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Evidence for the Involvement of a Specific Cell Wall Layer in Regulation of Deep Supercooling of Xylem Parenchyma

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ABSTRACT

Current theory indicates that the structure of the cell wall is integral to the ability of a tissue to exhibit deep supercooling. Our previous work has indicated that the structure of the pit membrane and/or amorphous layer (protective layer), rather than the entire cell wall, may play a major role in deep supercooling (21, 22). The present study indicates a shift in the low-temperature-exotherm of current year shoots of peach can be induced by soaking twigs in water over 3 to 10 days. Alternatively, these shifts can be inhibited by exposing tissues to 10^{-4} molar cycloheximide. Ultrastructural observations indicated a marked alteration of the amorphous layer in xylem parenchyma of water-soaked tissue. Alterations consisted of an apparent loosening or partial dissolution of portions of the amorphous layer. Changes in the density or uniformity of the amorphous layer in cycloheximide-treated tissues were not as readily apparent. The appearance of the protoplast in tissue soaked in water for up to 10 days was characteristic of deacclimated cells. However, in tissue soaked in cycloheximide for the same period these changes were not evident. These observations further support our contention that the structure of the amorphous layer may play a key role in establishing and regulating the ability of a cell to exhibit deep supercooling.

Many species of woody plants exhibit freezing avoidance by deep supercooling in response to exposure to low temperatures (6). It has been postulated that this attribute may play a major role in defining species distribution on both a latitudinal (8) and elevational gradient (2). For deep supercooling to occur, a tissue must exhibit several features: (a) a barrier to water movement must exist that prevents a 'rapid' loss of cellular water to extracellular ice in the presence of a strong vapor pressure gradient, (b) a barrier that excludes growth of ice crystals into a cell that would result in nucleation of intracellular water, and (c) cells which are free of heterogeneous nucleating substances which would trigger intracellular ice formation at relatively warm temperatures.

Based on theoretical calculations or on model systems, current theories indicate that restricted pore size (60–100 Å in diameter) in the cell wall would play a major role in defining this barrier (1, 7). These theories, however, do not indicate whether the entire primary and/or secondary wall need exhibit this structure or just specific sites. It is also unclear whether all cells of xylem tissue need possess this structure or just living cells.

In previous studies, Wisniewski *et al.* (21, 22) utilized lanthanum nitrate as a tracer to examine the permeability and porosity of xylem tissue. They observed that xylem parenchyma cell walls were impermeable to the lanthanum solution except in areas of the pit membrane and amorphous layer. This indicated that the pit membrane and/or amorphous layer rather than the entire cell wall (primary and secondary) may, in relation to deep supercooling, play a major role in regulating the permeability of a cell. The amorphous layer is a cell wall layer lining the interior of the secondary cell wall of xylem parenchyma and may in some cases also lie sandwiched between layers of secondary cell wall (3–5, 15, 16).

In the lanthanum nitrate studies, shifts in deep supercooling to warmer temperatures were observed in water-soaked tissue, whereas the freezing profiles of samples treated with lanthanum nitrate remained stable (21). The lanthanum-treated tissues, however, exhibited marked ultrastructural aberrations, indicating cytotoxicity.

The present study was conducted to monitor cellular changes in xylem tissue that had been water soaked, resulting in a shift of the LTE,[1] and to compare these changes with the ultrastructure of tissue treated with cycloheximide, where the freezing profiles remained relatively stable. This was done to determine if any observed structural changes might logically account for the observed shifts in deep supercooling to warmer temperatures.

MATERIALS AND METHODS

Current season twigs of peach [*Prunus persica* (L.) Batsch], 'Loring,' were collected on the premises from September 1987 through January 1988, trimmed to a length of 10 cm and the base of the stems were immersed to a depth of 3 to 4 cm in either deionized water or 1×10^{-4} M cycloheximide. Ends of twigs exposed to air, were sealed with paraffin to prevent excessive desiccation. Samples were placed in a lighted incubator at 20°C for up to 10 d, and water or cycloheximide solution was added to maintain the depth of immersion.

