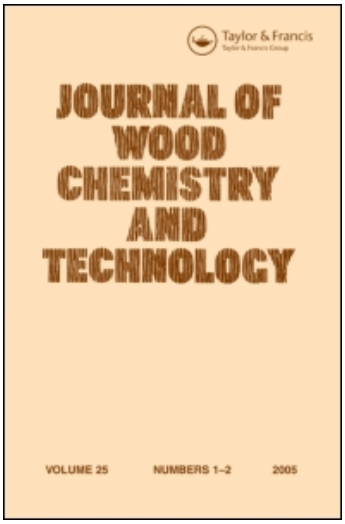

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Kazuhiko Fukushima[a]; Noritsugu Terashima[a]
[a] Faculty of Agriculture, Nagoya University, Nagoya, Japan

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HETEROGENEITY IN FORMATION OF LIGNIN. XIII. FORMATION OF p-HYDROXYPHENYL LIGNIN IN VARIOUS HARDWOODS VISUALIZED BY MICROAUTORADIOGRAPHY

Kazuhiko Fukushima and Noritsugu Terashima
Faculty of Agriculture, Nagoya University, Nagoya, 464, Japan

ABSTRACT

The heterogeneous nature of hardwood lignin was shown by visualizing the deposition process of p-hydroxyphenyl (H) lignin in the differentiating xylem of magnolia, beech, lilac and poplar. When p-glucocoumaryl alcohol-[arom. ring-2-^{3}H], an efficient precursor of H units in lignin, was administered to the differentiating xylem of these trees, radioactivity was incorporated in the compound middle lamella region of vessel and fiber cell walls. The deposition of H units occurs only in the early stage of cell wall formation when the outer layer of the secondary wall is formed. H lignin deposits mainly within pectic substances and hemicellulose gel to form a highly condensed structural moiety.

INTRODUCTION

It has been shown that the ratios of syringylpropane units to guaiacylpropane units in birch lignin vary in different morphological regions: vessel lignin is guaiacyl rich and fiber lignin is syringyl rich. This topochemical heterogeneity has been studied by UV-microscopy[1-3], bromination-TEM-EDXA[4] and by chemical characterization of various tissue fractions[5,6]. Recently, it was shown that a combination of selective radio-

labeling of a specific unit in lignin and visualization of the labeled unit by microautoradiography is useful for the study of formation and structure of poplar and magnolia lignins with respect to their location in the wood tissue[7,8]. In this method, ^{3}H-labeled coniferin and syringin are administered to label the guaiacyl and syringyl units in lignin. The distribution of both units as visualized by microautoradiography is similar to the results obtained by the other methods described above.

However, most papers dealing with hardwood lignin pay little attention to p-hydroxyphenylpropane (H) units in lignin. Most hardwoods give only small amounts of compounds derived from H units upon oxidative degradation, but this does not constitute proof that H unit is an unimportant building stone of hardwood lignin. It has been shown that H units in lignin cannot be estimated quantitatively by oxidative degradation[9,10]. In pine differentiating xylem, tritiated p-glucocoumaryl alcohol, an effective precursor of H lignin, was incorporated into compound middle lamella (CML) of tracheids[11]. According to Whiting and Goring the CML lignin of black spruce tracheids contains less methoxyl per C_9 than secondary wall (SW) lignin[12]. We therefore expect H units to exist also in hardwood lignins as one of the main building stones.

In this study, p-glucocoumaryl alcohol-[arom. ring-2-^{3}H] was administered to differentiating xylem of magnolia, beech, lilac or poplar, and the incorporation of H units into cell wall lignin was visualized by microautoradiography. Our results support the proposal that H lignin is one of the main structural moieties of hardwood lignin.

EXPERIMENTAL

Materials

Two-year-old shoots were obtained in July from *Magnolia kobus* DC., *Syringa vulgalis* L. and *Populus Maximowiczii* X

Populus berolinensis grown on the campus of Nagoya University and from Fagus crenata Blume grown on the experimental forest of Nagoya university.

p-Glucocoumaryl alcohol-[arom. ring-2-^{3}H], coniferin-[arom. ring-2-^{3}H] and syringin-[arom. ring-2-^{3}H] were synthesized as described previously[11]. The specific activity of each tritiated glucoside was 8-10 μCi/mg. Coniferin-[β-^{14}C] and syringin-[β-^{14}C] were synthesized also as described previously[13]. The specific activity of each glucoside labeled with ^{14}C was 0.7 μCi/mg. UDP-glucuronic acid-[glucuronyl-U-^{14}C] was obtained from New England Nuclear. Mass., U.S.A. p-Hydroxybenzoic acid-[arom. ring-2-^{3}H] was synthesized from p-hydroxybenzaldehyde-[arom. ring-2-^{3}H][11] according to the method described by Pearl[14]. The specific activity of this acid was 20 μCi/mg.

Administration of precursors

A water solution of each [^{3}H]-glucoside (1mg, 8-10 μCi), [^{14}C]-glucoside (1mg, 0.7 μCi), [^{3}H]-p-hydroxybenzoic acid (0.5mg, 10 μCi) or UDP-glucuronic acid-[glucuronyl-U-^{14}C] (3 μg, 2 μCi) was added dropwise to a groove made on the shoot according to the previous paper[8]. The shoot was metabolized for 3 hours, and labeled parts of the shoot were embedded in epoxy resin by the same procedure described previously[8].

Microautoradiography

Two μm thick transverse sections were cut on a Reichert-Jung Supercut 2050 microtome equipped with a glass knife from the embedded xylem tissue. They were mounted on glass slides and covered with Kodak AR-10 stripping film. The glass slides were stored in a refrigerator for 2 months to 1 year. They were then developed with Kodak D-19 and fixed with Fuji-Fix. The sections of magnolia, lilac and poplar were stained with toluidine blue O

and the sections of beech were stained with safranin O. Photomicrographs were taken with a ZEISS photomicroscope III. A polarization microscope Olympus POS was used for observation of deposition of cellulose microfibrils during the secondary wall formation.

Alkaline nitrobenzene oxidation

Xylem tissue of Magnolia kobus DC. was milled (80 mesh) and extracted thoroughly with ethanol-benzene and hot water. The dried wood meal (20 mg) was subjected to alkaline nitrobenzene oxidation, and aromatic aldehydes and acids were determined by high performance liquid chromatography (HPLC) according to the method described by He and Terashima[15]. Egg albumin (Katayama Chem. Co. Tokyo) was also subjected to alkaline nitrobenzene oxidation according to the same method for use as control.

