SUMMARY
The cellular distribution of heartwood substances and the structure of the pathways for their diffusion were studied in *Acacia mangium* Willd. Apart from ray parenchyma cells, axial parenchyma cells also are involved in the formation of heartwood substances. Heartwood substances were unevenly distributed in the heartwood. A closer inspection of interfibre pit pairs revealed that, although many pit membranes were completely covered with encrusting materials, some pit pairs had many small openings on their pit membranes. The openings possibly function as intercellular diffusion pathways for heartwood substances. The sizes of the pits varied considerably, ranging from 0.4 to 2.3 µm in diameter. These structural variations in the interfiber pits might be one of the factors contributing to the uneven distribution of the heartwood substances. A large number of blind pits were present in the ray parenchyma cells and faced the intercellular spaces, into which heartwood substances from the ray parenchyma cells were released via these blind pits. Resin-cast replicas demonstrated that the intercellular spaces and the blind pits formed a three-dimensional network that is considered to serve as an extracellular diffusion pathway for heartwood substances.

Key words: Heartwood substances, diffusion pathway, *Acacia mangium*, interfiber pit, blind pit, intercellular space, resin-cast replica, three-dimensional network.

INTRODUCTION
Currently, *Acacia mangium* Willd. is one of the most important plantation tree species in tropical regions. It grows fast and forms heartwood at a comparatively early stage of growth. *Acacia mangium* wood is also known as a multipurpose hardwood with the potential of using for wattle timber, construction, boat building, moldings, particle board, firewood, charcoal, furniture and for paper making (Lemmens et al. 1995). A medium brown color of the heartwood, which is due to the presence of chromophoric extractives, is one of the visually appealing features of this wood. Generally, heartwood...
is more resistant to decay; thus, knowledge regarding the composition of heartwood substances as well as knowledge regarding their location and concentration would facilitate understanding of wood quality parameters such as natural durability and color. Therefore, various studies have been conducted on heartwood formation as well as on the transportation and distribution of heartwood substances (El Sherbeiny et al. 1971; Hillis 1971, 1987; Kuo & Arganbright 1980; Nobuchi et al. 1984; Nobuchi & Harada 1985; Nobuchi 1985; Magel et al. 1991; Kleist & Bauch 2001; Zhang et al. 2004, 2006; Koch et al. 2006; Mayer et al. 2006; Carrillo et al. 2008). Kleist and Bauch (2001) reported that the composition and the concentration of the extract in fiber tissue affect the natural resistance of Sapelli (Entandrophragma cylindricum) heartwood to fungal decay. Zhang et al. (2004) found that the heartwood substances of Albizia julibrissin are not evenly deposited throughout the heartwood. They also discovered that not all interfiber pit pairs permit the diffusion of heartwood substances. Further microscopic examination of the interfiber pit pairs revealed considerable structural variations, which were inferred to be the causes for the uneven distribution of the heartwood substances (Zhang et al. 2006). Koch et al. (2006) provided strong evidence for impregnation of pit membranes and canals by phenolic extractives in merbau heartwood, and Mayer et al. (2006) demonstrated that the phenolic substances responsible for coloration were mainly located in ray and longitudinal parenchyma cells in American black cherry. Here we report on a study of Acacia mangium attempting to reveal the structure of the diffusion pathways and the distribution of heartwood substances.

MATERIAL AND METHODS

Plant material

A 6-year-old Acacia mangium tree (diameter at breast height: 10.4 cm; height: 1084 cm) growing in the Malay Peninsula was used in this study. Fresh wood blocks were obtained from the trunk at breast height and fixed immediately with 3% glutaraldehyde in 0.07 M phosphate buffer at pH 7.2. These fixed specimens originated from juvenile wood and were used for the following examinations.

Light microscopy

Transverse sections (30 µm thick) of the outer sapwood, sapwood-heartwood transition zone, and inner heartwood were cut and mounted in gum syrup without staining.

