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ORIGINAL ARTICLE

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Profile of bordered pit aspiration in *Cryptomeria japonica* using confocal laser scanning microscopy: pit aspiration and heartwood color

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Abstract Nine trees of *Cryptomeria japonica* from six elite tree clones with a broad range of heartwood colors were selected. The profiles of pit aspiration percentage (ASP) of earlywood and latewood from pith to bark for green and air-dry conditions were determined to study the relationship between heartwood color and pit aspiration. Confocal laser scanning microscopy (CLSM) observations showed that the ASP of earlywood was low in sapwood and high in heartwood in the green condition. Pit aspiration increased in intermediate wood when compared with sapwood. On the other hand, latewood pits did not aspirate during heartwood formation. Comparing the air-dry condition with the green condition, sapwood pits aspirated during drying in both earlywood and latewood; however, there was no significant difference in pit aspiration of heartwood. There was no significant difference between samples with red and black heartwoods for ASP. The difference in ASP between individual trees was larger than that by heartwood color. The general advantage of CLSM over light microscopy is that serial optical sections along the Z axis can be obtained for any moisture condition, without the need for thin sectioning or embedding.

Key words Pit aspiration · Confocal laser scanning microscopy · CLSM · Black heartwood · *Cryptomeria japonica*

Introduction

It is well known that the state of bordered pits on tracheid walls affects the gas and liquid permeabilities of softwoods. Côté[1] described three changes in pits in relation to heartwood formation. First, pit aspiration, in which the torus is held against the pit border, mechanically blocks the pit aperture. Second, pit occlusion can occur due to extractives. Third, pit encrustation can occur with lignin-like substances that are insoluble in both hot water and alcohol. Pit aspiration also occurs when green sapwood dries up to the fiber saturation point.[2] Hart and Thomas[3] proposed that pit aspiration is caused by capillary tension. According to Siau,[4] the factors that contribute to pit aspiration are: (1) pit membranes must have small openings and low rigidity, such as in earlywood, (2) the evaporating liquids must be capable of forming hydrogen bonds and must have both donor and acceptor properties, (3) the liquids must swell wood nearly as much as or more than water, and (4) the surface tension and contact angle relationship must be such that the capillary force is sufficient to cause pit membrane displacement. These studies may indicate that there is a causal relationship between green moisture content (GMC) and pit aspiration.

Variation of heartwood color between the cultivars of *Cryptomeria japonica* is large from pale red to black. Black color restricts the use of heartwoods for decorative purposes. Because of the high GMC, drying and transport costs of such logs are high. In general, black heartwood has much higher GMC than normal red heartwood,[5] the GMC being nearly as much as in the sapwood, in spite of low moisture content in intermediate wood called the "white zone." The information available to date suggests a significant correlation between the brightness of heartwood and its GMC.[6–8] However, the exact reason for a high moisture content of black heartwood is not clear. Some researchers[9–11] studied the relationship between GMC and the percentage of aspirated pits, but conflicting results were obtained. This is probably because of the technical difficulties in observing and quantifying pit aspiration in a large number of pits. Consequently, the relationship between pit aspiration and heartwood color has not yet been clarified.

The purpose of this study was to explore whether there is any relationship between pit aspiration and heartwood color in *Cryptomeria japonica*. Six elite tree clones differing

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Table 1. Information on test trees

Sample no.	Clone name	Diameter at breast height (cm)	Tree height (m)	Heartwood brightness (mean ± SD)	Heatwood color
R-1	Obisho9	15	12	71.8 ± 2.5	red
R-2	Obisho9	13	11	70.6 ± 1.4	red
R-3	Kakutosho1	16	12	69.7 ± 2.4	red
M-1	Kenaira1	17	13	64.3 ± 5.5	middle
M-2	Kakutosho1	19	14	64.3 ± 1.8	middle
M-3	Kenhioki6	15	12	62.7 ± 2.8	middle
B-1	Kenkoyu3	26	15	52.6 ± 1.5	black
B-2	Obisho5	14	11	47.6 ± 3.0	black
B-3	Obisho5	14	11	46.3 ± 1.0	black

SD, standard deviation

in their heartwood color were selected. Confocal laser scanning microscopy (CLSM) was used to obtain the percentage of aspirated pits for each growth ring from pith to bark, and to develop the profile in the radial direction. The validity of CLSM for observing aspirated pits was also examined because this method has not been previously used for this purpose.