RESULT AND DISCUSSION

Lignification process in magnolia

It was shown by Terashima et al.[8,11] that p-glucocoumaryl alcohol, coniferin and syringin are suitable precursors of p-hydroxyphenyl (H), guaiacyl (G) and syringyl (S) propane units in lignin respectively. In microautoradiography, the localization of radio-labeled lignin is visualized by silver grains. Therefore, the silver grains in Fig.1**a**, 1**b** and 1**c** show the deposition of H, G and S moieties of protolignin in the cell wall respectively. Fig.1**a**, the microautoradiogram of differentiating xylem of magnolia administered with p-glucocoumaryl alcohol-[arom.ring-2-^{3}H], shows that the deposition of H units of lignin in differentiating xylem occurred mainly during the formation of the S_1 layer. The H units were distributed mostly in the CML region of vessels and fibers (Fig.2). Fig.1**b** shows the deposition of G units. It is

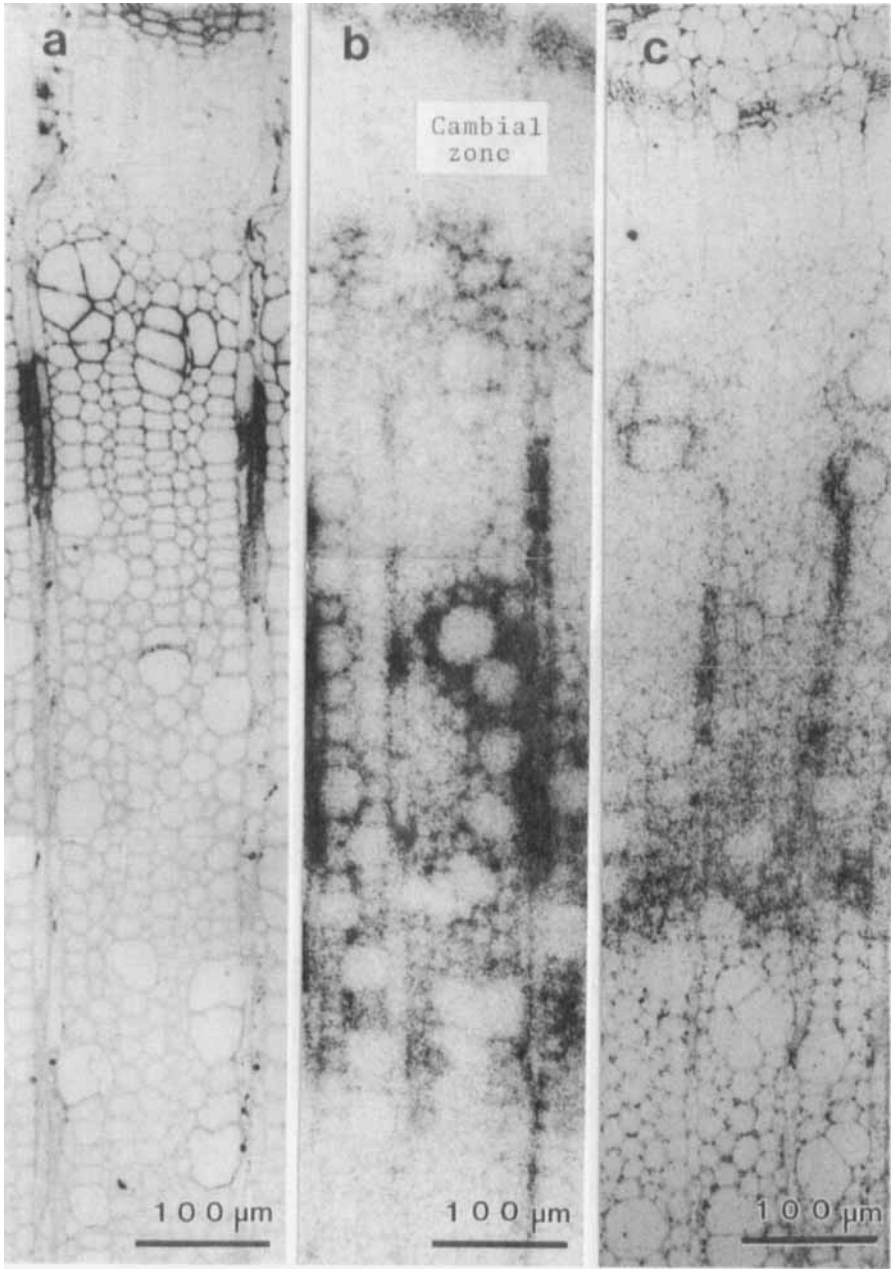


FIGURE 1. *Microautoradiograms of differentiating xylem of magnolia administered with* **a:** p-glucocoumaryl alcohol-[arom. ring -2-^{3}H], **b:** coniferin-[β-^{14}C] or **c:** syringin-[β-^{14}C].

