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# XYLEM TRACHEID DEVELOPMENT IN *PINUS RESINOSA* SEEDLINGS IN CONTROLLED ENVIRONMENTS

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## ABSTRACT

Progressive tree-ring xylem cell size changes may reveal the influence of changing environment during the growing season. This study examines xylem tracheid cell growth in red pine (*Pinus resinosa* Ait.) seedlings grown in cabinets under controlled environment, where single parameters (temperature, light, soil moisture and $CO_2$) were varied step-wise in each chamber at *ca.* 30-day increments for *ca.* 6 months. Control and temperature treatments were replicated. Cross-sections (20 $\mu$m thick) sliced with a sliding microtome from each of four seedling stems from each cabinet were mounted on glass slides. Lumen diameters and cell-wall thickness were measured on 4 orthogonal tracheid radial files on 4 radii of each stem. Mean cell sizes were 11–17 $\mu$m among treatments and growth periods, whereas numbers of cells formed averaged 0.2–1.3 cells per day. Cell size increased throughout the experiment in most of the treatments, including one of the control treatments and those with the greatest potential to limit growth (decreasing temperature, light and soil moisture). Soil moisture was the only environmental parameter that tended to cause late declining growth, and $CO_2$ up to 500 ($\mu$mol mol$^{-1}$ did not appear to influence cell development. Despite a substantial range of environmental shifts in the chambers (100 $\mu$mol mol$^{-1}$ $CO_2$; 125 $\mu$Einsteins m$^{-2}$ s$^{-1}$ light; 8°C temperature; 35% relative humidity; watering every day to every 5th day), the continued stem elongation and cell-size increases indicate that conditions never became significantly limiting to growth in most treatments. Although the range of environmental variability is undoubtedly much greater in most natural red pine systems, these results indicate that fairly large variations in environment during development of juvenile wood in seedlings may not leave an imprint retrievable from cell-size measurements made on the earliest rings of mature trees.

## INTRODUCTION

In addition to genetic contributions, xylem growth will be affected by climate and exogenous and endogenous disturbances that control light, temperature, precipitation, relative humidity, soil moisture, soil nutrients, and $CO_2$ (Cook 1987; Kozlowski and Pallardy 1997). Growth has customarily been quantified by ring width, but there is increasing interest in micro-characteristics of tree rings such as the number and size of cells and cell-wall thickness produced during the growing season (Vaganov *et al.* 1985; Schweingruber *et al.* 1993; Telewski *et al.* 1999). Jagels and Dyer (1983) reported very rapid decrease in cell lumen area under dry conditions, while under wet conditions large cell lumen areas are sustained longer. Saß and Eckstein (1992) found that average vessel area in tree rings of beech trees shows a stronger response to precipitation (+) and temperature (−), than did ring width. Orcel *et al.* (1992) determined that the number of pores of Swiss oaks correlated to spring temperature (+) and sunlight (−), and pore size correlated to winter rainfall (+) and early spring sunlight (+). Jagels and Telewski (1990) summarized recent advances in cell parameter measurements, and noted smaller cell-wall thickness and lower ratio of cell-wall thickness to lumen diameter in *Pinus taeda* seedlings under drought-stressed conditions. Modeling of xylem cell growth in response to climate is being actively pursued using daily climate information for mature

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trees in field stands (Fritts *et al.* 1991, 1999; Deleuze and Houllier 1998).

This study examines radial tracheid growth of red pine (*Pinus resinosa* Ait.) seedling stems under controlled environmental conditions to determine the respective influences of light, temperature, $CO_2$, soil moisture and relative humidity. Red pine is widespread in the central and eastern parts of southern Canada and northern United States, and has a reputation for genetic uniformity (Fowler and Morris 1977). Comparisons are made within and among trees under the same growing conditions, and among seedlings under different growing conditions. These results inform us about the nature of xylem tracheid development in red pine seedlings exposed systematically to a range of environmental conditions, although the environmental range is probably less than that found in most natural red pine systems over the growing season. The results may be transferable to juvenile rings in mature trees.

## METHODS

Two sets of red pine seeds from a common seed lot were germinated (in July 1989 and June 1990) and the ensuing seedlings were raised in pots with homogeneous bulk potting soil in controlled growth cabinets at the University of Wisconsin's Biotron facility for two separate simulated growing seasons. For the first group ("Phase I") of experiments the growing season continued for 177 days (August 10, 1989 through February 2, 1990); the second group ("Phase III") continued for 186 days (July 23, 1990 through January 21, 1991). An environmental control failure prematurely terminated the "Phase II" plants.

