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Micromorphological Characteristics of Frost Rings in the Secondary Xylem of *Pinus radiata*[*1]

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ABSTRACT

Frost ring formed in the secondary xylem of *Pinus radiata* was examined using various microscopic techniques. Cell walls in a frost ring were poorly developed, lacking in the proportion of wall components. Formation of secondary cell wall was imperfect and thickness of secondary wall was varied. Cytochemical examinations provided the evidence that the synthesis of structural polysaccharides and lignin was inhibited, resulting in the malformation of secondary cell walls. Judging by the highly irregular nature of the cell wall, it appears that cellulosic/hemicellulosic framework was affected and the template for lignification by frost.

Keywords : frost ring, *Pinus radiata*, secondary cell wall, cellulose, lignin, ultrastructure

1. INTRODUCTION

Adverse effects of environmental stresses limit the productivity of forest trees. Secondary xylem (wood) can be changed anatomically as well as chemically in response to environmental stresses (Kubacka-Zebalska and Kacperska 1999; Nakamura *et al.*, 2003; Zabotin *et al.*, 1998), resulting in alteration of wood qualities. Considerable research has been done on the impact of environmental stresses on enzyme systems and metabolic pools in plants (Pearce 2001; Sakai and Larcher 1987). Despite high economic value and importance of wood to the society, however, there is a very little information available on the effect of environmental stresses on the development and composition of wood cell wall. Much of our understanding of how trees respond to environmental stresses derives from the studies of nonwoody model plants, such as *Arabidopsis thaliana*, *Zinnia elegans*, and *Nicotiana tabacum* (Cano-Delgado *et al.*, 2003; Janas *et al.*, 2000; Stefanowska *et al.*, 2002; Zabotin *et al.*, 1998). Understanding the responses of forest trees to the environmental stresses is crucial for rational engineering to produce the hardier trees and for appropriate use of those materials in industrial production.

Frost is a major environmental stress, inflicting economic damage on forest trees and

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limiting the distribution of tree species on the earth. Frost, i.e. the temperature range below 0°C, leads to freezing of water in the plant. In this range of temperatures, metabolism of the cell is reduced to a minimum; thus many physiological functions cease (Brummell *et al.*, 2004; Egierszdorff and Kacperska 2001; Fujikawa and Kuroda 2000). The damage by frost to the forest trees in active growth season is very huge, although its period was extremely short such as overnight in early spring (Sakai and Larcher 1987). When forest tree becomes injured from a sudden exposure to freezing temperature, frost-ring develops in tree trunks in response to an abrupt drop of temperature during the growing season. Not much is known about the features of cells that comprise frost rings beyond the knowledge that cells are abnormal in their form, tend to collapse and stray from the normal course of linear files. The present work describes the composition and ultrastructure of cell walls within a frost ring produced in radiata pine.

2. MATERIALS and METHODS

Two or three year old branches of radiata pine (*Pinus radiata*) were collected from Whakarewa forests in Rotorua, New Zealand Forest Research Institute. The frost ring was formed in response to a sudden drop in the temperature from the usual range of around 20°C during the growing season in the Rotorua region of New Zealand where the trees were growing to about 1°C in the spring of 2003. Light microscopy (LM) and transmission electron microscopy (TEM) were used to examine the structural differences of frost-ring. Ultraviolet (UV) microscopy was also employed. Small pieces of wood were fixed overnight in formalin, acetic acid, and ethyl alcohol (FAA) fixatives or 2% paraformaldehyde and 2% glutaraldehyde in 0.05 M cacodylate buffer (pH 7.2) at room temperature. Some pieces were also post-fixed with 1% osmium tetroxide at 4°C for 2 hrs and embedded in Spurr's low viscosity resin for TEM observation. Semi-thin sections (2 μm thick) repared by glass knife using ultramicrotome were stained with toluidine blue and phloroglucinol HCl for examination of general aspect and lignin, respectively. Some wood blocks were treated with hydrogen peroxide and acetic acid (1 : 1, v/v) for the maceration of tracheids.

2.1 Ultraviolot (UV) Microscopy

The wood samples without post-fixation with osmium tetroxide were embedded in Spurr's resin and processed for UV-microscopy. Semi-thin sections (1 μm thick) were cut, fixed to quartz slides, immersed in non-UV absorbing glycerol and covered with quartz coverslips. The sections were analyzed using a ZEISS UV microscopy (Carl Zeiss AG, Germany). Images were taken at a wavelength of 280 nm.