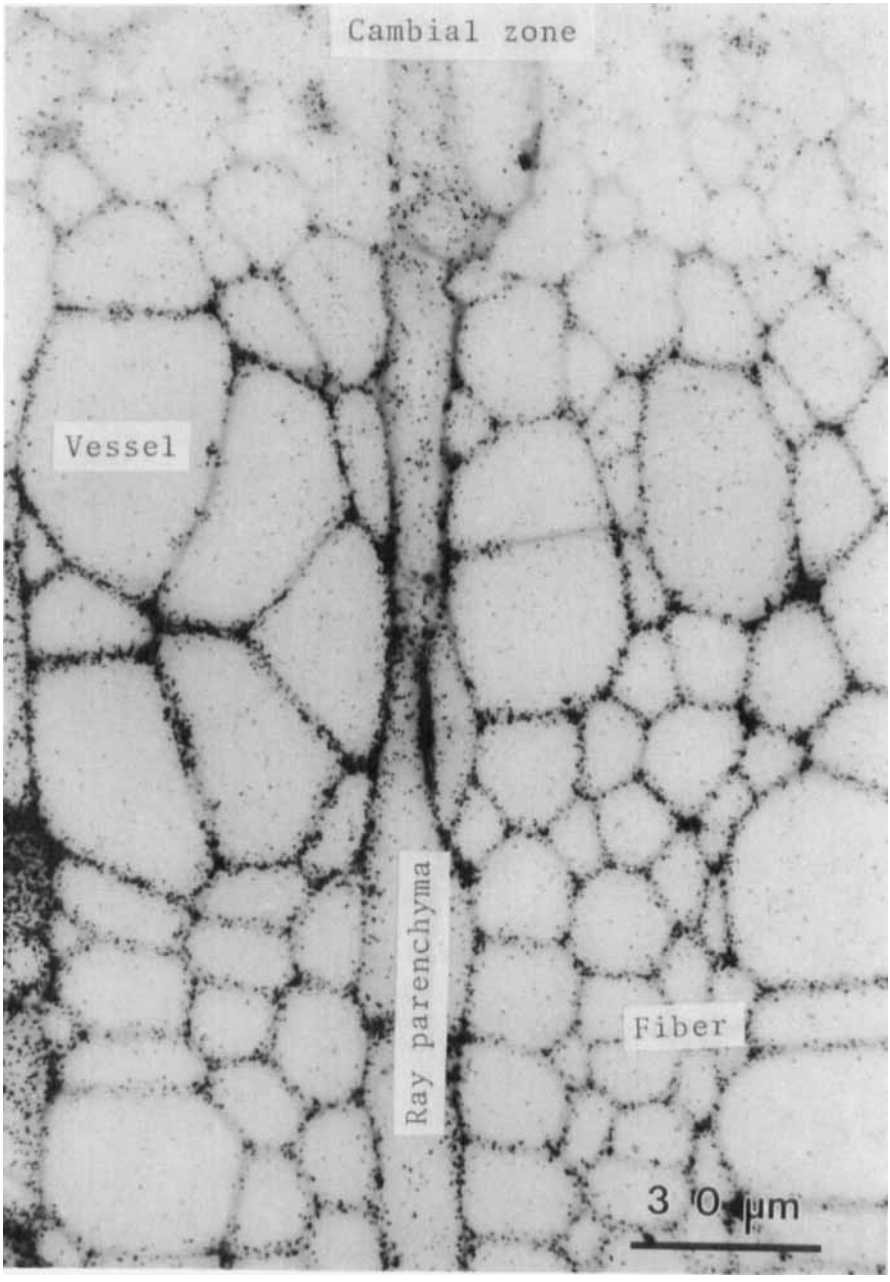


FIGURE 2. Microautoradiogram of differentiating xylem at the initial stage of lignification of magnolia administered with p-glucocoumaryl alcohol-[arom. ring-2-^{3}H]

noticeable that deposition of G lignin occurred in two peaks. The first deposition of G lignin occurred during the formation of the S_1 layer (outer layer of secondary wall) and the later deposition occurred after the start of S_3 layer (inner layer of secondary wall) formation. A similar lignification pattern has been observed in a microautoradiogram of pine administered with coniferin-[β-^{14}C][13] and also pine administered with phenylalanine-[G-^{3}H][16]. The early stage deposition mainly corresponds to the formation of CML lignin. Lignification during this period is particularly noticeable at the cell corners of vessels and fibers. The later deposition corresponds mainly to the formation of secondary wall (SW) lignin. The period of lignification of SW is longer than that of CML lignification (Fig.1**b**). Fig.1**c** shows the deposition of S units. S lignin was mostly formed at the late stage of cell wall differentiation (Fig.1**c**). The deposition patterns of the three units shown in these microautoradiograms indicate that CML lignin consists mainly of H and G units and that SW lignin consists mainly of G and S units. However, the exact contents of the three units could not be estimated by this method.

These microautoradiograms further suggest that more than two kinds of isozymes of β-glucosidase are present in differentiating xylem. The isozyme(s) existing in the early stage (S_1 formation stage) is/are able to hydrolyse p-glucocoumaryl alcohol and coniferin, and another isozyme(s) produced in the later stage (after the start of S_3 formation) is/are able to hydrolyse coniferin and syringin. In other words, a cell in differentiating xylem may produce isoenzymes of different substrate specificities depending on the age of the cell. It is known that hydrolytic activities of β-glucosidase for p-glucocoumaryl alcohol, coniferin and syringin vary widely with the source of the enzyme[17,18].

Fig.3 shows the HPLC profile of nitrobenzene oxidation products of magnolia xylem. In addition to the compounds derived

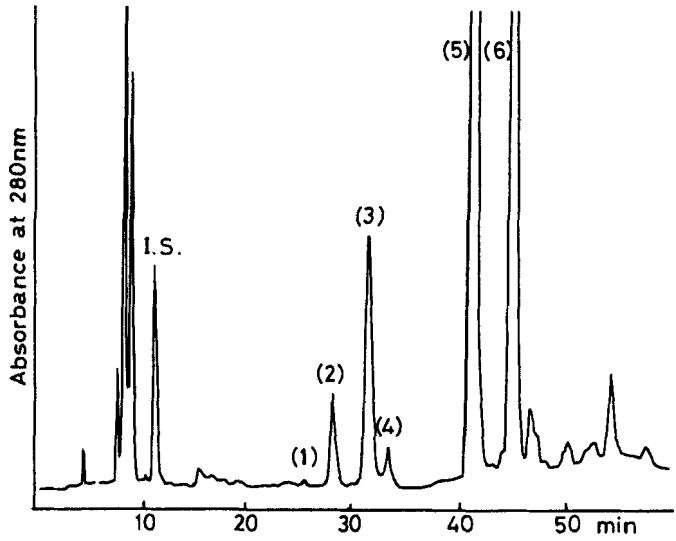


FIGURE 3. HPLC profile of products from nitrobenzene oxidation of xylem of magnolia.(1): p-Hydroxybenzoic acid, (2): Vanillic acid, (3): Syringic acid, (4): p-Hydroxybenzaldehyde, (5): Vanillin, (6) Syringaldehyde, I.S.: Internal standard, 3,5-dihydroxybenzoic acid

from guaiacyl and syringyl units, a peak corresponding to p-hydroxybenzaldehyde can be seen. However, the amount of this compound is very low. The yields and their molar ratios are shown in Table 1. The total amount of p-hydroxybenzaldehyde and p-hydroxybenzoic acid was only 0.06 μmol from 20mg of wood meal, and the molar ratio was only 0.8%. On the other hand, Fig.1a suggests that H units are one of the main components of CML-lignin. If the H units are highly condensed, they will give low yield of p-hydroxybenzaldehyde on oxidation. This suggestion is supported by the facts that nitrobenzene oxidation of birch gave p-hydroxybenzaldehyde from only the differentiating xylem in the initial stage of lignification, but mature wood did not give p-hydroxybenzaldehyde[19].

TABLE 1

Yield* of Degradation Products from Nitrobenzene Oxidation of Magnolia Wood.

