SEASONAL CHANGES IN THE DISTRIBUTION OF WATER IN THE OUTER GROWTH RINGS OF FRAXINUS MANDSHURICA VAR. JAPONICA: A STUDY BY CRYO-SCANNING ELECTRON MICROSCOPY

by

Yasuhiro Utsumi, Yuzou Sano, Jun Ohtani & Seizo Fujikawa

Faculty of Agriculture, Institute of Low Temperature Science, Hokkaido University, Sapporo 060, Japan

SUMMARY

Seasonal changes in the distribution of water in the outer growth rings of Fraxinus mandshurica var. japonica were visualised by cryo-scanning electron microscopy using samples in which water was freeze-fixed in the living trunk. During the growing season from mid-May to late-July when formation of xylem progressed steadily, all cell lumina of the newly forming xylem elements were filled with water. From August to October, water was lost from the lumina of wood fibres in the current-year xylem. Loss of water from wood fibres began in August at the initial zone of the earlywood, and progressed toward the cambial zone. By November, water had disappeared from the lumina of current-year earlywood vessels, and water reappeared in the lumina of earlywood fibres around the current-year earlywood vessels. Our results indicate that cavitation in lumina of current-year earlywood vessels occurred during the period from October to November.

Key words: Cavitation, water transport, cryo-SEM, ring-porous tree, Fraxinus mandshurica var. japonica, xylem physiology.

INTRODUCTION

The transport of water and its dysfunction in woody plants have been discussed for many years. In early studies, causes of water-transport dysfunction in plants were designated cavitation and embolism. In plants, cavitation refers to formation of a cavity or a hollow within a body of water, such as plant sap, and embolism refers to an obstruction to the flow of sap (Milburn 1991). When cavitation occurs in xylem conduits (vessels and tracheids) in which water is under tension, air bubbles tend to emerge and occupy the entire lumen of the conduits (Zimmermann 1983). This event interrupts the transport of water and often results in the dysfunction of xylem conduits.

According to findings obtained in previous studies, there are two types of cavitation: cavitation caused by water stress and cavitation caused by freezing. Cavitation caused by water stress has been explained by the assumption that tension inside xylem...
conduits exceeds a certain critical point and air is sucked into the lumina of water-filled conduits from adjacent gas-filled conduits through pores of intervascular pit membranes (Zimmermann 1983; Sperry & Tyree 1988). Cavitation caused by freezing seems to occur as a result of the low solubility of gases in ice: gases dissolved in water freeze out to form bubbles (Zimmermann 1983).

In ring-porous species, conduction of water in earlywood vessels is generally confined to the outermost growth ring, which is formed during the current growth season (Chaney & Kozlowski 1977; Ellmore & Ewers 1986). It is suggested that dysfunction of water conduction in the earlywood vessels of the outermost ring occurs by the end of winter (Zimmermann 1983). Recently, Cochard and Tyree (1990) determined seasonal changes in hydraulic conductivity for an estimation of the occurrence of embolisms in Quercus, and they concluded that the first hard frost induces irreversible dysfunction of water transport of the earlywood vessels formed in the current year. However, details of the mechanism responsible for the dysfunction due to cavitation in ring-porous species are still speculative and some aspects remain that clearly require further study.

To date, various methods have been used in investigations of water transport and its dysfunction in conduits. Patterns of water movement in trees have been delineated by staining techniques (Greenidge 1958; Chaney & Kozlowski 1977). Seasonal changes in water transport and the mechanism of such changes have also been studied by determination of hydraulic conductivity (Sperry et al. 1988; Cochard & Tyree 1990). Cavitation has even been detected without surgical treatment by an acoustic emission technique (Grace 1993; Milburn 1993).

In this study, we attempted to visualise seasonal changes in the distribution of water and the seasonal progression of xylem cavitation in the outer sapwood in Fraxinus mandshurica var. japonica by cryo-scanning electron microscopy (cryo-SEM) using freeze-fixation of living trunk. Cryo-SEM observation allows us to fix water as ice crystals in the cell lumina and to visualise the location of water in the xylem of living trees (Ohtani & Fujikawa 1990).