2.2 Transmission Electron Microscopy (TEM)

The embedded tissues were sectioned on an ultramicrotome using a diamond knife. The ultrathin sections (80~100 nm thick) were examined with Jeol 1010 TEM after staining with 1% potassium permanganate (prepared in citrate buffer) for lignin. For visualization of polysaccharides, sections were also treated with periodic acid-thiosemicarbazide-solver proteinate (PATAg) (Tierry, 1967). Some sections were stained also with uranyl acetate and lead citrate.

3. RESULTS

Examination of frost rings with a microscope showed that both axial (Fig. 1) and radial sys-

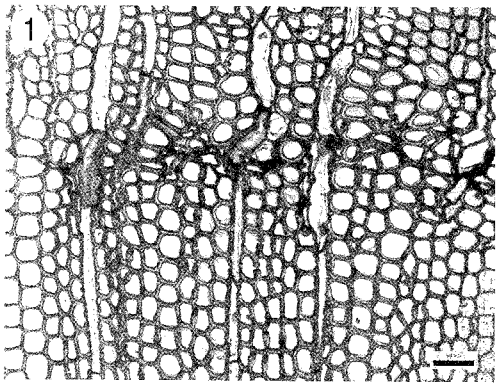

Fig. 1. Transverse section of the frost ring showing collapse in axial tracheids and expansion a ray. LM. Toluidine blue staining. Scale bar = 50 μm.

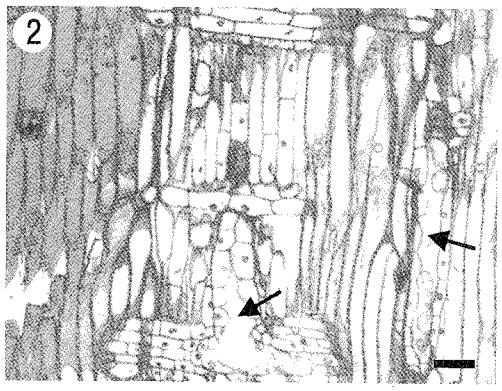

Fig. 2. Radial section of frost ring showing malformation of ray parenchyma (arrows). LM. Toluidine blue staining. Scale bar = 100 μm.

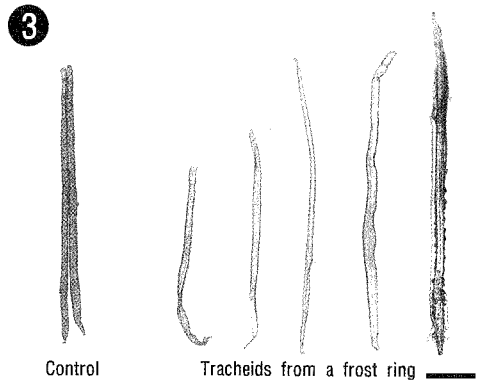

Fig. 3. Malformed tracheids in the frost ring. LM. Toluidine blue staining. Note the dimension of tracheids in a frost ring. Scale bar = 200 μm.

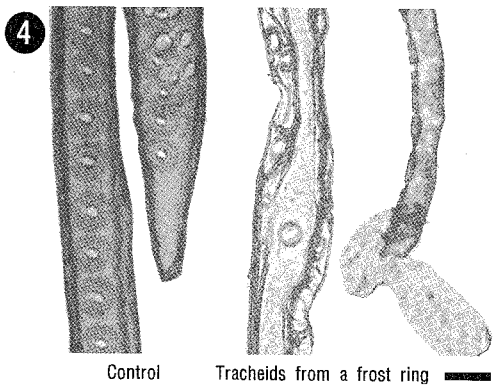

Fig. 4. Malformation of tracheids in the frost ring. Macerated with Franklin solution. LM. Toluidine blue staining. Scale bar = 20 μm.

tems (Fig. 2) in the secondary xylem of *P. radiata* were affected by an abrupt drop in the temperature. A frost ring was consisted of 2 to 10 layers of abnormal axial tracheids in radial direction. The frost ring was localized to the earlywood zone, indicating that the frost ring was formed in the spring time when the cambium is already activated and the first xylem elements of the earlywood have been developed. The rays in the frost ring were distended and distorted (Fig. 2).

The tracheids in a frost ring was short and crooked when compared to unaffected tracheids (Fig. 3). In addition, the staining intensity of tracheids with toluidine blue was very weak or nil in comparison with unaffected tracheids. The distortion and alteration of tracheids in a frost ring was diverse. The mildest form of distortion was just a deformation of individual cells. The greatest distortion was characterized by twisted and totally compressed tracheids (Fig. 4), exhibiting the incomplete wall formation.