Compounds	μmol	%(mol)**
p-Hydroxybenzoic acid(HA)	0.02	
Vanillic acid(VA)	0.30	
Syringic acid(SA)	0.45	
p-Hydroxybenzaldehyde(Ha)	0.04	
Vanillin(Va)	2.39	
Syringaldehyde(Sa)	4.56	
HA+Ha	0.06	0.8
VA+Va	2.69	34.7
SA+Sa	5.01	64.5

* Yields are expressed as μmol of degradation product per 20mg of extracted free wood meal.
** Based on the total yield of six compounds.

It should be noted that proteins contained in the cell wall may give rise to p-hydroxybenzaldehyde and p-hydroxybenzoic acid on oxidation. The content of proteins in magnolia wood estimated from the nitrogen content (0.17%), is about 1.06%, and albumin gave 0.2% and 0.1% of p-hydroxybenzoic acid (HA) and p-hydroxybenzaldehyde (Ha) respectively on nitrobenzene oxidation. This results means that the amount of HA and Ha derived from the proteins in the magnolia wood may be 0.005 μmol from 20mg of wood, corresponding to about one tenth of actual yield (0.06 μmol). Therefore a large part of the HA and Ha must be derived from uncondensed H units in magnolia lignin.

Fig.4 is a microautoradiogram of newly formed xylem of magnolia administered with UDP-glucuronic acid-[glucuronyl-U-^{14}C]. It is known that UDP-glucuronic acid is the common precursors of pectic substance and xylan in woody plants[20]. Two

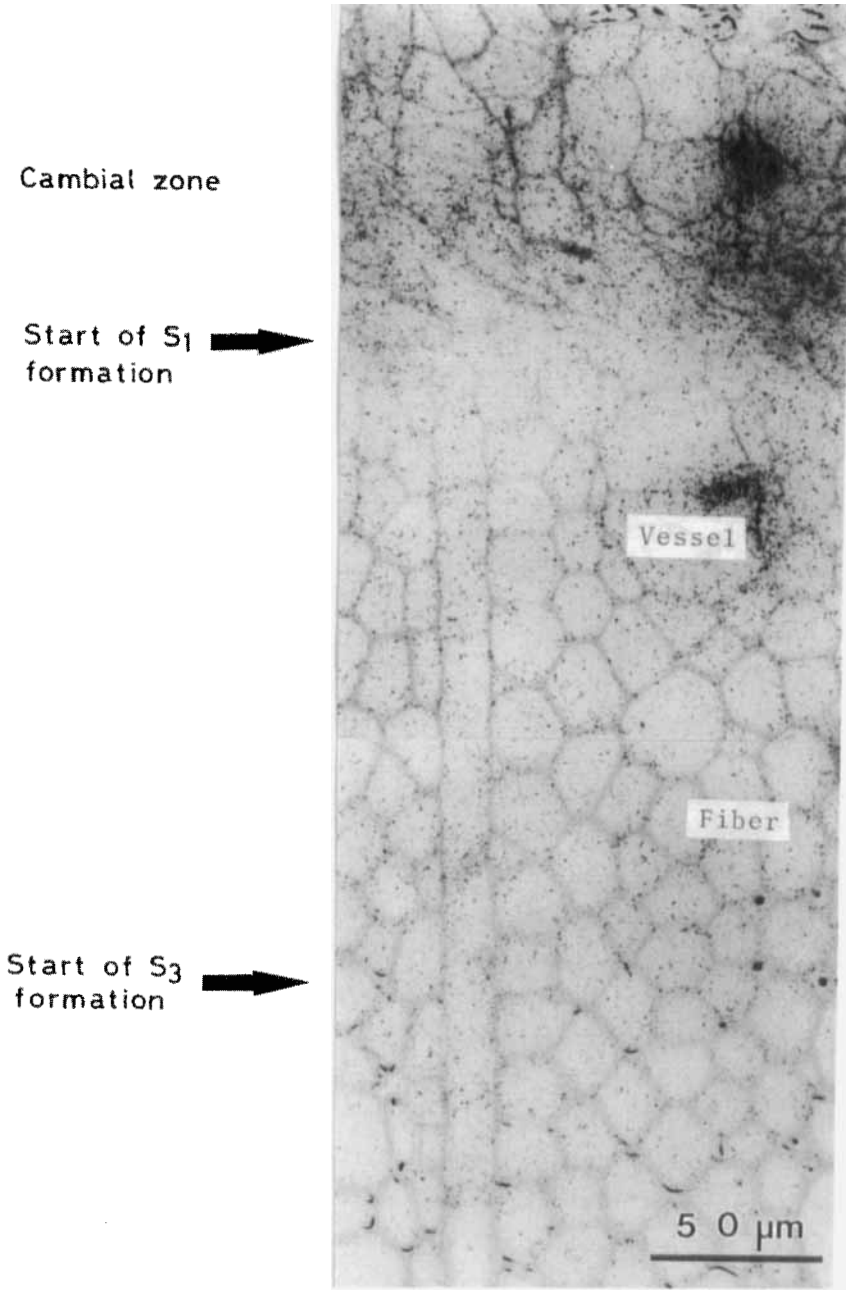


FIGURE 4. Microautoradiogram of differentiating xylem of magnolia administered with UDP-glucuronic acid-[glucuronyl-U-^{14}C].

peaks of incorporation of radioactivity in the xylem can be seen; one at the earliest stage of cell wall formation and the other at the stage of secondary wall formation. The former incorporation is considered to be deposition of pectic substance, and the latter is deposition of xylan. This result is similar to those observed in the case of pine[13]. It is very interesting that lignification always occurs after the deposition of carbohydrates. It seems that CML-lignin is formed in a pectin gel and SW-lignin is formed in a xylan gel.

It is well known that the structure of CML-lignin is different from SW-lignin[1-8,11,12]. This can be explained by the fact that the lignification in CML and SW proceeds under different conditions. Each lignification gives rise to different H/G and S/G ratios of lignin. In addition to the ratios, bonding patterns of each unit are different in the early stage lignification and the late stage lignification. This is supported by the results of dehydrogenative polymerization of coniferyl alcohol under different conditions, in which more condensed structures are formed within a pectin gel than within a xylan gel[21].

Lignification process in lilac

Fig.5a shows the microautoradiogram of newly formed xylem of lilac administered with p-glucocoumaryl alcohol-[arom. ring-2-^{3}H]. Most of the radioactivity is incorporated into CML during the formation of S_1 layer. This indicates that H units deposit mainly in the early stage of cell wall differentiation in CML. While, G and S units deposit not only in CML but also in SW (Fig.5b and Fig.5c).

