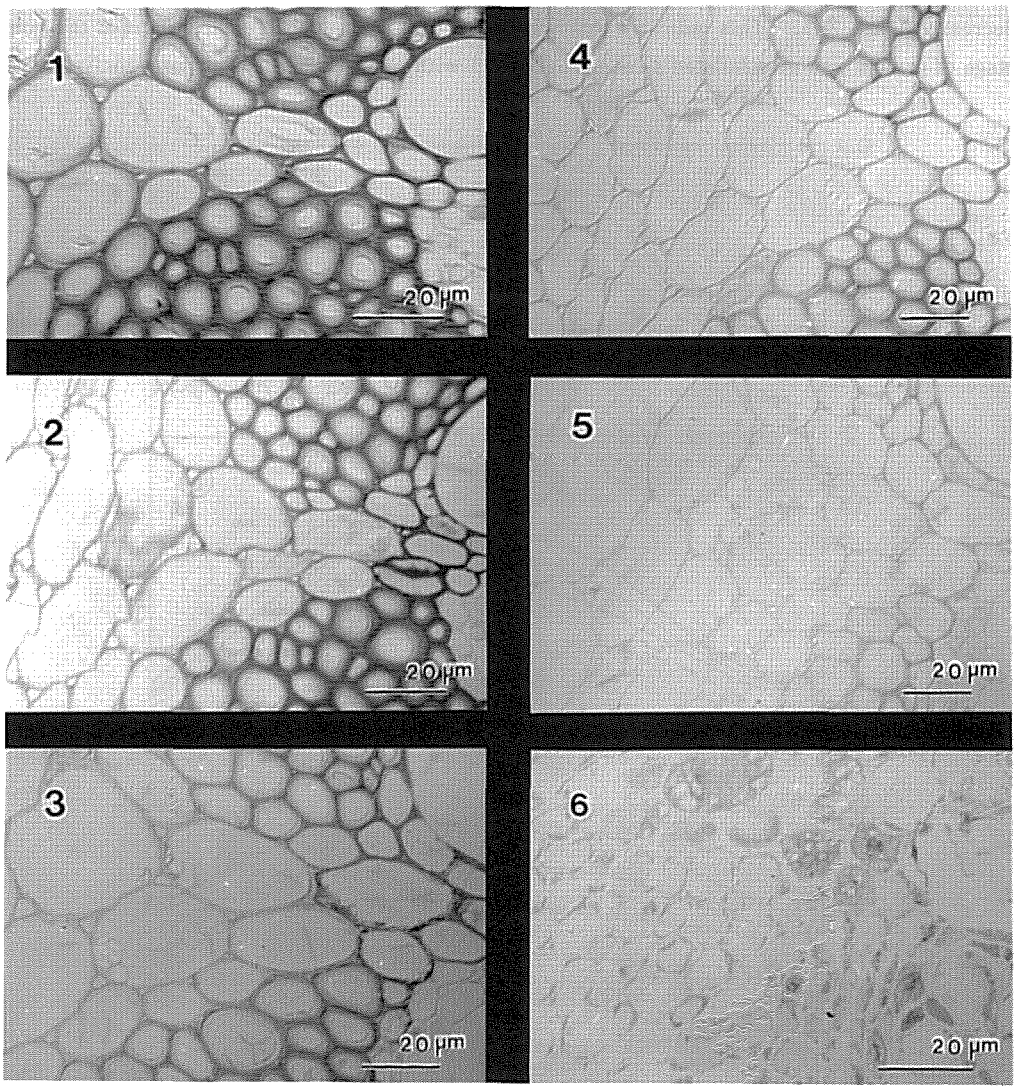

Fig. 2 Ultraviolet photomicrographs of the six different portions from the same culm of immature moso-bamboo.

Photomicrographs of 1-6 corresponded directly to the samples No. 1-6, respectively, as shown in Fig. 1.

tion increased rapidly as approaching to the metavessel cell wall. At No. 5, only cell walls of vessel and fibers adjacent to vessel showed weak UV absorption. At the top portion of the culm (No. 6), no distinctive UV absorption occurred in the cell walls, but it showed substantially strong UV absorption probably due to proteins.