**MATERIALS AND METHODS**

**Plant material**

Sample trees were selected from a plantation of 15-year-old specimens of Fraxinus mandshurica var. japonica (height, about 8 m; diameter at breast height, about 8 cm) in the Nakagawa Experimental Forest of Hokkaido University, which is situated in Northern Hokkaido. Two trees were felled at intervals of two to eight weeks from spring (mid-May 1994) through winter (mid-February 1995).

**Collection of samples**

During periods when the trees were in leaf (June–October) and in November, samples were collected after xylem sap had been freeze-fixed by freezing the trunks at breast height in living trees. Watertight collars were made with plastic funnels and fitted around the trunk of each sample tree at breast height. These collars were filled
with liquid nitrogen (LN$_2$), and the trunks were allowed to freeze for approximately 15 minutes. Discs of about 2 cm in thickness were immediately removed from the frozen area of each trunk, and they were divided into blocks that included outer sapwood and bark. The blocks were stored in a container with LN$_2$.

In May and following February, samples were collected without pre-freezing and then stored as described above.

**Cryo-SEM**

The sample blocks that had been stored in LN$_2$ were transferred to a low-temperature room kept at $-20^\circ$C. The sample blocks were equilibrated at $-20^\circ$C exactly to prevent contamination by frost during subsequent treatments. The blocks were further cut into small blocks (5 x 5 x 5 mm$^3$) that included one to two outer annual rings and phloem. The transverse or tangential surfaces of the blocks were cleanly cut with steel blades on a sliding microtome or with glass knives on an ultramicrotome to expose cell lumina (Sano et al. 1993, 1995). Then the blocks were attached to specimen holders, immersed in LN$_2$ and transferred to a system for cryo-SEM (JSM 840-a; JEOL Co. Ltd., Tokyo, Japan) equipped with a freeze-etching unit, as developed by Fujikawa et al. (1988). The specimen holder with the sample was fixed on the cold stage of the freeze-etching unit, which was maintained under a vacuum of approximately $1 \times 10^{-4}$ Pa and equilibrated to $-110^\circ$C. The specimen was freeze-etched under these conditions for about 5 minutes to eliminate contamination by frost, and then it was rotary-shadowed with a platinum-carbon pellet. The sample was transferred to the cold stage of the SEM, which was kept at about $-160^\circ$C, and secondary electron images were observed at an accelerating voltage of 3 to 5 kV.

**Temperature data**

Daily minimum and maximum temperatures were obtained from Otoineppu Meteorological Station of the Japan Meteorological Agency, located about 10 km from the study site.

**RESULTS**

**Seasonal changes in the distribution of water in outer growth rings**

In the middle of May, when the leaves had not yet unfolded, the formation of current-year xylem was observed (Fig. 1). In the differentiating zone, wood fibres, ray parenchyma and earlywood vessels (Fig. 1, large arrow) could be distinguished. The cell lumina of the differentiating cambial zone were filled with water or cytoplasm, irrespective of cell type. In the xylem formed in the previous year, latewood vessels (Fig. 1, small arrow) were filled with water, although almost all the fibres and earlywood vessels had no water in their lumina. Changes in the location of water in the previous year's xylem were not observed from May to the following February.

During the period from early June to late July, wood formation progressed steadily. Changes in the location of water were not observed in the newly forming xylem during this period; all the cell lumina were filled with water or cytoplasm, resembling samples
collected in May. Figure 2 shows the newly forming xylem collected in early June. The earlywood vessels (v) contained water in their lumina. The thin-walled fibres (f) and parenchyma cells, which were probably in the process of developing, contained cytoplasm in their lumina.

During the period from August to October, water was present in both the earlywood and the latewood vessels in the current-year xylem (Fig. 3–8). However, water gradually disappeared from the wood fibre lumina in the current-year xylem during this period (Fig. 3–8). In August, when xylem differentiation was still occurring, fibres without water in their lumina were found in the earlywood wood zone only (Fig. 3). In September, when xylem formation had almost entirely ceased, the number of such cavitated fibres increased and fibres with water (or cytoplasm) were found only in the outer layer of the current-year xylem (Fig. 4). In October, when most of the studied trees had lost many of their leaves, most of the wood fibre lumina of the current-year xylem lost water. The lumina of earlywood vessels had been filled with water till October (Fig. 5 & 6). These results were also confirmed in tangential sections (Fig. 7 & 8).