Internodal sections from the treated twigs (4–5 mm in diameter) were obtained from submerged portions of twigs and subjected to DTA on the day of collection or after 3, 7, and 10 d. Samples were debarked and cooled at a linear rate of 20°C/h as previously described (19). At least five samples were used from each time period and for each treatment. No

[1] Abbreviations: LTE, low temperature exotherm; DTA, differential thermal analysis; AL, amorphous layer.

attempt was made to correlate the LTE with the killing temperature of the twig.

For electron microscopy, internodal sections of twigs were fixed in 5% (V:V) glutaraldehyde in 25 mM sodium phosphate buffer (pH 6.8) and post-fixed in 2% (W:V) osmium tetroxide in buffer. Samples were dehydrated in a graded ethanol series and embedded in Spurr's epoxy resin (17). Sections were stained with uranyl acetate and lead citrate and viewed with a Hitachi H-600 transmission electron microscope.

RESULTS

DTA Profiles

The extent of deep supercooling of xylem tissue collected in September was relatively minimal (midpoint at −20°C) compared to samples obtained in January (Fig. 1A). Soaking twig samples in water resulted in a shift of the LTE to warmer temperatures (midpoint at −15°C). In contrast, cycloheximide treated tissues remained more stable, although a broadening of the LTE was observed (Fig. 1A).

The extent of deep supercooling of samples collected in January was much greater (Fig. 1B) with a midpoint at −32°C. Soaking twig samples, collected in September, in water shifted the midpoint of the LTE to −18°C. Soaking twig samples in water caused the LTE to appear bimodal and shift the midpoint of the major portion of the exotherm to −18°C. The LTE of cycloheximide-treated samples remained relatively stable although a slight broadening and bimodality was observed.

Ultrastructural Observations

Marked alterations in cell ultrastructure were observed during shifts in the LTE. These changes were most dramatic in samples collected in January when the degree of deep supercooling was more marked. For this reason, the micrographs presented are from samples collected and treated during that time period.

A general view of a xylem parenchyma cell is visible in Figure 2. Cells had a centrally located nucleus and were densely cytoplasmic with a few small vacuoles. The amorphous layer which lies between the secondary cell wall and the protoplast, appeared uniform in density (Fig. 3). Immersion of twigs in water resulted in marked changes in the structure of the amorphous layer and the appearance of the protoplast. Changes were observed within 3 d after treatment, became prominent within 5 to 7 d, and were quite extensive after 10 d.

Initial change consisted of a marked swelling of the amorphous layer in the vicinity of the pit membrane (Fig. 4). This was followed by the appearance of numerous invaginations of the plasma membrane, containing both fibrillar wall material derived from the amorphous layer, and vesicles (Figs. 5–7). These invaginations often appeared bulging into the vacuole where they were pinched off, indicating recycling of wall and membrane material (Figs. 7 and 8). Within the cytoplasm, increased vacuolation became apparent as well as greater Golgi activity and an increase in the number of cytoplasmic vesicles was observed.

In later stages of alteration, a distinct loosening and extraction of cell wall material within the amorphous layer was observed (Figs. 8–11). In some cells, vesicles were apparent within the amorphous layer (Fig. 9) and features interpreted as intrusion of the protoplast into loosened or digested wall material were also seen (Figs. 8 and 9). Initially, these appeared as dark patches within the wall which then later assumed a granular, cytoplasmic appearance.