Temperature, relative humidity, light, soil moisture and $CO_2$ were varied step-wise in separate cabinets in approximately 30-day increments, with a single parameter varied in each chamber to isolate its effects (Table 1). Control cabinet parameters for each phase were maintained at a $CO_2$ level of 400 ppm, a light level of 12 hours @ 320 $\mu$Einsteins $m^{-2}$ $s^{-1}$, a temperature of 25°C ($T_{day}$)/19°C ($T_{night}$), and a watering regime of 4 times daily (2 minutes per watering). In Phase I, five cabinets were utilized: (1) temperature decreased step-wise from 25°C ($T_{day}$)/19°C ($T_{night}$) to 17°C ($T_{day}$)/11°C ($T_{night}$) in cabinet #106; (2) $CO_2$ increased from 400 $\mu$mol $mol^{-1}$ to 500 $\mu$mol $mol^{-1}$ in cabinet #112; (3) relative humidity increased from 50% to 85% in cabinet #102; (4) light decreased from 320 $\mu$Einsteins $m^{-2}$ $s^{-1}$ to 195 $\mu$Einsteins $m^{-2}$ $s^{-1}$ in cabinet #108; (5) all conditions remained constant in the control cabinet #110. In Phase III, three cabinets were used: (1) temperature decreased from 25°C ($T_{day}$)/19°C ($T_{night}$) to 17°C ($T_{day}$)/11°C ($T_{night}$) in cabinet #110; (2) soil moisture decreased from watering four times daily initially to alternating four days of no watering with one day of standard watering in cabinet #108; (3) cabinet #106 was maintained as the control. With the exception of the variable being altered, the other environmental conditions were identical

**Table 1.** Schedule of step-wise temperature, light, relative humidity (RH), soil moisture and $CO_2$ changes in experimental chambers. Standard conditions in all cabinets for Period 1 in Phase I (177 days) and Phase III (184 days): 25°C temperature in light period, 19°C in dark period, 50% relative humidity (RH), ~320 $\mu$Einsteins $m^{-2}$ $s^{-1}$, 400 $\mu$mol $mol^{-1}$ $CO_2$, four 2-minute watering per day. Cabinet #110 in Phase I and cabinet #106 in Phase III are control chambers with constant conditions managed as in all cabinets for Period 1. Length of daily light period was changed from 12 to 16 hours midway through Period 2 in both phases in all cabinets.

**Phase I**

| Period/Days | RH [%] | Temperature [°C, Day/Night] | Light [$\mu$Einsteins $m^{-2}$ $s^{-1}$] | $CO_2$ [$\mu$mol $mol^{-1}$] |
|---|---|---|---|---|
| Cabinet # | 102 | 106 | 108 | 112 |
| 1/1–54 | 50 | 25/19 | 12 hr/320 | 400 |
| 2/55–100 | 58 | 23/17 | 12/16 hr/290 | 425 |
| 3/101–128 | 66 | 21/15 | 16 hr/255 | 450 |
| 4/129–157 | 76 | 19/13 | 16 hr/225 | 475 |
| 5/158–177 | 85 | 17/11 | 16 hr/195 | 500 |

**Phase III**

| Period/Days | Temperature [°C, Day/Night] | Soil Moisture [Daily Watering Sequence] |
|---|---|---|
| Cabinet # | 110 | 108 |
| 1/1–66 | 25/19 | wet |
| 2/67–114 | 23/17 | wet/dry |
| 3/115–143 | 21/15 | w/d/d |
| 4/144–172 | 19/13 | w/d/d/d |
| 5/173–186 | 17/11 | w/d/d/d/d |

to control cabinets. Radial growth was measured with a micrometer caliper every two weeks during the growing season to determine incremental growth within each period.

The first growth period in each phase, representing uniform and equivalent environmental conditions in all chambers, proceeded for 54 days in Phase I and 66 days in Phase III to assure adequate seedling establishment. In Phase I the seedlings appeared to go dormant within the second growth period prompting an increase in photoperiod from 12 hours to 16 hours on day 74. The second growth period was then extended to 46 days to ensure the seedlings were growing normally. The second growth period in Phase III was extended to 48 days to duplicate the length of time in Phase I, with photoperiod likewise changed to 16 hours at an equivalent time. The remaining growth periods were approximately 28 to 29 days in length for both phases. Additional detail of experimental design and procedures are included in a separate paper on isotopic results from these seedlings (Leavitt 2000).