Transmission electron microscopy

Small blocks (T × R × L, approximately 1 × 1 × 2 mm³) of the sapwood and heartwood were washed with distilled water and stained with 2% aqueous ferric sulfate solution for 3 days at room temperature, as described by Kuo and Arganbright (1980). After washing with distilled water, the stained blocks were dehydrated in a graded ethanol series, infiltrated with propylene oxide, and then embedded in epoxy resin. Ultrathin sections (about 90 nm thick) were cut using a diamond knife; the sections were stained with 2% aqueous uranyl acetate, post-stained with Reynolds’ lead citrate, and examined under a transmission electron microscope (TEM; JEM-1220; JEOL, Tokyo, Japan) at an accelerating voltage of 100 kV.
Field-emission scanning electron microscopy
Small cubes (about 5 mm³) were cut from the sapwood. To maintain the natural structure of the paired pits as much as possible, the cubes were dried with a freeze-drying apparatus. These freeze-dried blocks were split along the radial-longitudinal plane as described by Sano et al. (1998, 1999), mounted with carbon cement on specimen stubs, and coated with platinum by vacuum evaporation. Both faces of the split pit pair were identified by comparing the images on two monitors of field-emission scanning electron microscope (FE-SEM; JSM-6301F; Jeol Co. Ltd., Tokyo, Japan) at an accelerating voltage of 2.5 kV and at a working distance of 8 or 15 mm, and then photographed.

Preparation of resin cast replicas
Small sapwood cubes (about 3 mm³) were dehydrated in a graded ethanol series. Further, a styrene monomer resin with 1% benzoyl peroxide was also used for the preparation of resin-cast replicas. The cubes were then infiltrated with increasing concentrations of the resin in ethanol (25%, 50%, 75%) for 1 hour per solution; and with 100% resin, 3 times for 1 hour each. Finally, the cubes were placed in gelatin capsules filled with the resin, and the resin was polymerized at 60 °C for 3 days. The resin-embedded cubes were trimmed to expose the xylem tissues on 5 surfaces and then treated with a mixture of hydrogen peroxide and glacial acetic acid (v:v, 1:1) to remove lignin, followed by treatment with 97% sulfuric acid to remove cell-wall polysaccharides (Kitin et al. 2001). The resin-cast replicas were then carefully rinsed with distilled water, freeze dried, and observed under a scanning electron microscope (SEM; JSM-T330A; JEOL, Tokyo, Japan) at an accelerating voltage of 10 kV.

RESULTS AND DISCUSSION
Cellular distribution of heartwood substances
Acacia mangium has colored heartwood and a distinct boundary between the sapwood and heartwood. This feature enables distinguishing between the sapwood, sapwood-heartwood transition zone, and heartwood. A light microscope was used to observe these three regions.

Unstained transverse sections of the sapwood (Fig. 1a) appeared transparent and colorless, whereas the sections prepared from the transition zone (Fig. 1b) and the heartwood (Fig. 1c) appeared yellowish brown; the color gradually darkened toward the inner heartwood. It is well known that heartwood substances are generally synthesized in the ray parenchyma cells present in the sapwood-heartwood transition zone, and then diffuse into the neighboring heartwood tissues. Wood color is ascribed to the composition and structure of extractives. Mayer et al. (2006) reported that the coloration of American black cherry heartwood is caused by phenolic substances located in parenchyma cells and vessels. Therefore, the above mentioned natural color of the transition zone and the heartwood is most probably due to colored heartwood substances, and the intensity of the color is indicative of the amount of colored heartwood substances deposited there.
Furthermore, the color intensity was uneven in the heartwood, as shown in Figure 1c. This irregularity of color indicates that colored heartwood substances are not uniformly distributed in the heartwood. Similar uneven distribution of the heartwood substances was also observed in *Albizia julibrissin* (Zhang et al. 2004, 2006). Mayer et al. (2006) described that inhomogeneity in color of American black cherry impairs decorative applications and causes depreciation.

In the outermost part of the transition zone, colored heartwood substances appeared in axial parenchyma cells, particularly in the cell walls and the intercellular spaces (arrowheads in Fig. 2), before appearing in ray parenchyma cells. As shown in Figure 1b, the color in the axial parenchyma cells mostly in the cell walls and the intercellular spaces (arrowheads) were stronger than that in the ray parenchyma cells (arrows). These findings suggest that besides ray parenchyma cells, axial parenchyma cells are also involved in the formation of heartwood substances in *A. mangium*.