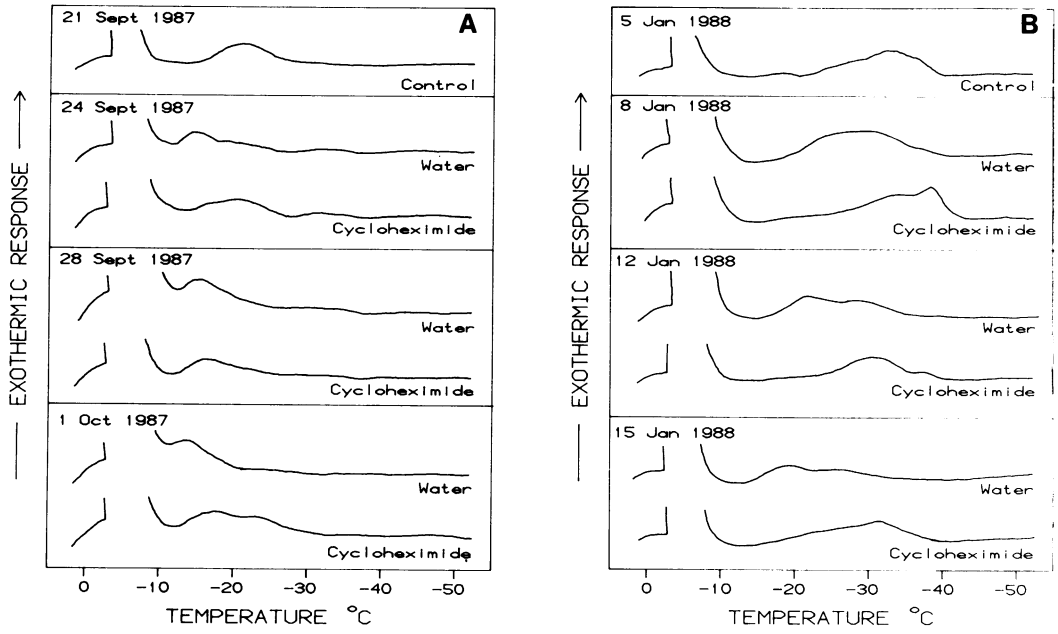


Figure 1. Differential thermal analysis profiles of 'Loring' peach twigs immersed in water or 1×10^{-4} M cycloheximide for 3, 7, and 10 d. A, Twigs collected on September 21; B, twigs collected on January 5. The broken portion of the curves represents the high-temperature exotherm.