After the growing season of each phase, the seedlings were harvested and oven dried. Four seedlings were selected from each cabinet for further analysis. The stems were soaked in water overnight after which a 20 m-thick cross-section was sliced from the base of each stem with a sliding microtome. The thin sections were then mounted on a glass slide and stained with safranin (a red dye) so that contrast between cell walls and lumen was more clearly defined. In Phase I, cell measurements were made visually to 0.5 $\mu$m with a micrometer ocular. Cells of stems from Phase III were measured to 0.3 $\mu$m by image analysis using NIH-Image, public domain image processing software from the National Institute of Health, customized for this application (Munro *et al.* 1996), which permits rapid measurement of dimensions with the software subroutines. Student t-tests comparing mean cell measurements from micrometer ocular and image analysis methods indicate no significant difference between the two methods. Beginning at the pith, successive cells were measured along four radial directions in the stem cross-sections from 4 trees per cabinet. A single, cell diameter measurement consists of the sum of one-half of the cell-wall thickness on each side of the lumen plus the lumen diameter as measured in the radial direction. Cell-wall thickness was found to show little variability, so that most of the variability in cell diameter is a result of lumen diameter changes. Therefore, no further analysis was done with cell-wall thickness measurements.

For each radius of each growth period, the number of cells was tabulated, the mean cell size was calculated, and the total radial growth was determined by summation, the latter to apportion cells to their respective growth periods based on stem diameter measured biweekly during the experiments. These three parameters were then averaged per plant and per cabinet. Cell size was compared within plants, among plants within a cabinet, and among cabinets in each phase for each growing period. ANOVA was used to compare results among treatments for a given time period, and repeated-measures ANOVA was employed to determine differences in results of the 5 progressive growth periods (Anderson 1971).

## RESULTS AND DISCUSSION

For both Phase I and Phase III, mean cell size within a plant varied over a range of up to 6 $\mu$m among radii. Figures 1A and 1B demonstrate interradial variability for single seedlings under variable light and temperature conditions, respectively, showing a range of *ca.* 4 $\mu$m in Period 5 for Tree #341. Among plants in a cabinet, differences in the mean cell size among the four radii varied by up to 4 $\mu$m. Figure 2 shows representative cell-size variability among seedlings in the soil moisture treatment of Phase III, showing a range of *ca.* 3 $\mu$m among seedling mean cell size in Periods 3 and 4.

Two treatments (temperature and control) were repeated in Phase III, switching growth chambers to better assess overall reproducibility of the experiment. Comparison of mean cell size in cabinets in which temperature was altered (#106 in Phase I and #110 in Phase III, Figure 3A) shows no significant differences for the first four periods of the experiment, but a significant difference in Period 5 (one-way ANOVA F-test, $p < 0.05$). The difference in mean cell size between control cabinets

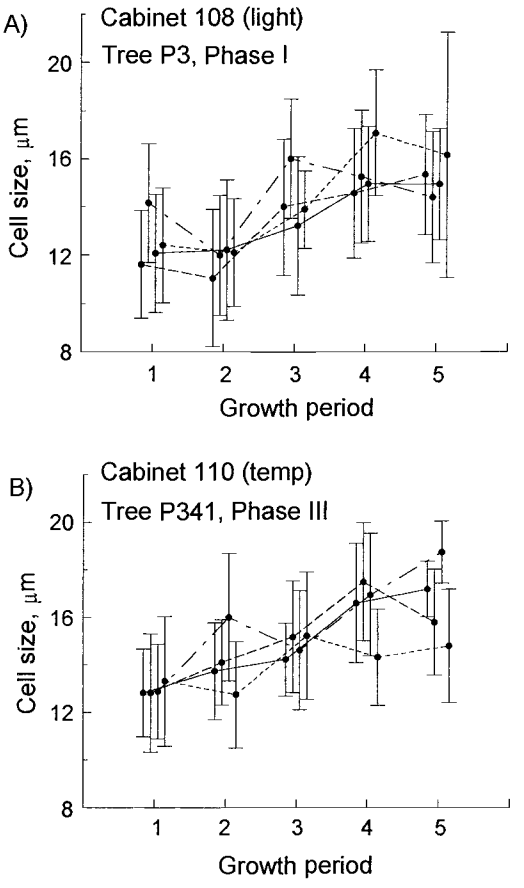


**Figure 1.** Examples of mean tracheid cell-size patterns along four orthogonal radii of individual red pine seedling stems in (A) Phase I, cabinet #108 (tree P3) in which light was varied, and (B) Phase III, cabinet #110 (tree P341) in which temperature was varied. Plants were not rotated during the experiment and moved only briefly during maintenance and measurement activities in the cabinets. Vertical bars represent 1 standard deviation.