Following to UV absorption microspectrometric analysis was carried out to characterize the chemical nature of UV positive compounds. Figure 3 shows variation of UV absorption spectra. As to the vessel cell walls, the absorption became weaker with ascending height. The absorption around 312 nm of No. 1 seemed to be due to the *p*-coumaroyl group. With ascending height, the absorption at 325 nm due to feruloyl group appeared, while the absorption of lignin at 280 nm decreased (Fig. 3a). This indicates that vessel cell wall contained feruloyl groups at the young stage of growth and accumulated *p*-coumaroyl group with progress of lignification, confirming the

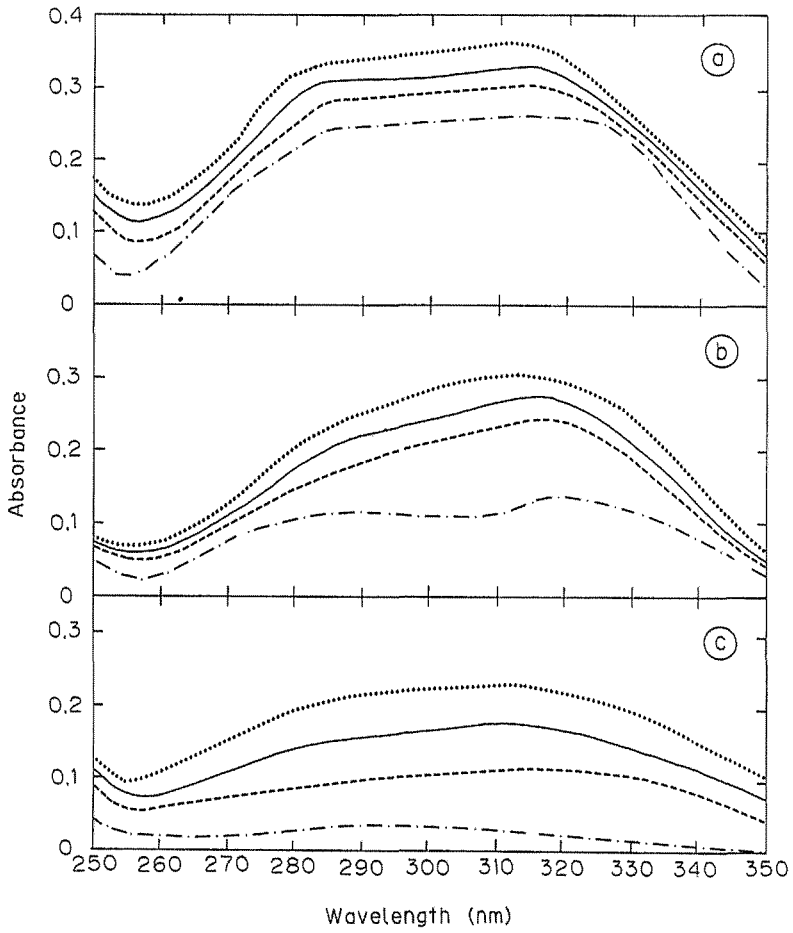

Fig. 3 Ultraviolet absorption spectra of metaxylem vessels (a), fibers (b) and parenchyma (c) cell walls of different portions from the same culm of immature moso-bamboo. Spectra shown by (·····), (——), (·····) and (—·—) corresponded to the samples No. 1, 2, 3, 5, respectively, as shown in Fig. 1.

results of chemical composition analysis (Table 1). In the case of fiber cell walls (Fig. 3b), absorption near 320 nm was stronger than that at 280 nm and it was predominant at the lower portions of the culm (No. 1-3). A rather strong absorption near 320 nm shows that the fiber cell walls are rich in ferulic acid. In comparison to these cell walls, parenchyma cell walls showed a weak UV absorption. This is in agreement with the previous finding that lignification of parenchyma cells did not start until the bamboo-shoot grew up about 500-580 cm in height[21,22] However, the presence of broad maximum between 310-330 nm at No. 1-3 indicates that esterification of parenchyma cell walls with phenolic acids proceeded slowly with growth. In contrast to the cell walls of vessel, fiber and parenchyma, degree of lignification and esterification with phenolic acids in the cell walls of phloem was concluded to be quite low because of extremely weak UV absorption (data not shown). Previously, Yoshinaga *et al.*[23] measured UV absorption spectra of various secondary cell walls in moso-bamboo harvested in December of the same year after sprout. The present spectra of the vessel cell walls in the lower portion of the culm were similar to their result. However, absorptions at 280 nm and 310 nm in their spectra of fiber and parenchyma, respectively, were much stronger than those in the present result. This indicates that deposition of lignin and phenolic acid in these cells proceeded in later stage of growth.