Lignification process in beech

Fig.6a shows the microautoradiogram of newly formed xylem of beech administered with p-glucocoumaryl alcohol-[arom. ring-2-^{3}H], which visualizes the localization of newly formed H units in

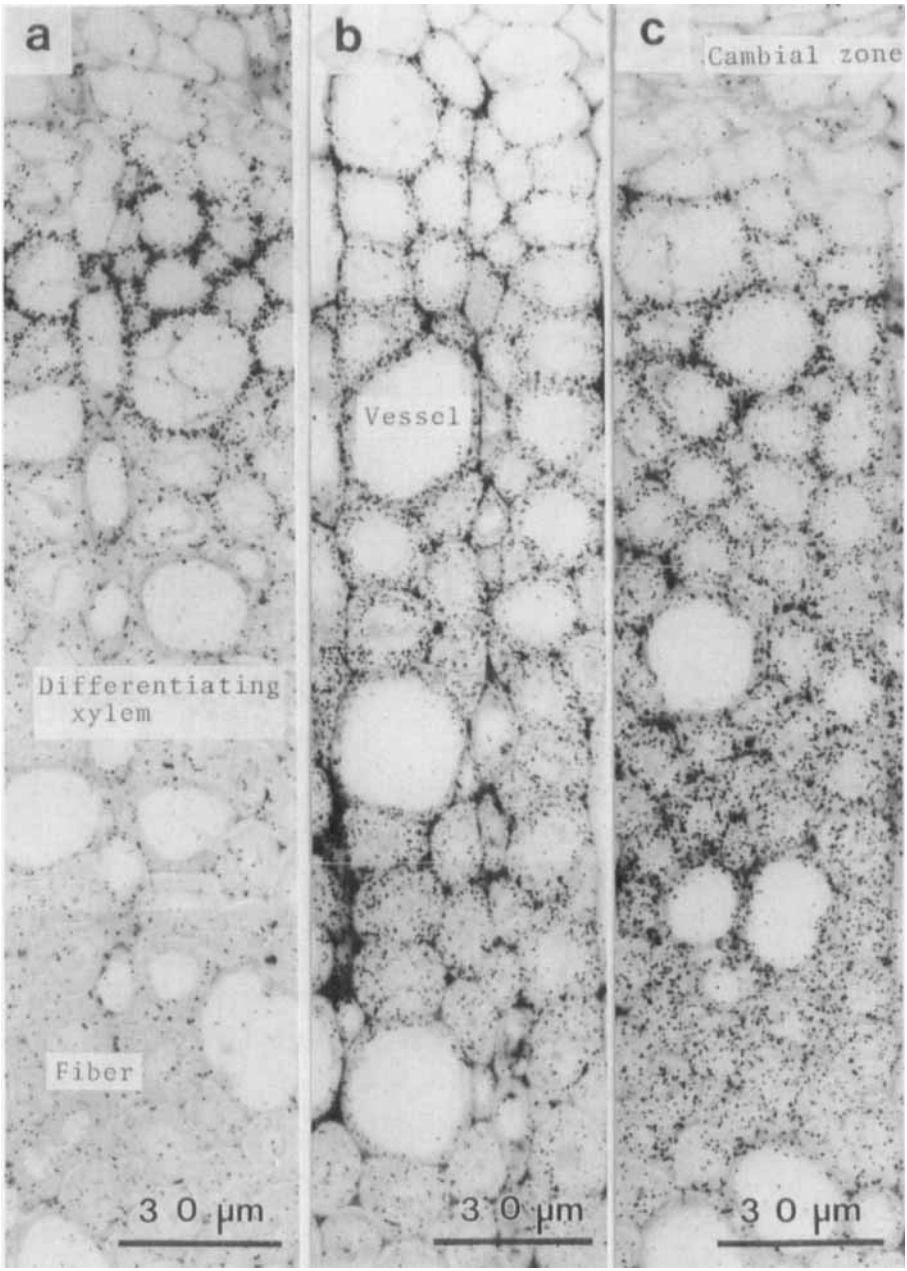
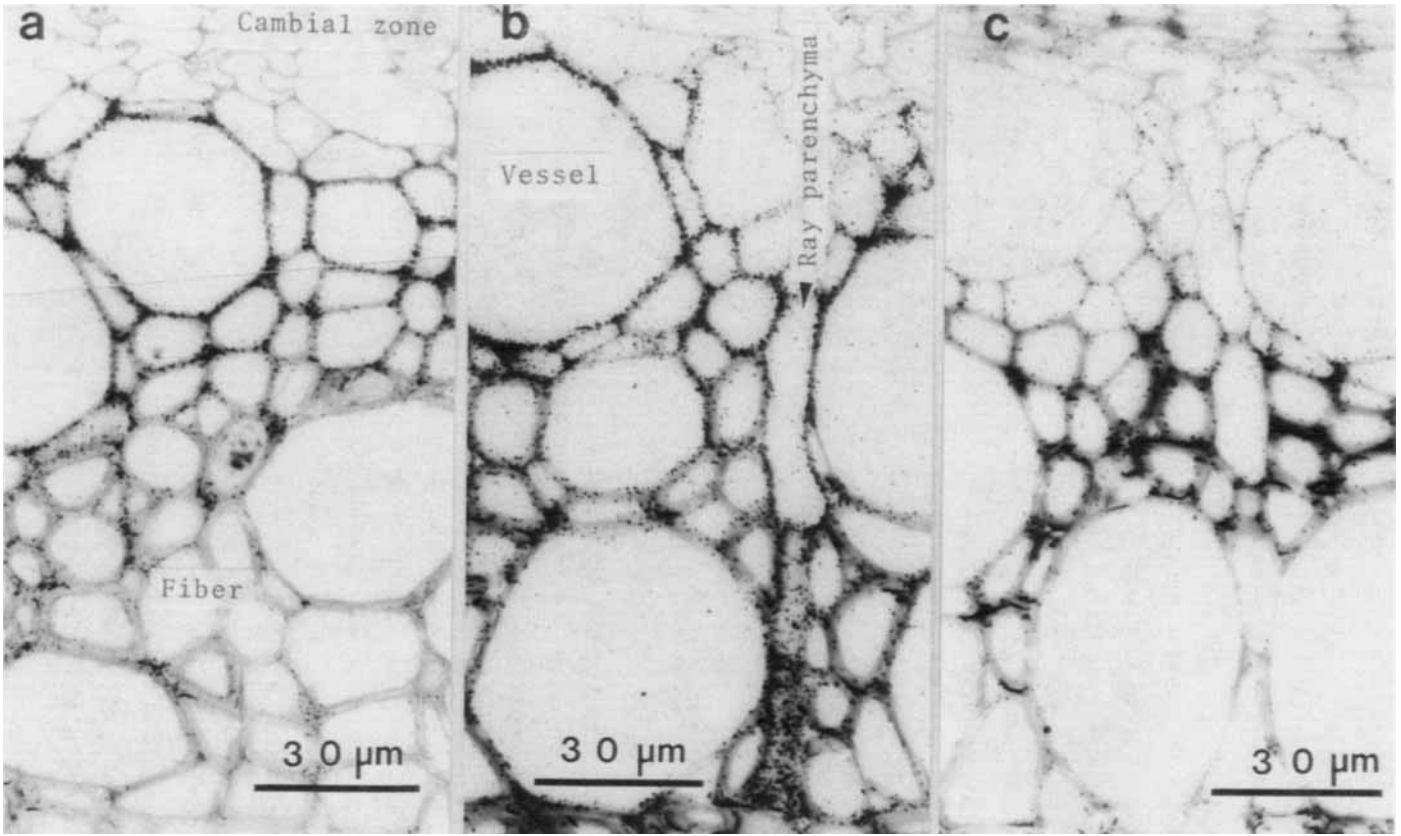