By November, when all the leaves had fallen, water had disappeared from the lumina of the current-year earlywood vessels, which had been filled with water in October, and water appeared in the lumina of current-year earlywood fibres (Fig. 9). Only the wood fibres surrounding current-year earlywood vessels were filled with water and the other fibres were empty (Fig. 10). Figures 11 and 12 show a tangential surface and it can be seen that all the vessels in the current-year earlywood were empty, even though the wood fibres surrounding the earlywood vessels were filled with water.

Fig. 1. Current-year xylem and xylem formed the previous year in May. Newly forming xylem has thin cell walls and the xylem formed the previous year has thick cell walls. The large arrow indicates a newly forming earlywood vessel and the small arrow indicates a latewood vessel that was formed the previous year. — Fig. 2. Newly forming xylem in June. All cell lumina of the cambial zone and newly forming xylem are filled with water or cytoplasm.; v, vessel; f, fibre. — Fig. 3. Earlywood of current-year xylem in August. Most of the wood fibre lumina in the earlywood are empty. Earlywood vessels are filled with water. — Fig. 4. Latewood of current-year xylem in September. Only wood fibre lumina near the cambial zone are filled with water. Arrows indicate latewood vessels that are filled with water.

Fig. 5–8. Current-year earlywood vessels (v) and fibres (f) in October. — 5: Most of the wood fibre lumina are empty. The earlywood vessel lumina are filled with water. — 6: Detail of the earlywood. — 7: Earlywood vessel in tangential section. Vessels are completely filled with water. — 8: Detail of the earlywood vessel, fibre and ray parenchyma.

Fig. 9–12. Current-year earlywood vessels (v) and fibres (f) in November. — 9: Most of the lumina of wood fibres that surround earlywood vessels are filled with water, while the earlywood vessel lumina are empty. — 10: A detail of Figure 9. Fibres near a vessel are filled with water (wf); fibres at a distance from a vessel are empty (ef). — 11: Earlywood vessel in tangential section. — 12: Detail of Figure 11.
When the formation of terminal parenchyma (Fig. 13, arrows) was complete, the lumina of wood fibres near the cambial zone contained no water. In February, water had disappeared from wood fibre lumina of all the earlywood in the outermost ring (Fig. 14) and remained only in lumina of latewood vessels.

The above-mentioned results are illustrated schematically in Figure 15.

**Temperature data**

In order to study the relationship between cavitation and first frost, we analysed daily change in temperature. Figure 16 shows the daily maximum and minimum temperatures in October and November, recorded at a meteorological station located about 10 km from the plantation. The first sub-zero minimum temperature was observed on October 18. The first sub-zero maximum temperature was observed on November 13 and the sub-zero temperatures continued for 3 days.

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*Fig. 13. Current-year latewood in November. The arrows indicate the terminal parenchyma. — Fig. 14. Earlywood of the outermost growth ring in February (next year, 1995). The fibre lumina in current-year earlywood contain no water.*

*Fig. 15. A schematic representation of seasonal changes in the presence of water in vessel and wood fibre lumina. * = newly formed annual ring.*
DISCUSSION

During the growing season, water rises from roots to leaves as a consequence of the negative pressure that is created in the leaf by transpiration. Zimmermann and Brown (1971) indicated that water in vessels is replaced by air when the pressure in these conduits is below atmospheric pressure and the conduits are exposed to air. We confirmed experimentally that water in earlywood vessels of newly formed growth rings in *Fraxinus mandshurica* var. *japonica* was replaced by air when stems were cut during summer (unpublished data). To prevent this phenomenon during cutting of trunks, we froze the trunks of living trees with LN$_2$. As a result, the water in vessel lumina was fixed as ice. Then samples were kept at sub-zero temperatures prior to cryo-SEM. Therefore, we can assume that the location of water in the living tree was unchanged from sampling to observation.