UV microscopy showed that lignin concentration and deposition in the tracheids were very

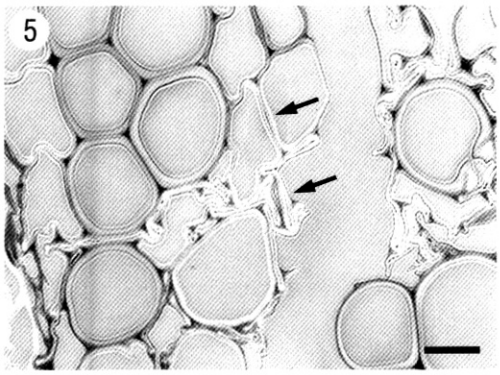

Fig. 5. Lack of UV (280 nm) absorption in the secondary cell walls (arrows). UV microscopy. Scale bar = 20 μm.

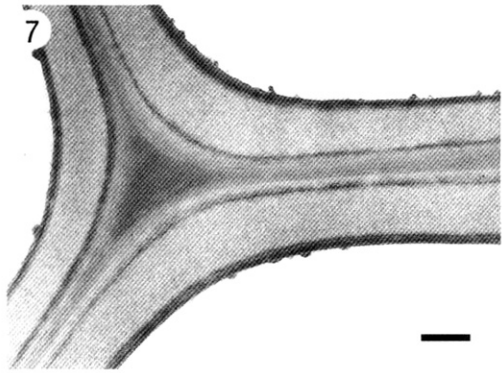

Fig. 7. Ultrastructure of tracheids of unaffected radiata pine (control). TEM. $KMnO_4$ staining. Scale bar = 1 μm.

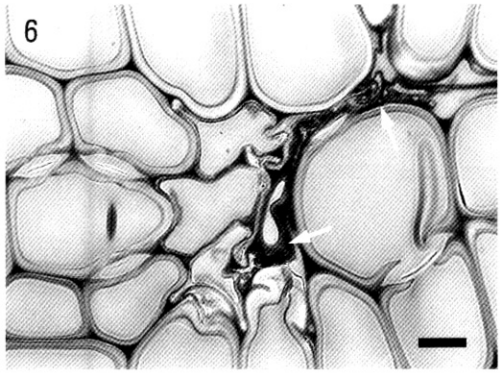

Fig. 6. Strong absorption of UV (280 nm) in the collapsed zone (arrow). UV microscopy. Scale bar = 10 μm.

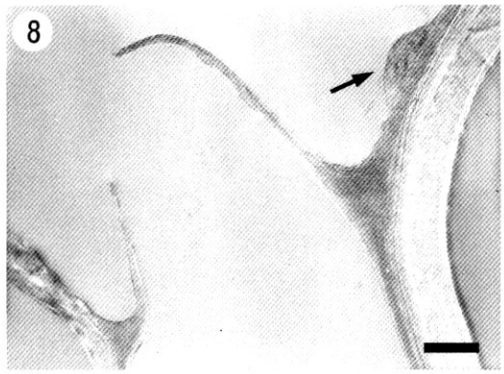

Fig. 8. A very thin cell wall has been formed apparently not beyond the primary wall development. A dense material is deposited irregularly along the lumen side (arrow). Note that the staining of middle lamella and secondary wall is uneven. TEM. Ruthenium red staining. Scale bar = 2 μm.

irregular depending upon the position in a frost ring. Some part of tracheids did not show the positive reaction with UV at 280 nm (Fig. 5) whereas some part of tracheids in the collapsed area showed strong absorption of UV at 280 nm, indicating the increased concentration of lignin (Fig. 6).

TEM micrographs prepared from ultrathin sections embedded in Spurr's resin show cell wall abnormalities in greater detail. The ultrathin sections were stained mainly with potassium permanganate, a stain widely used to contrast lignin in wood cell walls. TEM micrographs showed that diverse cell abnormalities were present in the frost ring (Figs. 8~12) when compared to the unaffected radiata pine (Fig. 7).