FIGURE 5. Microautoradiograms of differentiating xylem of lilac administered with **a**: p-glucocoumaryl alcohol-[arom. ring-2-^{3}H], **b** : coniferin-[arom. ring-2-^{3}H] or **c**: syringin-[arom. ring-2-^{3}H].



FIGERE 6. Microautoradiograms of differentiating xylem of beech administered with **a:** p-glucocoumaryl alcohol-[arom. ring-2-^{3}H], **b:** coniferin-[arom. ring-2-^{3}H], **c:** syringin-[arom. ring-2-^{3}H].

beech lignin. It is seen that H units deposit in the early stage of lignification. Remarkable incorporation can be seen in the cell corners of fiber and vessel walls during the formation of S_1 layer, but after this stage, incorporation cannot be recognized. Fig.6**b**, a microautoradiogram of beech xylem administered with coniferin-[arom. ring-2-^{3}H], shows the localization of newly formed G units. G units deposit in the cell corners and CML of vessel and fiber walls during formation of the S_1 layer. Also, deposition in the SW of vessels and fibers can be seen in the late stage of lignification. Deposition of G units in ray parenchyma walls also can be seen, and the timing of this lignification is delayed compared with that in fiber or vessel cell walls. Fig.6**c** shows a microautoradiogram of beech xylem administered with syringin-[arom. ring-2-^{3}H]. S units mainly deposit in SW of fibers, but the amount of deposition in cell corners and in the CML is lower than amounts of deposition of other units. These results are similar to those observed in magnolia and lilac.

Lignification process in poplar

Fig.7**a** shows the microautoradiogram of newly formed poplar xylem administered with UDP-glucuronic acid-[glucuronyl-U-^{14}C]. The results here is similar to that reported for magnolia (Fig. 4): radioactivity was mainly incorporated in the cell wall of the cambial zone, but small amounts were incorporated later during formation of secondary wall. Again, we interpret this to mean early formation of pectic substances which are deposited in the primary wall, and later formation of secondary wall xylan. Fig.7**b** shows the microautoradiogram of poplar xylem administered with p-glucocoumaryl alcohol-[arom. ring-2-^{3}H]. The incorporation of activity from this precursor can be seen in the early stage (S_1 formation) of lignification. This is similar to the results observed in the other hardwoods (magnolia and lilac, Fig.1**a** and