Cryo-SEM observations confirmed that seasonal changes in the location of water occurred in the outermost growth ring. Disappearance of water from the lumina of the wood fibres started in the earlywood in August and gradually progressed toward the terminal zone of the annual increment. This disappearance of water appears to correspond to the maturation of wood fibres. All the earlywood vessels, which had been completely filled with water through October, lost water in November. This observation means that cavitation occurred in these earlywood vessels during this period. In contrast, earlywood fibres that had no water in their lumina in October were filled with water in November.
What is the mechanism of induction of cavitation of earlywood vessels? Sperry (1993) reported that xylem embolisms occurred as a result of water stress and the freezing of xylem sap and that they had the immediate effect of reducing the hydraulic conductivity of the xylem. An embolism caused by water stress has been explained in terms of the entry of air through inter-conduit pit membranes as a result of a large difference in pressure between a gas-filled conduit with atmospheric pressure and a water-filled conduit with high negative pressure (Zimmermann 1983; Sperry & Tyree 1988). However, most leaves on the trees used in this study fell in October. Therefore, it is unlikely that transpiration generated a large difference in pressure between the adjacent conduits. An embolism caused by freezing occurs when dissolved air is displaced and then reappears as bubbles when water freezes (Zimmermann 1983; Tyree et al. 1994). Previous studies of seasonal changes in embolisms have shown that hydraulic conductivity decreases in the winter as a result of freezing-induced embolisms (Sperry et al. 1988; Sperry 1993). More recent studies have indicated that the first frost induces embolisms in ring-porous trees (Cochard & Tyree 1990; Sperry & Sullivan 1992).

In our study, cavitation was detected in late autumn but not in summer, when water stress would be expected. The weather data indicate that there was a period of low temperatures just before sampling in November (Fig. 16). Therefore, the cavitations detected in this study were deduced to be freezing-induced cavitations. However, in this case also, it is unclear why high xylem tension occurred.

What mechanism is involved in the enlargement of bubbles? Ewers (1985) studied the relationship between enlargement of bubbles and the size of conduits using glass capillary tubes of various diameters that were filled with water. When such capillary tubes were frozen and thawed, bubbles remained stable for days, weeks and, in some cases, months after thawing in the rather wide tubes (375 µm in diameter). That is to say, bubbles formed in large conduits (e.g., earlywood vessels in ring-porous tree) may exist for a long time in the absence of significant tension. There must be a relationship between vulnerability to cavitation and conduit size. Cavitation caused by freezing depends considerably on conduit size (Cochard & Tyree 1990; Tyree et al. 1994). In our study, no cavitation of latewood vessels (small conduits) was observed. Earlywood vessels and latewood vessels might play different roles in the transport of water in ring-porous trees.

Finally, we must ask why the fibres that surrounded current-year earlywood vessels were filled with water when cavitation occurred in November. The simplest hypothesis is that water moves from earlywood vessels to fibres when cavitation occurs. The location of water-filled fibres was restricted to the region around earlywood vessels when cavitation in earlywood vessels were observed in November (Fig. 9). This result supports our hypothesis. The passage along which water is transported and the mechanism of transport would be explainable on the physical aspects if all the vessels directly make contact with fibres. However, *Fraxinus mandshurica* var. *japonica* has both vasicentric parenchyma and scanty paratracheal parenchyma. Consequently, only some of the vessels are in contact with fibres, and this contact is limited (Sano & Fukazawa 1994). Therefore, water might move from vessel to fibre through axial paren-
chyma cells. Wheeler (1981) indicated that the intervascular pit membranes in the outer ring of *Fraxinus americana* appear to be filled with extraneous materials so as to yield an impervious-appearing structure in the winter. This phenomenon was also observed in *Fraxinus mandshurica* var. *japonica* in November (unpublished data). We suppose that these extraneous materials resulted from both vasicentric parenchyma and scanty paratracheal parenchyma. Therefore, seasonal physiological changes of axial parenchyma might be involved in the water movement.

In this study, we were able to visualise the progression of cavitation and seasonal changes in the location of water in xylem by cryo-SEM. The cavitation in earlywood vessels was probably caused by freezing. Fibres surrounding newly formed earlywood vessels were filled with water when cavitation had occurred. Since the mechanisms for this relocation of water are unclear, obviously more experiments are needed to examine these events in greater detail.

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REFERENCES