Materials and methods

Wood samples

Nine *Cryptomeria japonica* trees from six elite tree clones that were 30 years old and 13–26 cm in diameter at breast height (DBH) were selected in Shikayu National Forest in Koyu-gun, Miyazaki prefecture. Sample information is provided in Table 1. According to the brightness of heartwood, three trees with red heartwood, three trees with black heartwood, and three trees with heartwood color between red and black were classified.

Disks (15 cm thick) from each tree were obtained at 3 m height above the ground and were put into plastic bags to prevent them from drying, and then brought to the laboratory. Each disk was cut into a board (4 cm wide) including the pith and the external growth rings. Each board was sliced transversely and end-matched strips were prepared. One strip (3 cm thick) was for GMC and heartwood brightness, another (3 cm thick) was for pit aspiration in the green condition, and the other was for pit aspiration in the air-dry condition. The brightness in the tangential surface of each ring in the air-dry heartwood was measured with a colorimeter (Minolta CR-300). The averages of each tree are listed in Table 1.

Observation of bordered pits by CLSM

Four sticks were cut from each growth ring of a strip sample for measuring aspirated pits of earlywood and latewood. Transverse sections, 50 μm thick, were prepared from the green and dry sticks using a sliding microtome. The sections were mounted in a drop of immersion oil. Sections were observed by confocal laser scanning microscopy (CLSM) using Bio-Rad Radiance 2000 and an oil immersion lens (×60). Images were acquired using an argon laser at 512 × 512 pixel resolution and excitation wavelengths of 488 and 514 nm, with an emission filter (HQ500LP). The scan speed was 25 Hz. Sections were examined using the autofluorescence of the lignified cell walls.

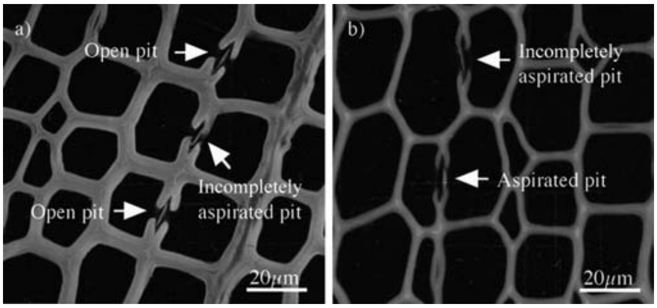

Fig. 1a,b. Images of the state of bordered pits obtained by confocal laser scanning microscopy (CLSM). *Bars* 20 μm

Sixty earlywood and 20 latewood bordered pits were observed in each growth ring. Aspirated pits, incompletely aspirated pits in which pit membranes were deflected to one side, and open pits (Fig. 1) were counted and the percentage of aspirated pit (ASP) was calculated by dividing the number of aspirated pits by the number of counted pits and multiplying by 100. Incompletely aspirated pits were not included as aspirated pits in this study.