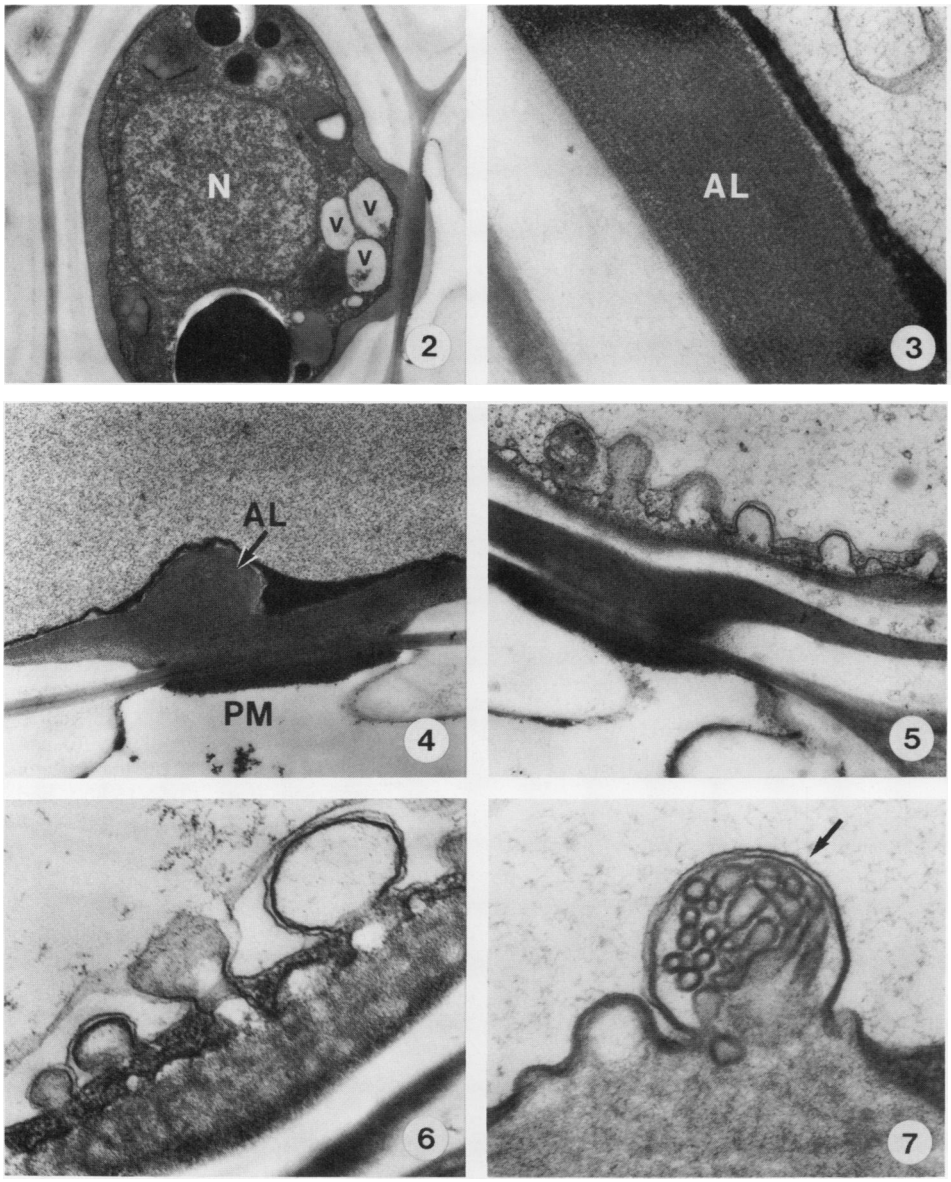


Figure 2–3. Xylem parenchyma in control tissue collected in January. Note centrally located nucleus (N), small vacuoles (V), smooth plasma membrane and uniform density of the amorphous layer (AL) ×8,800 and ×31,800, respectively.

Figures 4–7. Changes in the ultrastructure of xylem parenchyma of water-soaked samples after 5 to 10 d.

Figure 4. Swelling of AL (arrow) in area of pit membrane (PM) ×13,500.

Figures 5–7. Endocytotic vesiculation of the plasma membrane. Large vesicles contain both wall material derived from the AL as well as smaller, internal vesicles. Both plasmalemma and tonoplast membranes, visible in Figure 7 (arrow), apparently from the endocytotic vesicles, ×16,200, ×26,500 and ×56,400, respectively.

In contrast to the above, cycloheximide treated samples displayed little ultrastructural change (Figs. 12 and 13). Although some cells exhibited changes as described above, the majority of xylem parenchyma exhibited a smooth plasma membrane, free of any invaginations. The amorphous layer appeared uniform in density, with no apparent swelling or loosening. The marked difference in the structure of the amorphous layer in water-soaked tissue and cycloheximide-treated tissue can be readily seen by comparing Figs. 14 and 15.

DISCUSSION

The present research further supports our previous contention (21, 22) that the structure of the pit membrane and/or amorphous layer ('protective layer'), rather than the entire cell wall, plays a major role in deep supercooling. Pronounced shifts in the LTE of current year shoots of peach can be induced over a 3- to 10-d period by soaking twigs in water at 20°C. Shifts are characterized by the broadening of the original LTE and the appearance of additional peaks (Fig. 1, A and B). These changes are similar to those reported for other systems where naturally occurring or induced shifts in deep supercooling have been monitored (9, 10) and may represent the varying potential of different tissue types within the stem to deep supercool (10).