3. 4 Fluorescence microspectra of immature bamboo

The fluorescence microscopy has been effectively used for examination of phenolic materials localization in graminaceous plant tissues[10-12]. Since the esterified ferulic acid with carbohydrate could be differentiated from the esterified *p*-coumaric acid by a bathochromic shift to longer wavelength observed with alkaline treatment as shown in Table 2, we further analyzed distribution of the esterified phenolic acids in the bamboo tissue by fluorescence microscopy. When transverse sections from 6 different portions of the culm of moso-bamboo were investigated with UV illumination, all cell walls of the immature bamboo tissue exhibited a blue autofluorescence due to lignin and esterified phenolic acids. At the upper portion of the culm (No. 5, 6), the weak blue autofluorescence exhibited in all cell walls. With alkaline treatment, some alternation were observed: the blue color was changed into slightly greenish blue (Fig. 4a, b). This indicates that the ferulic acid esterified with carbohydrate was widely distributed in the young tissues of moso-bamboo. This result is in agreement with that obtained by UV microspectroscopic analysis as shown in Fig. 3.

Figure 4 (c, d) shows the fluorescence photomicrographs of No. 2 without and with the alkaline treatment. Different from No. 5, a remarkable color change of vessel, fiber and parenchyma cell walls with the alkaline treatment were not observed. In contrast to these cells, only phloem cell walls were turned into bright green and this color was strengthened with descending height from No. 5 to No. 2. The intensity of blue autofluorescence of No. 2 was stronger than that of No. 5. It may be due to lignin accummulation in cell walls.

In order to further analyze the bathochromic shift of No. 2, microfluorospectra were measured at vessel, fiber and phloem cell walls. The results are shown in Fig. 5. The emission maxima of cell walls of fiber at the outer layer of vascular bundle, vessel and phloem shifted from 435 nm, 440 nm and 460 nm to 465 nm, 475 nm and 510 nm, respectively. The emission maximum