The formation of cell walls in the frost rings was inhibited and imperfect. Tracheids which failed to develop to the normal cell wall architecture exhibited the compressed, collapsed, wavy, and granulated figures. Some tracheids had not been formed apparently beyond the

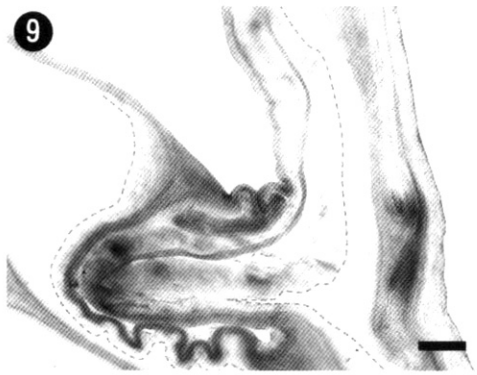

Fig. 9. Secondary cell walls negatively stained with PATAg. Note the distorted tracheid walls lacking S_3 layer (dotted). TEM. Scale bar = 1 μm.

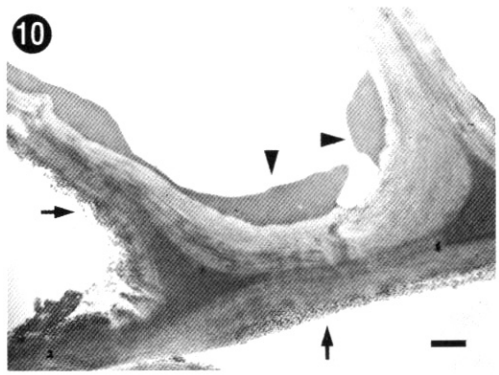

Fig. 10. Imperfect development of secondary wall. Note the uneven thickness of tracheids and the highly porous and granular materials (arrows) in the secondary cell wall layers. A dense material is irregularly deposited on the lumen face of the wall (arrow heads). TEM. $KMnO_4$ staining. Scale bar = 0.5 μm.

primary wall development. Fig. 8 showed that development of middle lamella/primary wall was blocked just prior to stages of secondary wall formation by frost. The tracheids just under developing the S_2 layer did not have a compact textures. The secondary layers in deformed tracheids were wavy and not stained with PATAg, which is a specific staining chemical for polysaccharides (Fig. 9).

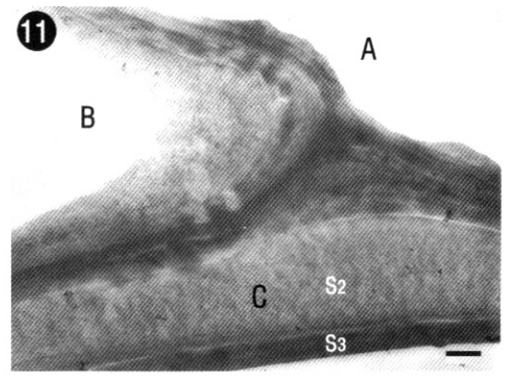

Fig. 11. Three tracheids with different development of secondary cell walls. (A) Apparently stopping of secondary cells, (B) highly porous and convoluted secondary wall lacking S_3 layer and (C) presence of thickened, highly lignified S_3 layer (C). TEM. $KMnO_4$ staining. Scale bar = 0.5 μm.

Fig. 10 shows that the secondary wall is not fully developed lacking the S_3 layer. Secondary cell walls were highly porous and granular. A dense material is irregularly deposited on the lumen face of the wall. In comparison to the cell walls of normal wood, the thickness and the density of cell walls in Fig. 10 are variable, resulting from irregular staining with $KMnO_4$. The collapsed tracheids were in part totally compressed. Fig. 9 showed that thin walled tracheids have been collapsed.

The tracheid wall with imperfect development of S_1 layer display the incomplete cell wall formation and the nodular, granulate, porous, and wavy shape (Figs. 11~12). TEM work shows how the formation of cell walls was affected in response to the frost at the different developing stage of secondary walls. Fig. 11 clearly shows that one tracheid did not developed apparently S_1 layer, the other lacked S_3 layer, and the apparently normal tracheid with S_1, S_2 and S_3 layers. The tracheid lacking S_3 layer was highly porous whereas the apparently normal tracheid has a thick and highly lignified

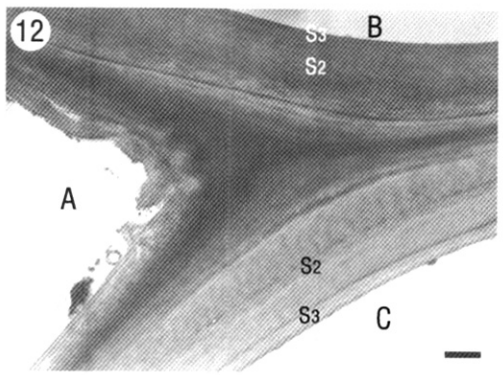

Fig. 12. Three tracheids showing the irregular deposition of lignin at the different development stage of cell wall formation. Note the weak staining intensity with $KMnO_4$ of tracheid C at S_3 layer in comparison with tracheid B. TEM. $KMnO_4$ staining. Scale bar = 0.5 μm.

