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Changes in Extracellular Polysaccharides During Cold Acclimation of Cultured Pear Cells

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Additional index words. freezing injury, hardiness, suspension culture, tissue culture, *Pyrus communis*, callose

Abstract. **Suspension-cultured pear (*Pyrus communis* 'Bartlett') cells cold-acclimated during exposure to 2°C. Cold acclimation was accompanied by changes in soluble extracellular polysaccharides and in the deposition of callose in the cell wall. Release of a relatively small neutral polysaccharide into culture medium was increased at 2°. However, low temperature decreased the extracellular accumulation of a larger molecular weight (M_r) pectic polysaccharide. The reduced amount of pectic polysaccharide may have been the result of both a low-temperature-enhanced degradation of existing polysaccharide and an inhibition of new synthesis or secretion. The effect of low temperature on callose deposition was observed using an aniline blue fluorescent staining procedure. Pear cells held at 2° showed far more intense staining than those at 22°, indicating increased deposition of callose or other β-1,3-glucans at the cell surface during cold acclimation.**

Recent attempts to explain cold acclimation have focused primarily on the plasma membrane, which is generally considered a site of critical freezing injury (18). However, the physical association of plasma membrane and cell wall (14) validates suggestions that the wall may contribute to injury (11, 19) and/or protection (15). In whole plant cells, unlike animal cells and plant protoplasts, the membrane shares close structural and functional relationships with the cell wall (6, 14, 24). During normal freezing, cellular dehydration causes cytorrhysis (11, 12, 19) rather than plasmolysis; i.e., the wall and membrane remain in contact. There is recent evidence (19) that supports Iljin's classic idea (11) that the wall may exert an injurious mechanical strain on the membrane during freeze-thaw stress. If cell wall-related strain causes injury, then changes that alter wall properties may be an important component of cold acclimation.

We have begun a study of possible cell wall involvement in cold acclimation and freezing injury. This report describes changes that occur in extracellular polysaccharides during exposure of cultured pear cells to cold-acclimating temperature. Polysaccharides released into the medium, assumed to represent component polysaccharides of the wall per se (2), were the primary subject of this study.

Materials and Methods

All experiments were conducted with suspension cultures of pear (*Pyrus communis* 'Bartlett'), which we used previously (22). The culture medium, maintenance, and growth conditions (22), as well as stress treatment and viability tests (23), were as described previously. Cultures were 110 ml of cell suspension in 500 ml Erlenmeyer flasks; they were aerated with gyratory shaking at 85 to 90 cycles per min. Pear cells were cold-acclimated by placing 7- or 8-day-old cultures on a platform shaker at 2°C. Freezing stress was imposed and injury measured essentially as described by Towill and Mazur (20).

The extracellular polysaccharide samples used in this study were filtrates obtained by passing cultures through Miracloth and washing the cells with deionized H_2O. Following dialysis to remove low M_r solutes, samples were concentrated (3 to 5 times) under reduced pressure at 50°C. Preliminary experiments showed that no oligosaccharides were present in culture media, so we used 6000–8000 M_r cut-off dialysis membranes for all experiments reported here. In certain experiments, extracellular polysaccharide solutions from control and cold-acclimated cultures were adjusted to equivalent osmolalities, measured with a Precision Systems osmometer. Gel filtration chromatography was performed with standard methods; 1-ml fractions were analyzed for neutral sugar (17) or uronic acid (5) content. The neutral sugar composition of pooled, lyophilized fractions was determined via GLC of alditol acetate derivatives of sugars solubilized by trifluoroacetic acid hydrolysis (3). The medium of suspension-cultured cells is a convenient source of extracellular polysaccharides. The concept of the suspending medium as an extended intercellular space (21) affirms the general assumption (2) that changes in soluble extracellular polysaccharides reflect those in the cell wall.

Callose was detected in walls of pear cells suspended in aniline blue (0.01% in 10 mM KPO_4, pH 8.5) as described by Smith and McCully (16). A Zeiss fluorescence microscope (excitation at 390 to 490 nm) was used to observe the yellow-green fluorescence characteristic of callose in aniline blue. None of the extracellular polysaccharides secreted by cultured pear cells were stained by aniline blue.