As to the colored heartwood substances which exist in the intercellular spaces, there is a possibility that heartwood substances are diffused into the intercellular spaces in colorless form, and that they turn brown on oxidation within the aerated environment.

Figure 1. Unstained transverse sections (30 µm thick) of *Acacia mangium*. The sapwood region (a) appears transparent and colorless, whereas the transition zone (b) and heartwood region (c) appear yellowish brown and become gradually darker towards the heartwood. Moreover, in the transition zone (b) the intensity of the color in the axial parenchyma cell walls and the intercellular spaces (arrowheads) is greater than that in the ray parenchyma cells (arrows). The color intensity in the heartwood region (c) is non-uniform. — Scale bar = 100 µm.

Figure 2. Unstained transverse section (30 mm thick) of the outermost part of sapwood-heartwood transition zone. Colored heartwood substances (arrowheads) appeared in the axial parenchyma cell walls and the intercellular spaces, while such substances are not apparent in the ray parenchyma cells. – F: wood fiber; V: vessel; R: ray parenchyma cell; AP: axial parenchyma cell. — Scale bar = 100 µm.
Our previous studies revealed that axial intercellular spaces are more spacious than radial intercellular spaces. This fact has been confirmed in this study also (Fig. 7a, b). Accordingly, coloration of the heartwood substances might show up more clearly in the axial system than in the radial system.

Kuo and Arganbright (1980) as well as Zhang et al. (2004, 2006) have reported that ferric sulfate is an effective reagent to fix and stain phenolic substances, enabling their use in the detection of heartwood substances. Hence, in this study, ferric sulfate-stained ultrathin sections were examined under a transmission electron microscope. In accordance with the results of the light microscopy, strongly-stained substances were absent in the sapwood (Fig. 3a) while they were present in the heartwood. As shown in Figure 3b & c, ferric sulfate-positive substances were detected in the luminal surface of wood fibers, axial parenchyma cells, and intercellular spaces. And furthermore, all the cell walls in the heartwood were darker than those in the sapwood.

Figure 3. Ultrathin transverse sections of ferric sulfate-stained sapwood and heartwood. Strongly stained substances (arrowheads) are not observed in the sapwood (a); however, they are abundant in the heartwood (b, c). Additionally, a large number of intercellular spaces are present among the axial elements. – I: intercellular space; F: wood fiber; AP: axial parenchyma cell. — Scale bar (a) = 5 µm, (b) = 1 µm; (c) = 200 nm.

Figure 4. Complementary pairs of radially split faces of a pit pair of Albizia julibrissin (a, b) that were photographed in the previous study, and of A. mangium (c, d). Arrow: centrally perforated pit membrane without incrusting materials; asterisk and arrowheads: pit membrane with incrusting materials. — Scale bars = 1 µm.
Field-emission scanning electron microscopy of interfiber pits

Zhang et al. (2004) and Wan et al. (2006) have reported the following two types of pathways for the diffusion of substances: intercellular and extracellular. Generally, a pit pair refers to two pits between adjacent cells, and three major types are regarded as intercellular diffusion pathways: pit pairs formed between adjacent parenchyma cells with simple pits (simple pit pairs), pit pairs formed between the parenchyma cells and adjacent cells with bordered pits (half-bordered pit pairs), and pit pairs formed between adjacent cells with bordered pits (bordered pit pairs). However, Zhang et al. (2004) found that not all bordered pit pairs served as intercellular diffusion pathways for heartwood substances. They considered this phenomenon to be the cause of the non-uniform cellular distribution of heartwood substances in Albizia julibrissin. What was the reason for this difference in function among bordered pit pairs? Sano et al. (1998, 1999), and Sano and Jansen (2006) investigated the structure of pits in both softwoods and hardwoods and reported that there are variations in the structure of intertracheary and interfiber pit membranes.

Figure 5. Bordered pits of a wood fiber of Acacia mangium showing different extents of incrustation. Some pit membranes are heavily covered with incrusting materials (arrow) and there are no visible openings (a), but some have many small openings (arrowheads) all along the incrusted pit membrane (b). — Scale bars = 1 µm.
In order to elucidate the reason for the uneven cellular distribution of heartwood substances in *A. mangium*, we examined the ultrastructure of interfiber pits in this species by field-emission scanning electron microscopy. A closer inspection of the outer sapwood revealed a considerable difference in the sizes of the bordered pits; the diameter of the pits ranged from 0.4 to 2.3 mm. This range is smaller than that of interfiber pits of *A. julibrisin* (0.6–4 mm) (Zhang et al. 2006).