S_3 layer when compared to unaffected S_3 layer (Fig. 6). In addition, the thickness of each tracheid was different. The staining intensity of cell wall with $KMnO_4$ was also diverse depending upon the developing stages of secondary walls in a frost ring (Fig. 12). Although tracheids were apparently normal, the staining intensity with $KMnO_4$ and the thickness of tracheid were different. One tracheid which did not developed apparently S_2 layer showed the nodular and porous features. Although tracheids showed the apparently normal wall structure with S_1, S_2 and S_3 layer, the staining intensity of cell wall with $KMnO_4$ in each layer was different, indicating the different lignification in the secondary cell wall layers by frost.

4. DISCUSSION

Environmental stresses influence the composition of cell wall, and lead to the modification of structural component. The present works show that both the axial and the radial systems in the secondary xylem of *P. radiata* trees were influenced by sub-zero temperature. The cell abnormalities in the frost ring of *P. radiata* were diverse; from the thin-walled cells which had apparently not progressed beyond the primary wall development to the irregular lignification of cell wall regions corresponding to middle lamella and secondary wall. Those abnormal features clearly suggest that even the brief freezing temperature had arrested the deposition of cell wall components.

A range of cell abnormalities in the frost ring tracheids of *P. radiata* observed in the present work suggests that frost had inhibited the deposition and restructuring of polysaccharides. Highly porous texture of the S_2 layer is an indication that synthesis of structural polysaccharides was inhibited by frost. Frost is known to lower the insufficient carbohydrate supply. Exposure of plants to a freeze treatment resulted in decreased cell wall contents, reduced the levels of non-covalently bound pectins, and decreased contents of hemicellulose fraction (Kubacka-Zebalska and Kacperska 1999). The depolymerization of actin microfilaments (Egierszdorff and Kacperska 2001), the regulation of cell wall extensibility (Nakamura *et al.*, 2003), and the alterations in the metabolism of cell wall polysaccharides (Brummell *et al.*, 2004) by freezing or chilling injury were well documented.

TEM micrographs showed that lignification at secondary wall was also affected by frost. The different pattern of lignification and the irregular deposition of lignin in the secondary wall were observed in the trachieds of a frost ring. The irregular deposition of lignin indicated that the control mechanism ensuring an orderly deposition of lignin monomer was inoperative. Abiotic stresses induce generally lignification (Jbir *et al.*, 2001). Solecka *et al.* (1999) observed the marked increase in the contents of soluble phenolic acids and anthocyanins in mesophyll cells of winter oilseed rape leaves by a

brief freezing. However, there are also reports of reduced lignification by drought (Donaldson 2002).

Furthermore, TEM micrographs indicated that cells which failed to develop a normal cell wall architecture by lignification, and affected the subsequent lignification process. It was reported that reduced cellulose synthesis invokes lignification in *Arabidopsis thaliana* (Cano-Delgado *et al.*, 2003). Wi *et al.* (2005) found that irregular lignin distribution in the middle lamella of alfalfa secondary xylem fibres has been related to the distribution pattern of pectin and peroxidase, which is also irregular. This may also be true for the irregular lignification observed. The architecture of cell walls prior to lignification must have a great influence on the extent and pattern of cell wall lignification.

The cell walls in frost ring were diverse, ranging from poorly lignified middle lamella and cell walls lacking a S_3 layer to cell walls endowed with an extremely thick S_3 layer (Fig. 12). The presence of wide-ranged cell wall features suggests that cellular response to frost stress was different depending upon the developmental stages of tracheids. Conclusively, the present TEM works clearly show that the control mechanism ensuring an orderly deposition of polysaccharides and lignin was inoperative by frost. Further studies are necessary to understand the effect of freezing temperature in detail on the synthesis and deposition of cell wall components in the forest trees.

5. CONCLUSION

Anatomical characteristics of frost ring formed in radiata pine were investigated. Collectively, cell walls in the frost ring were poorly developed, lacking the proper thickness and the proportion of wall constituents which confer the normal wood strength, stability, and resistance against collapse. Thus, the frost ring constitutes a serious defect in the timber. Further studies are needed to understand the different vulnerability of pine trees depending upon clones, and nutrient supplies.

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