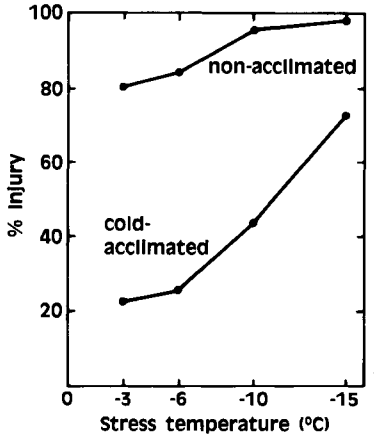

Fig. 1. Freezing injury to nonacclimated and cold-acclimated pear cells. Cold-acclimated cells were from cultures that were placed at 2°C when they were 7 days old; low-temperature treatment lasted 10 days. Injury measurement was based on TTC reduction.

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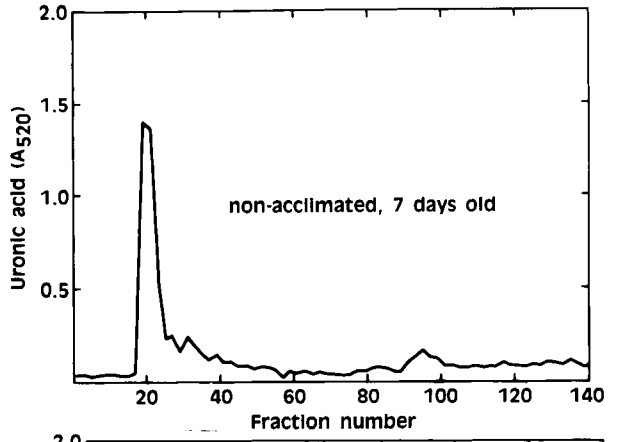

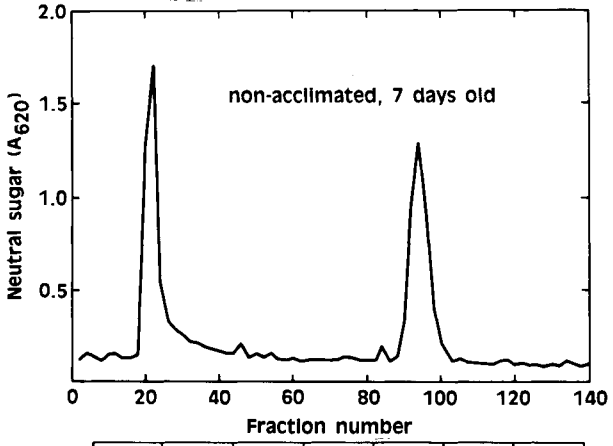

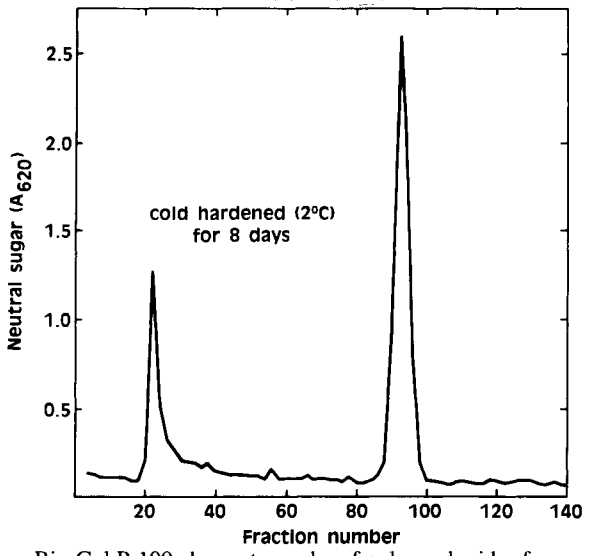

Fig. 2. Bio-Gel P-100 chromatography of polysaccharides from pear culture media. The void volume was 22 ml; 1-ml fractions were analyzed for neutral sugar or uronic acid content. (**A**) elution pattern for polyuronides from 7 days old, nonacclimated culture. (**B**) elution pattern for neutral polysaccharides from 7-day-old, nonacclimated culture. (**C**) elution pattern for neutral polysaccharides from culture that was held at 2°C for 8 days.

Results

Cold acclimation. Cultured pear cells developed increased freezing tolerance during incubation at 2°C (Fig. 1) (23). The low-temperature killing point (50% injury), determined on the basis of ability to reduce TTC, was about −13° after 10 days at 2°. Nonacclimated cultures showed 80% injury at −3°, the highest freezing temperature tested. Cold acclimation increased with additional time at 2° (23).