Results and discussion

Profile of pit aspiration from pith to bark

The profile of pit aspiration from pith to bark is shown in Figs. 2, 3, and 4. In the green condition, the ASP of earlywood was low in sapwood and high in heartwood. The profile indicates that bordered pits aspirated in the intermediate wood. This means that bordered pits aspirated during heartwood formation in the living tree, as reported earlier.[1,12,13] On the other hand, the profile for latewood shows that there may be no clear relationship with heartwood

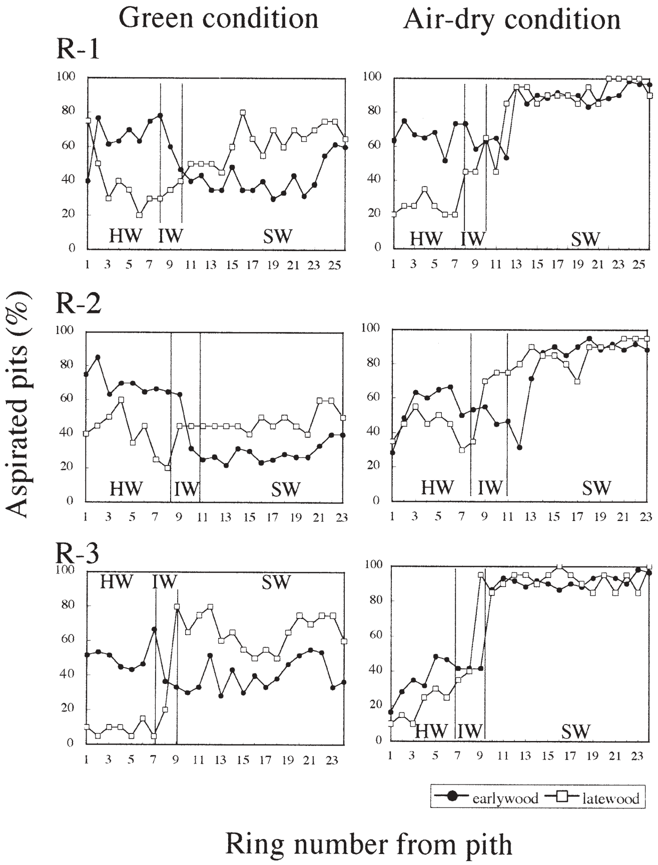

Fig. 2. The profiles of aspirated pits in the radial direction in *Cryptomeria japonica* with red heartwood (R-1–R-3). *HW*, heartwood; *IW*, intermediate wood; *SW*, sapwood

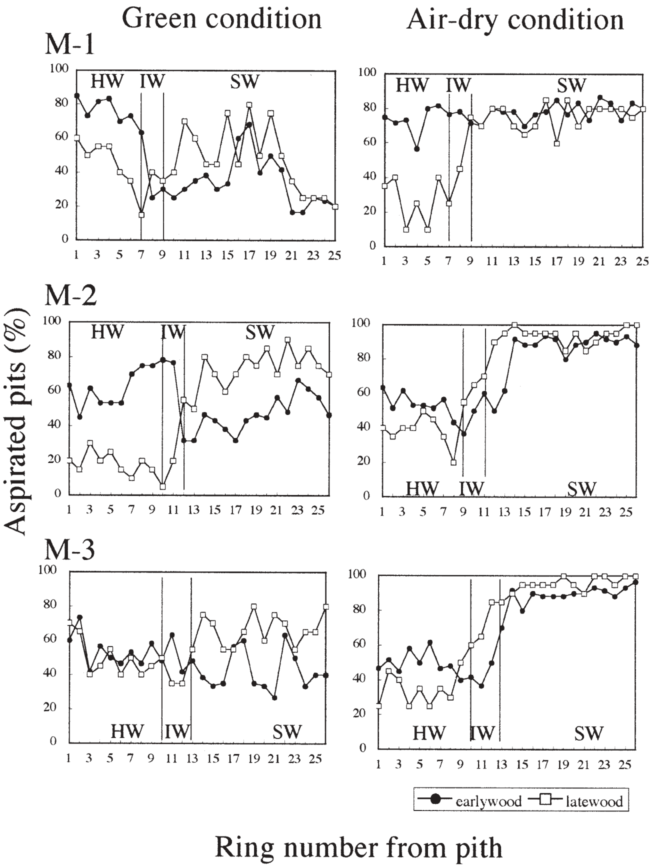

Fig. 3. The profiles of aspirated pits in the radial direction for green and air-dry conditions in *Cryptomeria japonica* with heartwood colors between black and red (M-1–M-3)

formation. This result is different from that reported[13] for Chinese yezo spruce, which indicated that 87% of latewood pits in heartwood aspirated in the green condition. Lin[14] reported that the percentage of aspirated pits in latewood in radiata pine heartwood was low and about 20%.