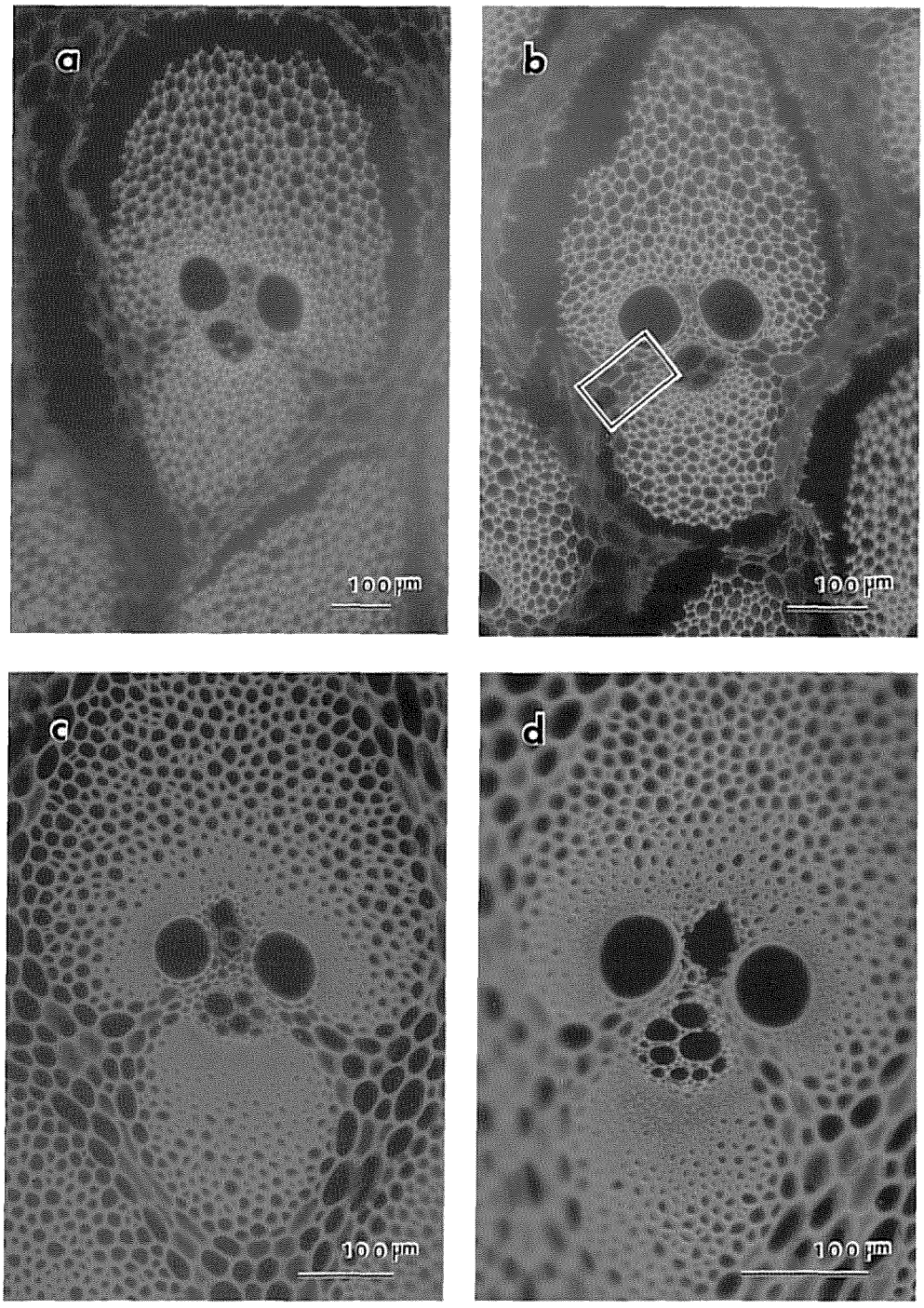

Fig. 4 Fluorescence micrographs of vascular bundles with and without alkaline treatment.

Micrographs a and b are respectively of No. 5 sample without and with alkaline treatment with 0.01 N NH_4OH, and c and d are the corresponding micrographs with No. 2.

The enclosed area in b indicates a part observed by ultraviolet spectroscopy. (See Fig. 2)

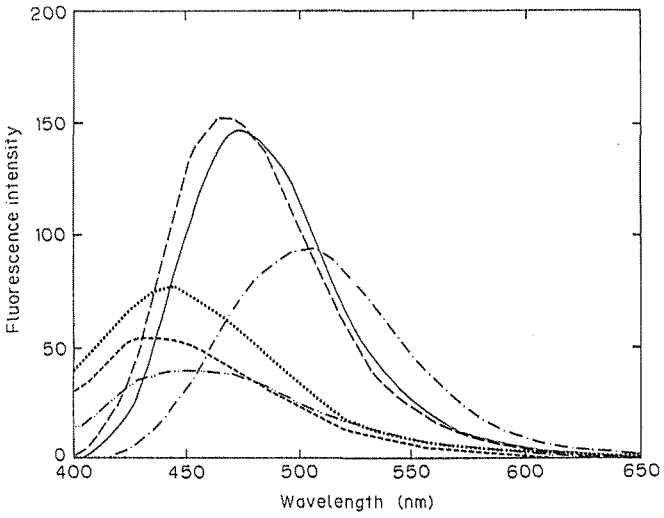

Fig. 5 Fluorescence microspectra of cell walls of metaxylem vessel, fiber and phloem of the No. 2 portions shown in Fig. 1.
Spectra shown by (·····) and (——) correspond to metaxylem vessel without and with alkaline treatment with 0.1 N NH_4OH, (·····) and (——) represent corresponding spectra of fiber, and (----) and (—·—) represent corresponding spectra of phloem, respectively.

shift of fiber cell walls was similar to that of model compound, FAX_2 (Table 2). Based on the bathochromic shifts and the UV microspectra (Fig. 3), the esterified ferulic acid is concluded to be rich in fiber cell walls. Vessel cell walls also contained the esterified ferulic acid, but its flourescence due to ferulic acid might be masked by contribution of lignin and *p*-coumaric acid. In the case of phloem cell walls, although color changed to green by the alkaline treatment, the emission maximum at 510 nm was different from that of FAX_2. This was identical to that of barley bran and similar to that of barley endosperm cell walls after alkaline extraction[11]. Together with UV spectrometric results, the results indicate low existence possibility of the esterified ferulic acid with arabinoxylan in phloem cell walls. But other esterified phenolic acid might be contained in phloem cell walls.

In summary, the present results demonstrated that UV and fluorescence microscopic techniques are useful to investigate distribution of phenolic acids in the immature moso-bamboo tissues.

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