The shifts in the LTE were associated with a distinct loosening and partial dissolution of portions of the amorphous layer. Additionally, cytological changes, marked by invaginations of the plasma membrane and increased vesicle activity were observed. As in other studies (11, 12, 14, 18, 20) these were interpreted as part of a process of cell wall and membrane turnover. Similar appearances in cell structure, although not as dramatic, have been reported to occur in cortical cells (12, 20) and xylem parenchyma (20) during periods of acclimation and deacclimation. Cortical cell walls, however, consist of

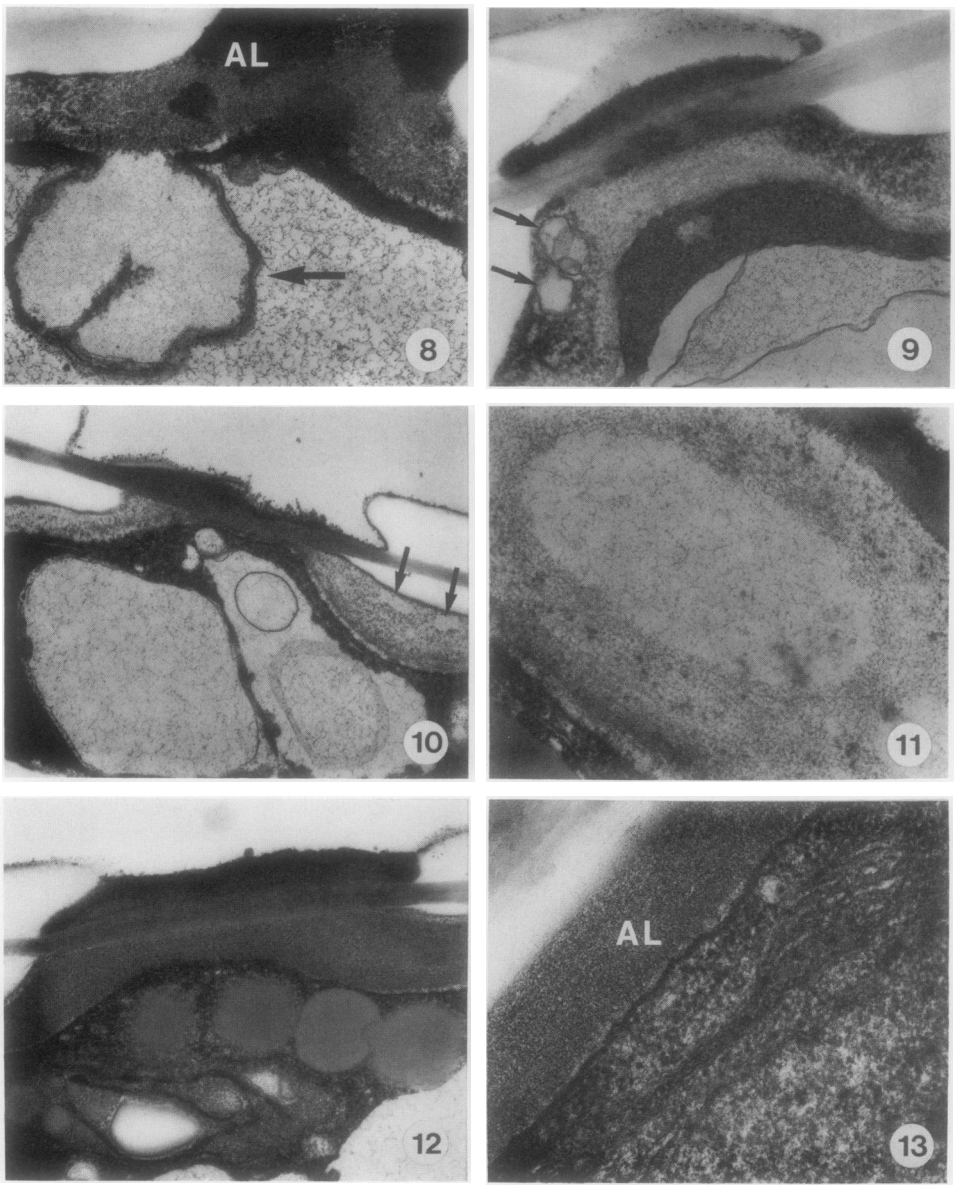
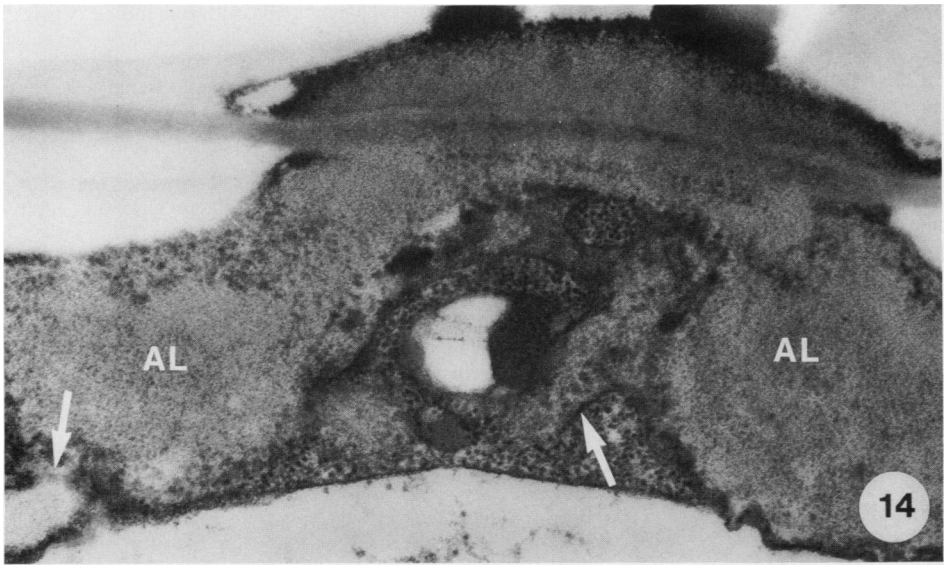