In our previous study on *A. julibrisin* (Zhang et al. 2006), we reported that many interfiber pit membranes were completely perforated in the center without any incrustation (arrows in Fig. 4a, b), although the other pit membranes were covered with incrusting materials to a different degree. In *A. mangium*, however, almost all interfiber pit membranes were heavily covered with incrusting materials (arrowheads in Fig. 4c, d & arrow in Fig. 5a). Although some incrusted pit membranes had some small openings (arrowheads in Fig. 5b), there were extremely few completely perforated pit membranes.
Figure 7. Resin-cast replicas of sapwood. In radial planes (a, b), intercellular spaces between the ray parenchyma cells and axial elements (narrow strings) form an anastomosing network structure, and the blind pits (arrows) of the ray parenchyma cells face the intercellular spaces. Furthermore, axial intercellular spaces are wider than radial intercellular spaces. In the tangential plane (c), intercellular spaces between wood fibers interconnect by forking at the ends. — Asterisk: forking intercellular space. See Figure 6 for other symbols. — Scale bars (a, c) = 10 µm; (b) = 50 µm.
Based on these results, we can infer that the pit pairs with heavy incrustation on their pit membranes could not function as intercellular diffusion pathways for heartwood substances; only those with slight incrustation (or without incrustation) and visible openings on their pit membranes could serve as intercellular diffusion pathways. Moreover, the size of a pit might also be one of the factors affecting the capacity of the pit for diffusing heartwood substances. We therefore assume that structural variations of a pit pair control the pit capacity for diffusing heartwood substances, and that these structural variations contribute to the non-uniform distribution of heartwood substances.

**Network structure of blind pits and intercellular spaces**

When a pit has no complementary pit in an adjacent cell, it is called a blind pit (IAWA Committee 1964). In this study, we also observed frequent blind pits in the ray parenchyma cell walls, all facing the intercellular spaces (arrows in Fig. 6).

According to previous studies (Kuo & Arganbright 1980; Nobuchi et al. 1984; Nobuchi & Harada 1985; Nobuchi 1985; Hillis 1987; Zhang et al. 2004), heartwood substances are generally synthesized in ray parenchyma cells and transported into adjacent axial elements through their pit pairs. In this study, TEM observations suggest that the heartwood substances formed in ray parenchyma cells are released into the intercellular spaces through blind pits. As shown in Figure 6a, strongly-stained heartwood substances (arrowheads) were detected in the ray parenchyma cells, in the blind pit structures, and in the intercellular spaces facing the blind pits. The same phenomenon was also observed in *A. julibrissin* (Zhang et al. 2004).

Additionally, our TEM observations of *A. mangium* revealed a large number of intercellular spaces among the axial elements (Fig. 3) that differ from those between ray parenchyma cells and axial elements (Fig. 6). In order to study the intercellular spaces in three dimensions, the resin-casting method was used, which is considered as an effective method for investigating apoplastic spaces (Fujii 1993; Mauseth & Fujii 1994; Kitin et al. 2001). The intercellular spaces appeared as very narrow strings that crossed (Fig. 7a, b), branched, or forked (asterisks in Fig. 7b, c). The strings of axial intercellular spaces were wider than those of radial intercellular spaces (Fig. 7a, b). The pits of the ray parenchyma cells appeared as minute spots, and some of them (arrows in Fig. 7a, b) were connected with the narrow strings. These findings suggest that the intercellular spaces between the ray and axial elements form an anastomosing network structure in the radial plane. Further, the blind pits of the ray parenchyma cells connect with this network structure, and the intercellular spaces between the axial elements also interconnect by forking. Thus, we infer that the intercellular spaces and blind pits in the xylem form a three-dimensional network, which might play an important role in the extracellular diffusion of the heartwood substances in *A. mangium*, as supported by the heartwood substances observed in the intercellular spaces.

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