Comparing the air-dry and green conditions, the ASP in the air-dry wood was larger in both earlywood and latewood in sapwood. This means that bordered pit aspiration occurs during drying. These results are basically consistent with previous findings.[12,13] For intermediate wood, the ASP of latewood increased during drying. For heartwood, there was little difference in ASP between green and air-dry conditions. However, there was a large variation in ASP among growth rings in the radial direction and the profiles were different among trees.

Green and air-dry woods

The ASP values under green and air-dry conditions are listed in Tables 2 and 3. These values were calculated from the profiles (Figs. 2–4). A *t*-test was carried out to determine the effect of drying on pit aspiration. For sapwood earlywood and latewood, there was a highly significant difference at the 0.1% level between the air-dry and green conditions. For intermediate wood, only latewood showed a highly significant difference at the 0.1% level. This means that latewood pits in intermediate wood aspirated during drying in spite of the rigidity of the latewood membrane. On the other hand, for heartwood earlywood and latewood, there was no significant difference in pit aspiration between green and air-dry conditions.

Bordered pit aspiration and heartwood color

For the green condition, the ASP of red heartwoods was 61.2% and 30.7% in earlywood and latewood, respectively. On the other hand, those of black heartwood were 67.1% and 36.4%. For heartwood with middle color they were 64.1% and 39.6% (Tables 2, 3). For all samples, the ASP of earlywood ranged from 48.6% to 70.7% in red heartwood and from 58.6% to 80.0% in black heartwood. These results mean that there was little difference in the percentage of aspirated pits between red heartwood and black heartwood for the green condition. Kubo et al.[11] reported that opening pits were frequently observed in typical black heartwood and that there was significant correlation at the 1% level between the opening rate and GMC in heartwood. On the other hand, Fujii et al.[10] concluded that pit aspiration was

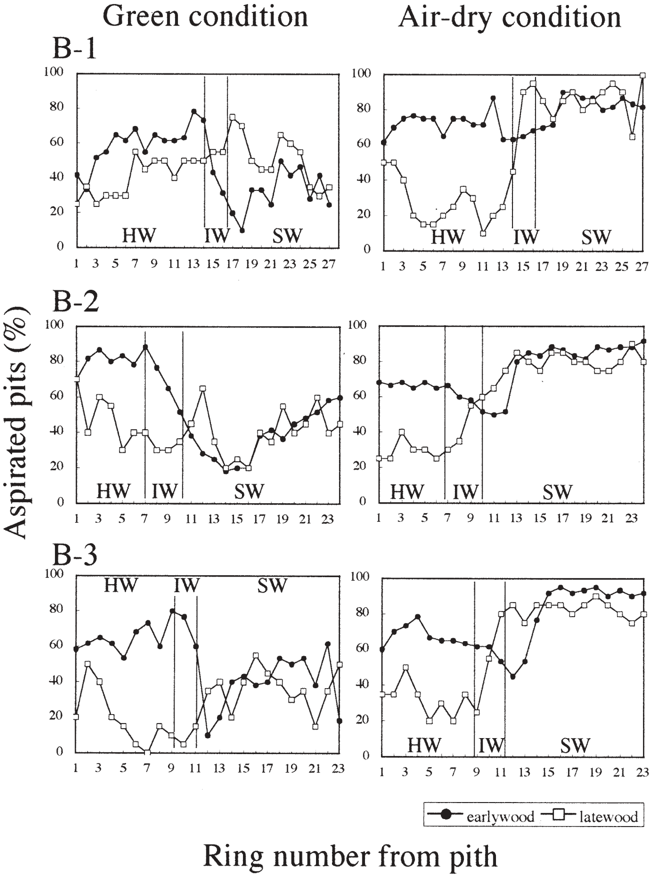

Fig. 4. The profiles of aspirated pits in the radial direction in *Cryptomeria japonica* with black heartwood (B-1–B-3)