Figure 8–11. Dissolution of the amorphous layer (AL) in water-soaked samples after 7 to 10 d.
Figure 8. Xylem parenchyma with endocytotic vesicle (arrow) and partial extraction of the AL, ×19,200.
Figure 9. Vesicles (arrow) within the AL, ×23,200.
Figure 10. Note partial extraction of wall material from AL (arrow) ×13,600.
Figure 11. Enlarged view of extracted area within the AL, ×26,600.
Figures 12–13. Xylem parenchyma in cycloheximide-treated tissue after 10 d. Note uniform density of the AL and smooth appearance of the plasma membrane (Fig. 13) ×15,800 and ×45,000, respectively.

primary walls only. In our studies, the greater response of tissues collected in January, compared to those collected in September, may have been due to the fact that final stages of xylem parenchyma maturation occur in early fall (15).

Saturation of the twigs with water rather than exposure to a warm temperature (20°C) was necessary to produce the observed shifts in deep supercooling. Although increases in water content were not measured, portions of twigs (stored at 20°C) taken from greater than 2 cm above the point of water immersion exhibited little shift in the LTE, whereas samples stored at 2°C and taken from submerged portions of the twig exhibited shifts in the LTE analogous to those detailed in this report (our unpublished data). Breakdown of the amorphous layer by microbial enzymes may also account for our observations, however, there was no indication of fungal contamination within the test period and shifts in the LTE were present in both water-soaked samples stored at 2°C and those containing 0.02% 8-hydroxyquinoline (W:V); (our unpublished data). Admittedly, the dramatic alteration of the amorphous layer may have been triggered by the saturation of the twigs with water and therefore represent an aberration of processes that occur during deacclimation. This, however, does not negate the idea that the structure of the amorphous layer may regulate the permeability of xylem parenchyma and hence the extent of deep supercooling. Regardless of how modification is achieved there is a strong association of changes in the amorphous layer (as opposed to other layers of the cell wall) with shifts in the LTE to warmer temperatures.