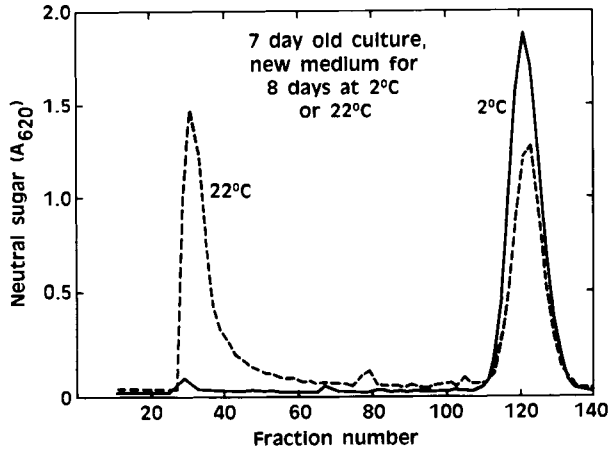

Fig. 3. Bio-Gel P-100 chromatography of polysaccharides released in medium replacement experiments. Cells were removed from growth medium, resuspended in fresh medium, and held for 8 days at 22° or 2°C. The samples were prepared as described in the text. The void volume was 26 ml; 1-ml fractions were analyzed for neutral sugar content.

Soluble extracellular polysaccharides. Polysaccharides released into culture medium during normal development included a neutral sugar and uronic acid-containing fraction that eluted in the void volume of a Bio-Gel P-100 column (Fig. 2). This large-M_r fraction resolved into multiple peaks (data not shown) when passed through Agarose AO.5 m (500 kDa exclusion limit). A relatively small (≈40 kDa) neutral polysaccharide also was released into pear culture medium. Component sugars of the small polysaccharide were glucose, galactose, arabinose, mannose, and xylose; glucose and galactose (2:1) accounted for >80% of this fraction. Although the molar ratio was quite different, the same 5 sugars were found to comprise an extracellular polysaccharide in tobacco culture medium (1).

Low temperature effected extracellular polysaccharide accumulation in pear culture media. This effect was first noticed as an increase in the relative size of the 2nd elution peak for neutral polymers on Bio-Gel P-100 (Fig. 2C). After cold acclimation (8 days at 2 °C), the low-M_r peak contained about twice as much neutral sugar as the void fraction. This result contrasted sharply with the elution pattern for samples from nonacclimated cultures (Fig. 2B).

Because cultures were placed at 2°C when they were 7 or 8 days old, the medium preparations (Fig. 2C) contained polysaccharides released at 22° as well as those released at 2°. Since the presence of control polysaccharides (Fig. 2A and B) may have partially obscured cold-induced change, we conducted medium replacement experiments (Figs. 3 and 4); that is, cells were removed from growth medium and resuspended in fresh medium immediately prior to low-temperature treatment. These experiments showed that the only extracellular polysaccharide present in significant amounts at 2° was the small neutral polysaccharide (Fig. 3). Pear cells returned to 22° in fresh medium released polysaccharides (Fig. 3) that showed the same elution pattern as before (Fig. 2B). The low-temperature effect was confirmed in time course studies of carbohydrate content in dialyzed medium preparations (Fig. 4). The release of neutral sugar-containing polysaccharides was enhanced at 2°, but pectic polysaccharide accumulation was greatly reduced compared to that at 22°.

The low concentration of extracellular pectic polysaccharides at 2°C could have resulted from inhibited release and/or removal