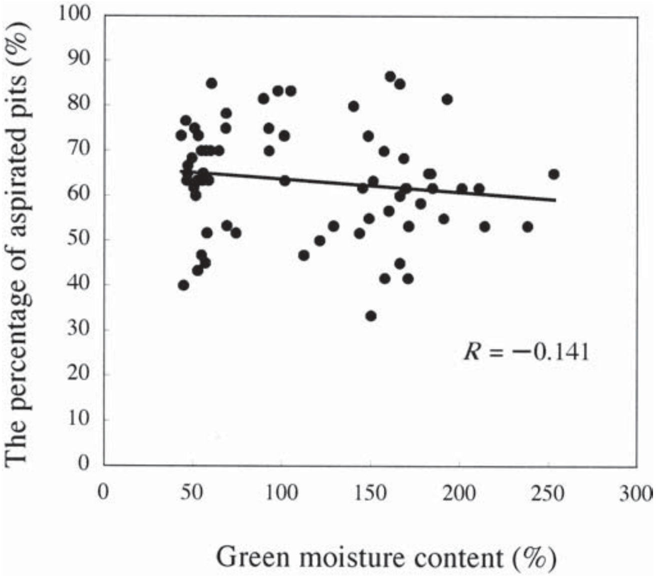

Fig. 5. Relationship between green moisture content (GMC) and the percentage of aspirated pits (ASP) in heartwood

not related to the heartwood color or to GMC. The results of analysis of variance (ANOVA) in this study indicated that there was no significant difference among red, middle color, and black heartwoods. Also, there was no significant correlation between ASP and GMC (Fig. 5). The correlation coefficient was −0.141 in earlywood and 0.183 in latewood. The difference among individual trees was larger rather than that by heartwood color. In both earlywood and latewood, there was a significant difference at the 0.1% level among individual trees. Therefore, we concluded that there is no relationship between pit aspiration and heartwood color formation.

Validity of CLSM for observation of pit aspiration

In the field of wood science, CLSM was initially used as a three-dimensional imaging technique for examining xylem cells[15] and pulp fibers.[16] Subsequently, its applications extended to analyzing wood cell structure[17–19] and fiber morphology,[20–22] and to visualizing liquid flow pathways in wood.[23,24]

Pit aspiration has generally been examined using optical microscopes. To judge the condition of pit aspiration under an optical microscope, it is necessary to make sections thinner than the diameter of the pit aperture and the preparations involve embedding of specimens in resin. During the process of solvent exchange after resaturation in water, the condition of pit aspiration can be affected. Furthermore, in obtaining a lot of data, mechanical sectioning with a microtome becomes very time consuming. Confocal laser scanning microscopy, which allows observation of internal structures along the Z axis in specimens, makes it possible to observe thick sections without embedding, and under a range of moisture conditions. Therefore, being a nondestructive tool, CLSM has distinct advantages over conventional optical microscopy in studying pit aspiration. However, when pit aspiration is observed by CLSM, it may be preferable to observe much thicker sections using a dry lens. The thinner a section is, the larger the effect of sectioning stress on the pit structure becomes. There is also the possibility that immersion oil affects the condition of aspirated pits. It is necessary to consider these problems. The preliminary experiment in this study showed that there was no difference in the results between 10-mm cubes and 50-μm and 100-μm-thick sections, and also between the observations with a dry lens (×60) and an oil immersion lens (×60).

When only one section, as in Fig. 6a, is seen, it is difficult to judge whether the torus is blocking the pit aperture, and therefore it is not possible to measure pit aspiration. However, because it is possible to obtain serial optical sections along the Z axis (Fig. 6a–d), measurements can be made on a large number of pits. Thus, CLSM is a powerful tool for studying pit aspiration.