In contrast to water-soaked tissues, dramatic shifts in the LTE accompanied by marked ultrastructural changes were not observed in cycloheximide treated tissues. Instead, the appearance of cycloheximide-treated cells were characteristic of fully acclimated cells (13, 18, 20). Although data obtained from tissues exposed to cycloheximide over a long period of time must be made with caution and may be of limited value, they serve as a starting point for the study of a complex



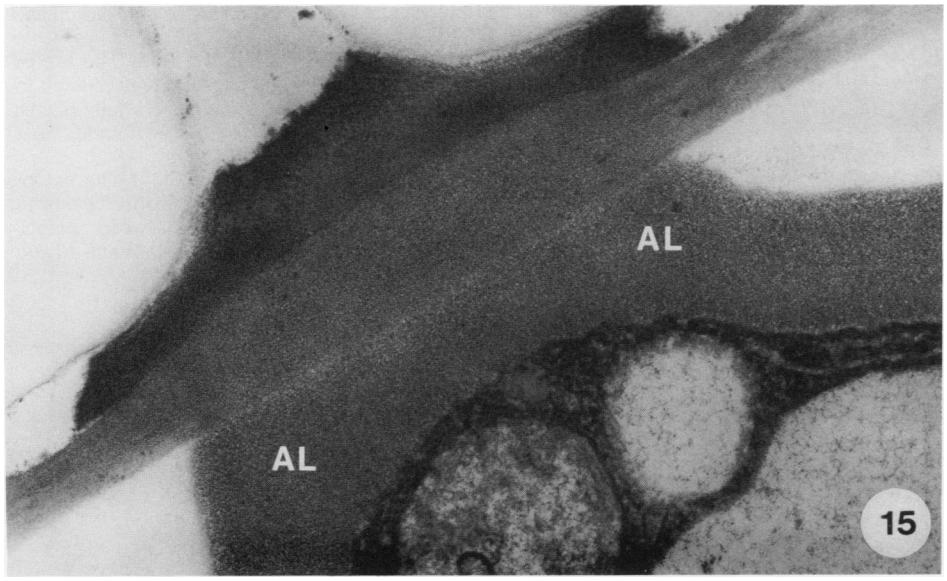


Figure 14–15. Comparison of xylem parenchyma in water-soaked (Fig. 14) and cycloheximide-treated (Fig. 15) tissue after 8 d of treatment.
Figure 14. Note swelling and partial extraction of AL as well as invagination of the plasma membrane (arrows) ×37,900.
Figure 15. Note uniform density of the AL ×44,000.

phenomenon. The fact that shifts in the LTE were inhibited indicates that hydration of tissues alone does not account for the loss of supercooling. Instead, active processes of cell wall, and perhaps membrane turnover, are necessary to account for the observed changes. Niki and Sakai (13) found that exposure of mulberry twigs to cycloheximide for up to 12 d inhibited the development of cold hardiness in cortical tissues. They postulated the need for the synthesis of functional protein during hardening. Based on our data, we postulate that the synthesis of cell wall degrading enzymes, responsible for altering the structure of the amorphous layer during deacclimation, was inhibited in cycloheximide-treated samples and prevented marked decreases in deep supercooling.

It is important to note that neither a complete loss of deep supercooling in water-treated tissues, nor an absolute stability of the LTE in cycloheximide-treated tissues was observed. This was also reflected in a population of cells with a mixed appearance in water-treated and cycloheximide-treated samples. Loosening and partial dissolution of the amorphous layer, with accompanying cytological changes, predominated in water-treated tissues whereas changes in the density or uniformity of this cell wall layer, along with cytological changes, were only occasionally observed in cycloheximide-treated tissues. Several factors may account for this response. Due to the complex structure of wood, all cells may not have been equally exposed to water or cycloheximide. At the time of this study the cells were also semi-dormant and may have been unresponsive to treatment. Alternatively, other wall components, such as the pit membrane or elements of structure at the tissue level, that are not affected by water-soaking, may play a role in defining the extent of deep supercooling.

The usage of the term amorphous or protective layer has been somewhat confusing and it has been reported to have several implied functions (15). This is the first report, however, of changes in this layer of the cell wall being associated with marked shifts in deep supercooling.

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