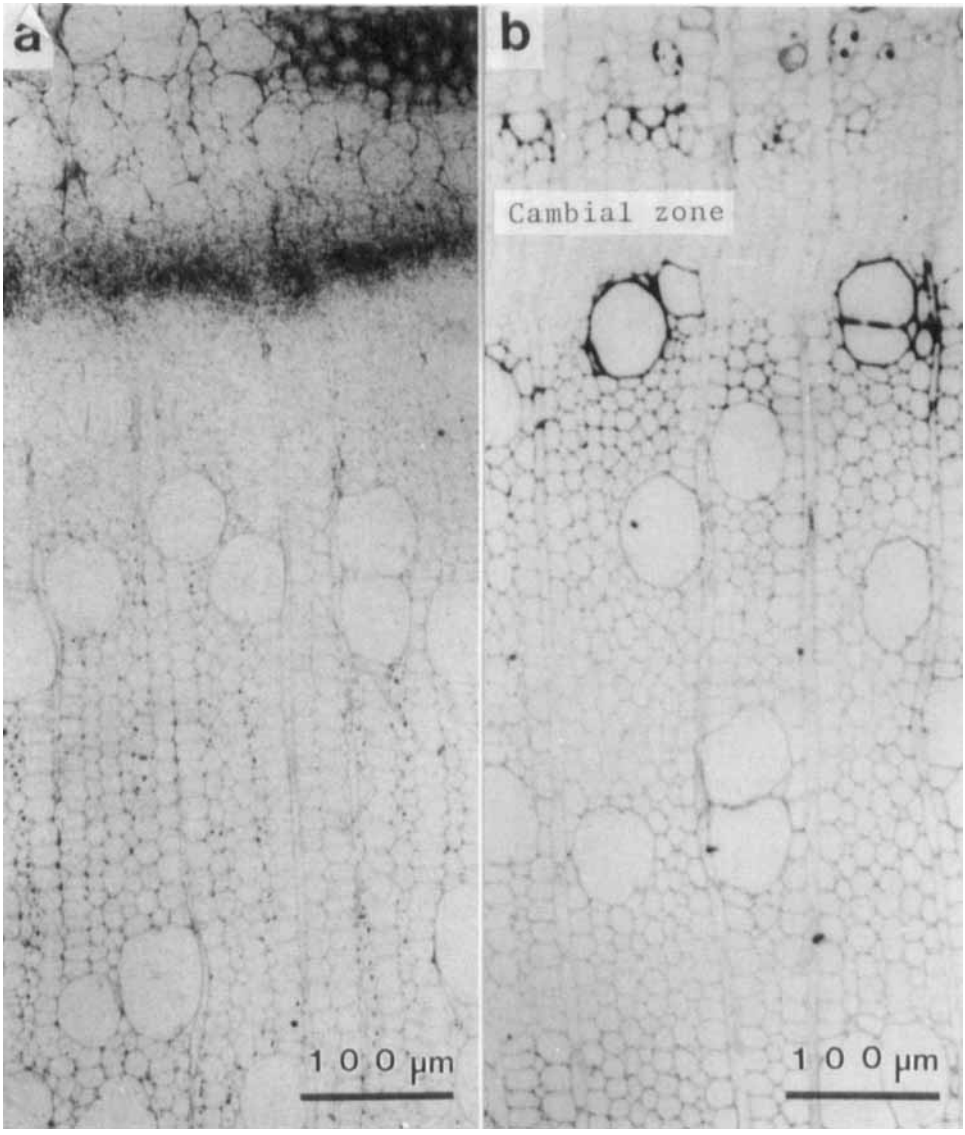


FIGURE 7. Microautoradiograms of differentiating xylem of poplar administered with **a:** UDP-glucuronic acid-[glucuronyl-U-^{14}C], **b:** p-glucocoumaryl alcohol-[arom. ring-2-^{3}H].

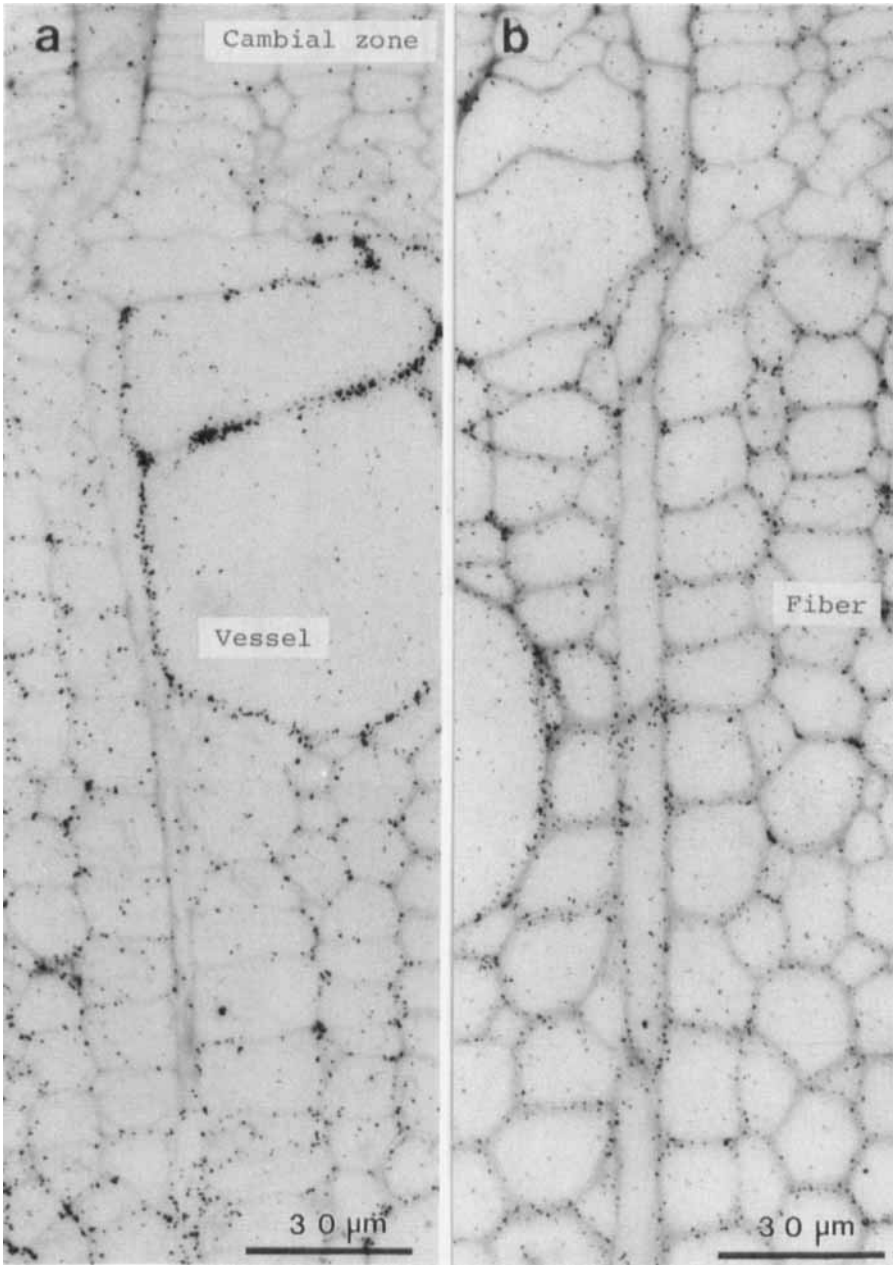


FIGURE 8. Microautoradiograms of differentiating xylem of poplar administered with **a:** coniferin-[arom. ring-2-^{3}H], **b:** syringin-[arom. ring-2-^{3}H].