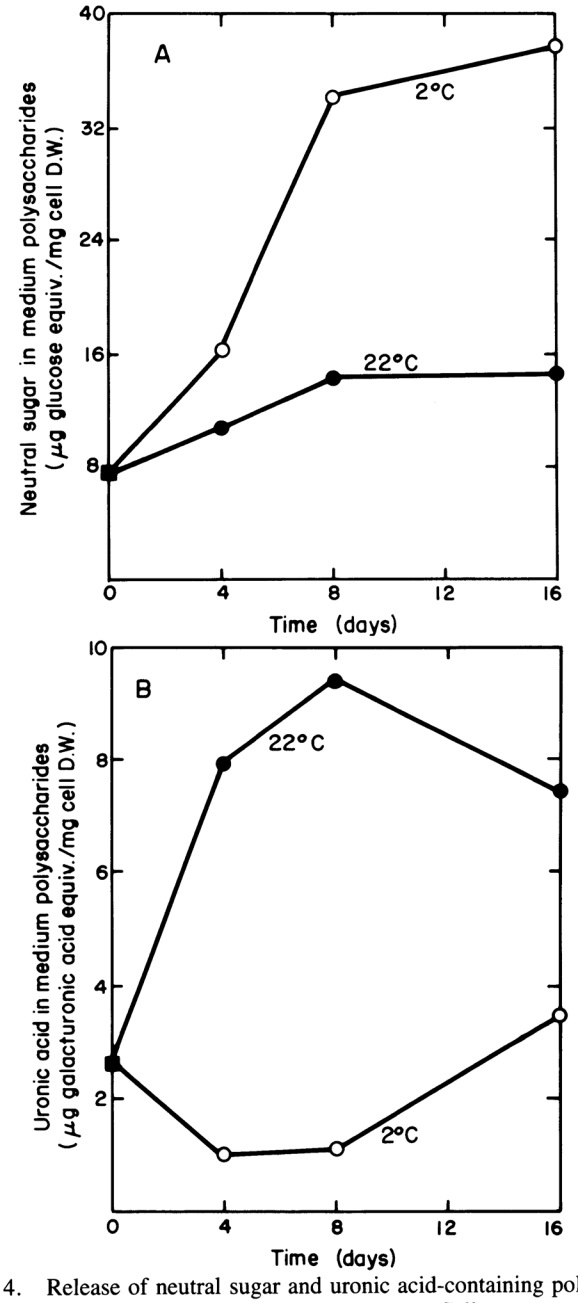

Fig. 4. Release of neutral sugar and uronic acid-containing polysaccharides in medium replacement experiments. Cells were removed from growth medium, resuspended in fresh medium, and held at 22° or 2°C. Replacement media were analyzed for neutral sugar and uronic acid-containing polymer at the indicated intervals.

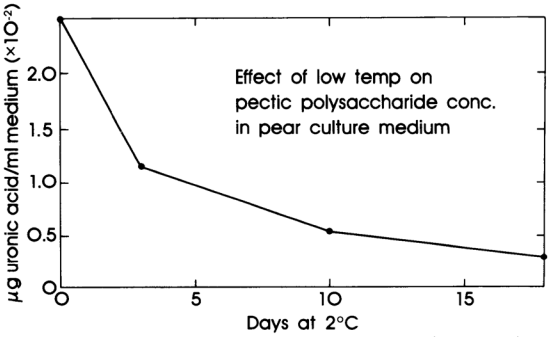

Fig. 5. Decline in pectic polysaccharide content of pear culture medium at 2°C. Cultures were placed at 2° when they were 7 days old and analyzed for uronic acid-containing extracellular polysaccharides at the indicated intervals.

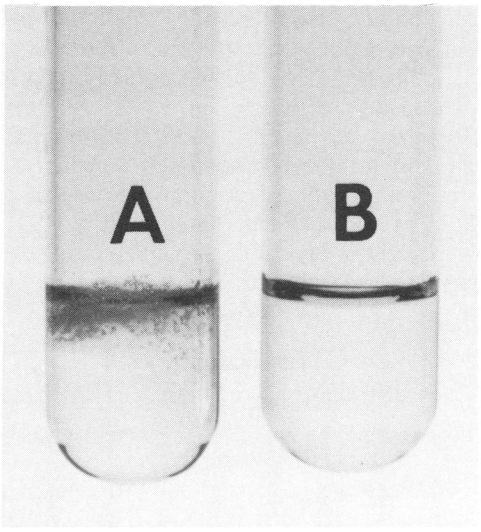

Fig. 6. Post-thaw gel formed during freezing to −20°C in 2-ml solution from medium of non-acclimated (**A**) pear cultures. The solutions from cold acclimated (**B**) cultures did not gel. The osmolality of both solutions was about 10 milliosmal per kilogram.

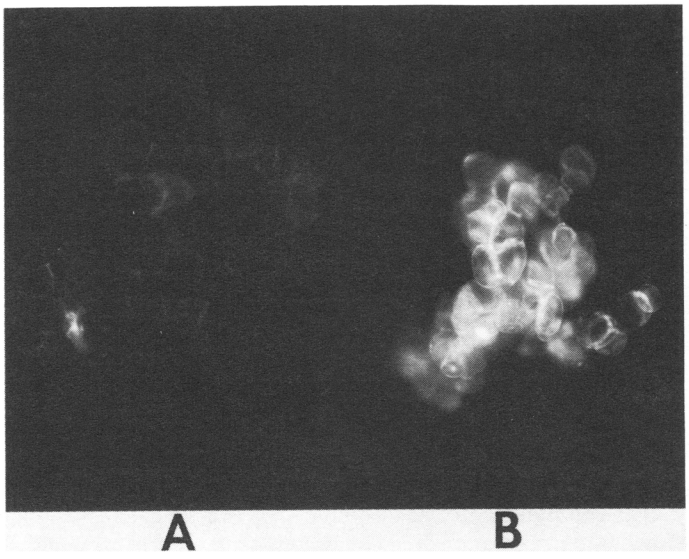

Fig. 7. Callose deposits in cell walls of pear cells from nonacclimated (**A**) and cold-acclimated (**B**) cultures. Nonacclimated cultures were 10 days old; cold acclimation was achieved by placing 7-day-old culture at 2°C for 12 days.