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Table 2. Percentage of aspirated pits for the green condition

	Heartwood		Intermediate wood		Sapwood	
	EW	LW	EW	LW	EW	LW
R-1	64.3	40.0	61.7	35.0	41.6	62.8
R-2	70.7	42.9	46.3	38.8	29.4	47.9
R-3	48.6	9.2	45.6	35.0	40.3	65.0
Rav	61.2	30.7	51.2	36.3	37.1	58.6
M-1	77.8	49.2	39.4	30.0	34.6	47.8
M-2	61.1	18.9	62.2	26.7	47.4	73.9
M-3	53.5	50.6	52.9	41.3	42.4	66.1
Mav	64.1	39.6	51.5	32.6	41.4	62.6
B-1	58.6	39.6	49.4	53.3	32.3	51.4
B-2	80.0	49.2	70.4	33.8	37.9	40.7
B-3	62.7	20.6	72.2	10.0	38.9	36.7
Bav	67.1	36.5	64.0	32.4	36.3	42.9

EW, earlywood; LW, latewood; Rav, average calculated from all rings of the red wood; Mav, average calculated from all rings of middle wood; Bav, average calculated from all rings of black wood

Table 3. Percentage of aspirated pits for the air-dry condition

	Heartwood		Intermediate wood		Sapwood	
	EW	LW	EW	LW	EW	LW
R-1	66.2	24.3	65.0	51.7	86.8	89.4
R-2	54.5	43.6	50.0	63.8	83.2	87.1
R-3	34.4	19.2	41.7	56.7	91.6	92.0
Rav	51.7	29.0	52.2	57.4	87.2	89.5
M-1	73.1	26.7	75.6	48.3	78.5	75.6
M-2	52.4	40.0	53.3	75.0	88.0	94.3
M-3	51.0	32.5	42.1	65.0	88.6	95.4
Mav	58.8	33.1	57.0	62.8	85.0	88.4
B-1	72.4	27.3	65.6	76.7	82.6	85.5
B-2	66.9	29.2	59.2	45.0	81.0	79.3
B-3	67.7	32.5	58.9	53.3	83.9	82.5
Bav	69.0	29.7	61.2	58.3	82.5	82.4

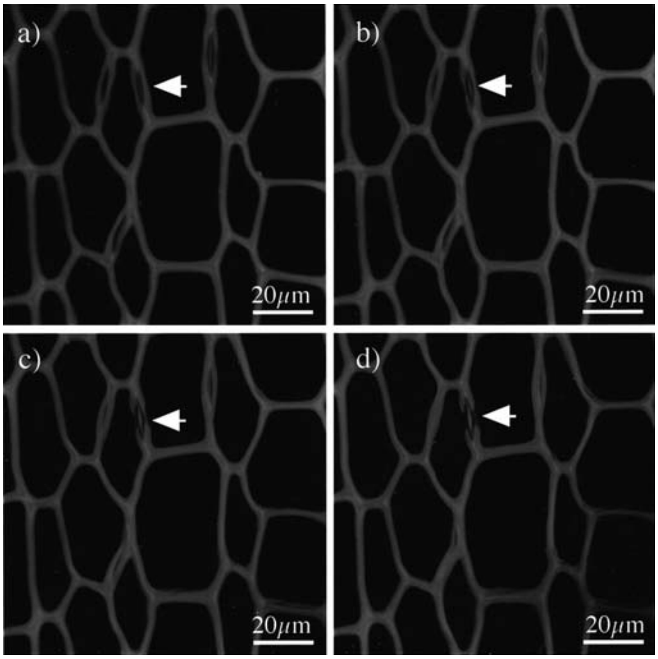

Fig. 6a–d. An example of serial optical images by CLSM. Note the change of the bordered pit (*arrows*). The images deepen by 1 μm along the Z axis from **a** to **d**

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