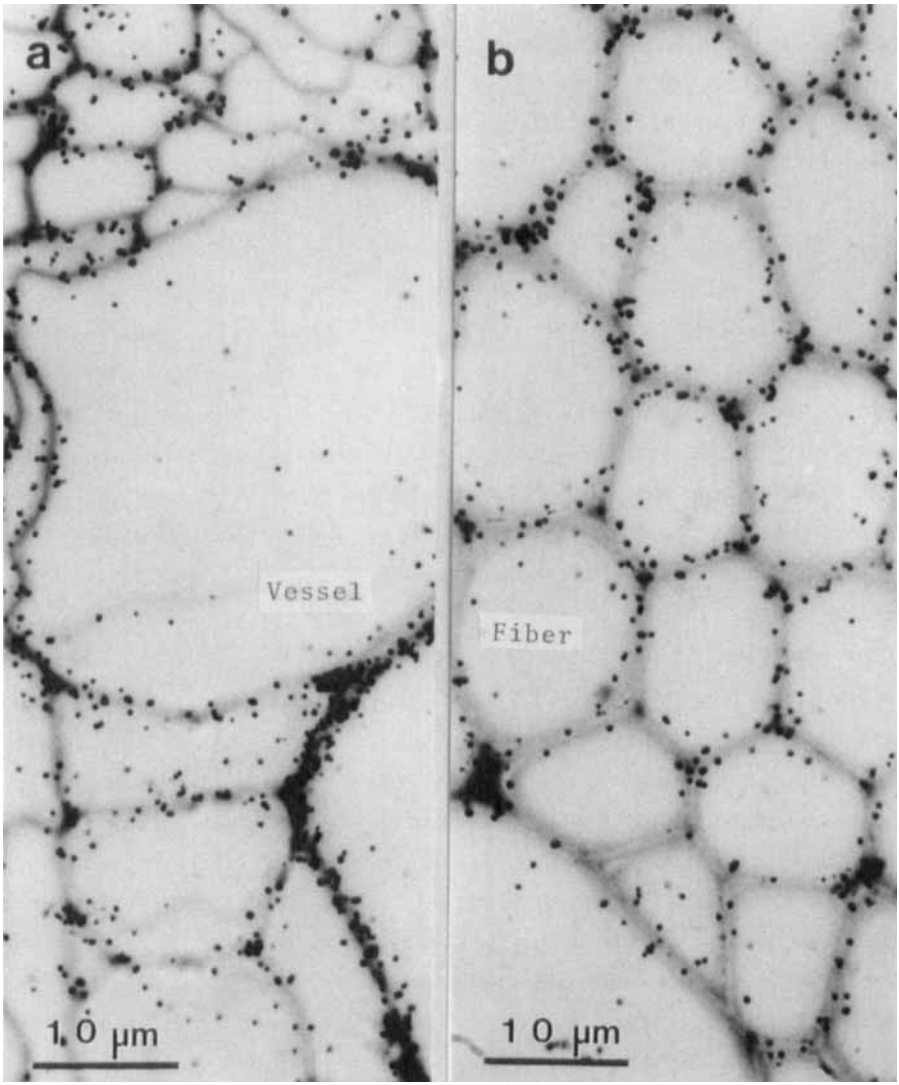


FIGURE 9. Microautoradiograms of differentiating xylem of poplar administered with p-hydroxybenzoic acid-[arom. ring-2-^{3}H]. **a:** Initial stage (formation of S_1 layer) of lignification. **b:** Late stage (after the start of S_3 formation) of lignification.

Fig.5**a**). In poplar, H units deposit almost exclusively in vessel walls, and the lignification of vessel walls occurs earlier than that of fiber. G units in vessel walls are also formed earlier than in fiber (Fig.8**a**). A similar pattern has been observed in the newly formed xylem of poplar administered with ferulic acid-[β-^{14}C][7]. S units mainly deposit in SW of fiber (Fig.8**b**). These results are similar to the case of magnolia, lilac or beech.

It is well known that poplar xylem contains p-hydroxybenzoic acid esterified to other cell wall components. So, it is important to make it clear that how the morphological distributions are different between esterified p-hydroxybenzoic acid and p-hydroxyphenyl propane units in lignin. Fig.9**a** and 9**b** show the microautoradiograms of xylem of poplar administered with p-hydroxybenzoic acid-[arom. ring-2-^{3}H]. The radioactivities from this precursor were incorporated both in CML and SW of differentiating xylem. These deposition patterns are quite different from that observed in the microautoradiogram of poplar xylem administered with p-glucocoumaryl alcohol-[arom. ring-2-^{3}H]. The reason why these two precursors are incorporated in different morphological regions is explained based on its biosynthetic pathway. It has been shown that p-hydroxybenzoic acid moiety in poplar lignin is biosynthesized in the later stage than phenylpropanoids, because the acid is formed from p-coumaric acid by splitting off of C_{β}-C_{γ} side chain carbons[22]. The radioactivity observed in the earliest stage of cell wall differentiation (Fig.7**b**) would not be assigned to esterified p-hydroxybenzoic acid but to H units. The distributions of the two kinds of H units are summarized in Fig.10.

CONCLUSION

p-Glucocoumaryl alcohol, the effective precursor of p-hydroxyphenyl(H) lignin, was radio-labeled and administered to differentiating xylem of four hardwoods, magnolia, beech, lilac

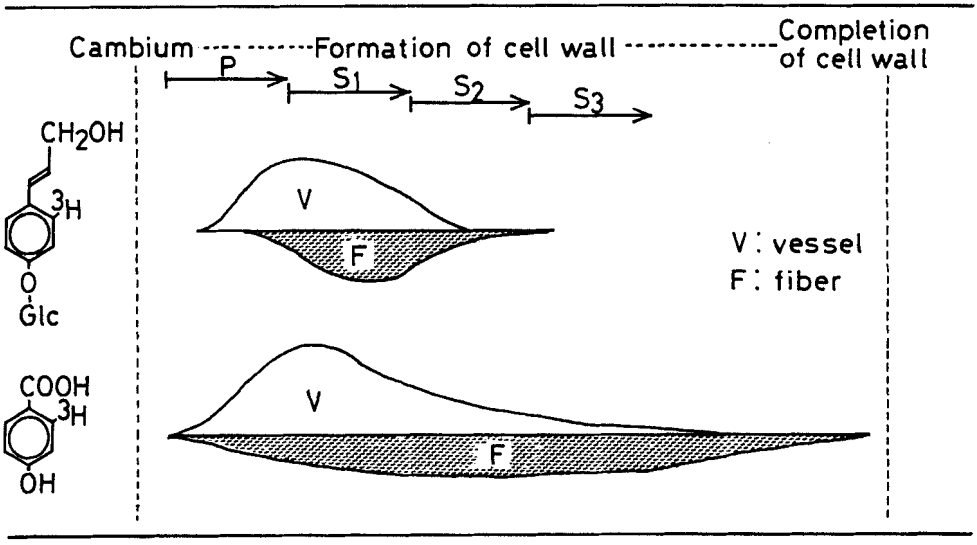


FIGURE 10. Distribution of silver grains in microautoradiograms of differentiating xylem of poplar administered with p-glucocoumaryl alcohol-[arom. ring-2-^{3}H] and p-hydroxybenzoic acid-[arom. ring-2-^{3}H]. P, primary wall; S_1, S_2 and S_3, outer, middle, and inner layer of secondary wall.

and poplar. The distribution of radioactivity in various morphological regions was visualized by microautoradiography. It was shown that H units are formed in CML during the formation of S_1 layer. These results strongly suggest that H units exist in hardwood lignin as one of the main building stones of CML lignin. H lignin is formed after the deposition of carbohydrate, perhaps pectic substance, in the early stage of cell wall formation. Thus, p-coumaryl alcohol polymerizes within a pectin gel to form highly condensed structures which are difficult to degrade by nitrobenzene oxidation.

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