by degradation or surface binding. Evidence for removal of pectic polysaccharides is presented in Fig. 5. In this experiment, pear cells were left in original growth medium when they were placed at 2°. During 8 days at 22°, the cells had accumulated 250 mg uronosyl residues per ml of medium (day 0 in Fig. 5). During the first 3 days at 2°, the amount of pectic polysaccharides was reduced by more than 50%; the decline continued and at 18 days the medium contained only 30 mg uronosyl residues per ml.

Cold-induced biochemical changes were accompanied by changes in other properties of solutions prepared from culture media. For example, during freeze–thaw treatment, polymers from the medium of nonacclimated cultures underwent irreversible gel formation; those from cold-acclimated cultures did not (Fig. 6).

Callose deposition. Exposure to 2°C increased the accumulation of callose in pear cell walls (Fig. 7). This increase was

observed using aniline blue, a stain that fluoresces on reaction with β-1,3-glucan. Callose was present in the wall of nonacclimated pear cells (Fig. 7A), but was greatly increased at 2° (Fig. 7B). The pattern of deposition was also influenced. Individual cells in nonacclimated cultures had different levels of callose, and highly localized deposits were sometimes observed (Fig. 7A) in contrast with the uniform presence of callose in walls of cells from cold-acclimated cultures (Fig. 7B). Increased callose was apparent 3 days after the initiation of low-temperature treatment and appeared to reach a maximum by 8 to 10 days at 2°.

Discussion

One requisite for involvement of the cell wall in cold acclimation is the capacity for adaptive change in response to inductive conditions, including low temperature. Capacity for cold-induced change is well-illustrated by the pear culture extracellular polysaccharide differences at 2° and 22°C (Figs. 3, 4, and 7). Accumulation of large-M_r soluble polysaccharide was inhibited, while that of a smaller, neutral fraction, containing mainly galactose and glucose, was increased at 2° (Figs. 3 and 4). Low temperature also has been shown to modify wall metabolism in other plant tissues (4, 8, 10). Low-temperature-induced changes in membranes, especially at the plasmalemma–cell wall interface, are likely to affect enzymes involved in the synthesis and/or assembly of extracellular polysaccharides.

The reduced amount of large-M_r pectic polysaccharides in culture medium at 2°C (Fig. 2B vs. Fig. 2C; Figs. 3 and 4) may have been due to inhibition of synthesis/release or to low temperature-stimulated turnover. Pectic polysaccharides that accumulated at 22° were lost rapidly when cultures were placed at 2° (Fig. 5). There was no evidence that precipitation occurred, and it also seems unlikely that much of the change (Fig. 5) was due to surface binding (surface extractions with high salt and preliminary analyses of cell wall uronide content did not support this possibility). Another possibility is that polygalacturonase hydrolyzed soluble pectic polysaccharides to low-M_r products that were lost via metabolism or during dialysis.

One consequence of the soluble polymer changes was the effect on gel formation (Fig. 6). Even when adjusted to equivalent concentrations of polyuronide, solutions from cold-acclimated cultures did not gel during freeze–thawing. Molecular aggregations involving cell wall polysaccharides would be relevant to freezing stress response if membrane interactions are affected or if the wall itself is a site of freezing injury.

Our observation that callose deposition increased at 2°C (Fig. 7) is consistent with the apparent involvement of this polysaccharide in plant response to many different stresses (7, 9, 16). Protective roles have been proposed, but never well-defined, for callose. For example, Musolan et al. (13), who described heat-induced callose synthesis, suggested that the hygroscopicity of callose may protect against water loss at high temperatures. The results of Jaffe and Telewski (9) indicate that callose deposition is a critical element of the mechanical stress responses that lead to increased cold and drought hardiness. Apparent localization of callose at the plasmalemma–cell wall interface in pear cells (Fig. 7) is consistent with the idea that it may stabilize this region against stress-induced disturbances.

Although present results are entirely descriptive, further study may demonstrate that cell wall polysaccharides are involved in plant response to low temperatures. The major point of this paper is that specific changes in extracellular polysaccharides do occur on exposure of pear cells to cold-acclimating temperature.

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Water Relations and Water Potential Measurements for Vegetative Poinsettia

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Additional index words. *Euphorbia pulcherrima*, stomata, leaf abscission, water vapor conductance

Abstract. **Pressure chamber and thermocouple psychrometer measurements of leaf water potentials in vegetative *Euphorbia pulcherrima* Willd. cv. Eckespoint C-1 Red were evaluated. The 2 methods agreed within 0.2 MPa between −0.3 and −1.8 MPa and were equal at −1.1 MPa. Minimum daily water potential for nonstressed plants reached −0.67 MPa by 1230 HR. Abaxial water vapor conductance and water potential varied little between 1230 and 1630 HR. When drought was imposed, incipient stomatal closure occurred at −0.8 MPa with full closure observed at −1.2 MPa. Complete loss of turgor pressure occurred at water potentials between −1.2 and −1.4 MPa. The linear correlation coefficient for conductance and leaf-air temperature differential was 0.96, with leaf and air temperature equal when conductance was 0.6 cm·s^{-1}. Xylem pressure potentials of upper leaves on drought-stressed plants declined to −1.7 MPa in 8 days and abscission of proximal leaves began. There was little change in xylem pressure potentials of upper leaves after leaf abscission began.**

Poinsettia water relations have been the subject of relatively little research. White and Holcomb (20) demonstrated with 2 cultivars that allowing plants to wilt prior to irrigation from planting until onset of bract coloration caused a 45% reduction in final plant height and a 20% decrease in inflorescence diameter. Gilbertz et al. (9) reported that leaf abscission resulted from drying flowering plants to water potentials of −1.3 to −1.1 MPa, and plants were more susceptible to stress after initiation of short days. Drought stress affected inflorescence size more when imposed after onset of bract coloration than before coloration. Plant height and time of flowering were affected more by drought prior to coloration than following. Barrett and Nell found that growth retardants reduced total water requirements for poinsettia crop production (4) and whole plant transpiration (3).

This research was conducted to evaluate pressure chamber and thermocouple psychrometer techniques for water potential measurements and to describe diurnal and drought stress effects on stomatal conductance and leaf water potential in poinsettia.

Materials and Methods

'Eckespoint C-1 Red' poinsettias were obtained as rooted cuttings from commercial sources, potted one per 15-cm pot, and grown under noninductive photoperiods (lighting provided between 2200 and 0200 HR). The tests were conducted on 5- to 10-week-old single-stem plants. Maximum greenhouse temperature was 36°C, minimum relative humidity was 60%, and maximum photosynthetic photon flux density was 1000 μmol·s^{-1}·m^{-2}.

Leaf position was determined by counting basipetally from the first expanding leaf >2 cm long. The sampled leaf was the most recent fully expanded leaf (number 8, 9, or 10), except where indicated. Each leaf used for pressure chamber determination of xylem pressure potential was enclosed in a plastic bag (that contained a wet paper towel) prior to being excised and rate of pressure increase was <1.5 MPa·min^{-1}. Chambers used were Soilmoisture Equipment Model 3005 or PMS Instrument Model 600. For psychrometer measurements of leaf water potential, a 15 × 40 mm section from midway of the blade and to one side of the midvein was excised, rolled, and enclosed in a J.R.D. Merrill Model 74-13C psychrometer and chamber. The thermocouple psychrometer assembly was placed in a 30°C water bath for 4 hr before the output was determined with a Wescor Model HR-33T micovoltmeter. The tissue then was frozen, thawed, re-equilibrated for 4 hr and the output again was determined to obtain osmotic potential. Turgor pressure was calculated as the difference betwen leaf water potential and osmotic potential. Abaxial water vapor conductance, air and leaf temperature, relative humidity, and photosynthetic photon flux density (PPFD) were obtained with a LI-COR Model LI-1600 steady state porometer. Porometer data and samples for use in the pressure chamber or psychrometer were taken between 1230 and 1430 HR, except where indicated.

Experiment 1. In order to determine diurnal patterns, leaf conductance and xylem pressure potential were determined for greenhouse poinsettias at intervals between 0600 and 2100 HR. Eight plants were sampled at each time. In order to determine the effect of leaf position, xylem pressure potentials were determined for leaves at node 10, 13, 16, and 19 on a separate

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