(Figure 3B) is statistically significant (ANOVA, $p < 0.05$) for all periods except Period 2. Repeated measures ANOVA indicates significant differences between successive growth periods for temperature treatments in both phases (F-test, $p < 0.001$) and control in Phase I ($p < 0.001$), but not for control in Phase III ($p = 0.58$). The outcome of the temperature replicates favorably supports minor indeterminate effects of cabinets on the treatments, but the control cabinet results indicate some large, unknown effects in operation during this replication. Cabinet attributes would not seem to

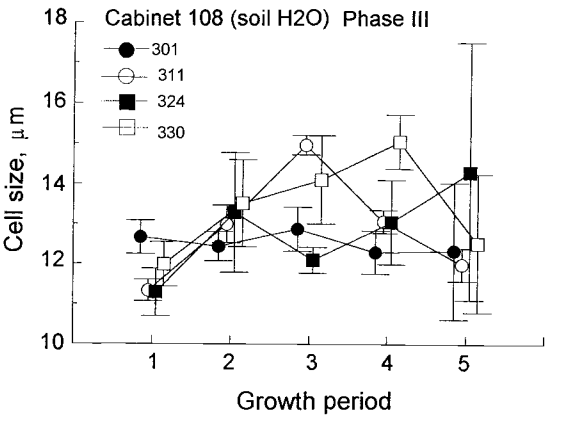


**Figure 2.** Mean tracheid cell-size patterns among stems of four red pine seedlings in Phase III, cabinet #108 (soil moisture). Vertical bars represent 1 standard deviation.

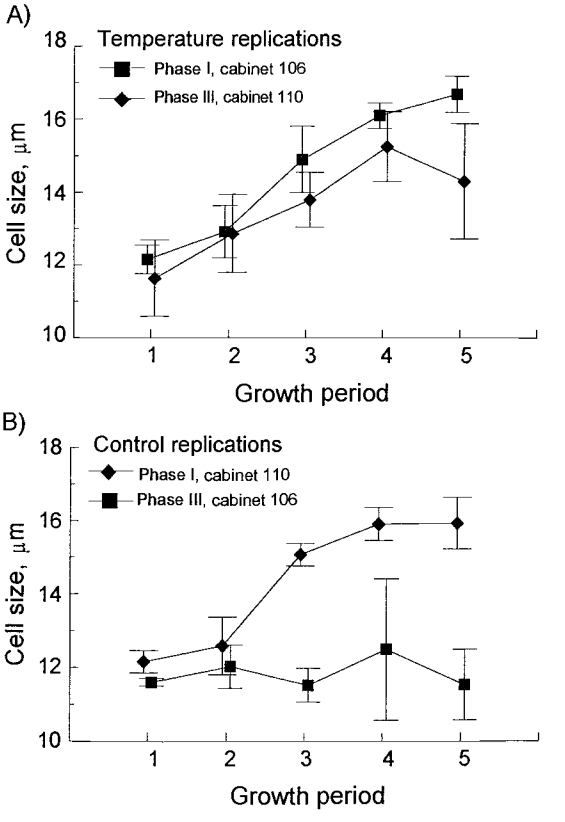


**Figure 3.** The mean tracheid cell-size of four red pine seedling stems from treatments replicated in Phase I and III. (A) Temperature-varied cabinets in Phase I (#106) and Phase III (#110), and (B) Control cabinets in Phase I (#110) and Phase III (#106). Vertical bars represent 1 standard deviation.

be a major factor because the same two cabinets were used for temperature replication. Perhaps ontogenetic differences (Denne 1976) among seedlings may have played a role, although this effect should be minimal because seedlings were distributed randomly and sampled randomly for cell-size analysis. Small differences in seedling age would not seem likely to make a difference, and would likely produce the same cell development trends with a lag. Mean final diameters in Phase III (2.5 and 3.6 mm, respectively, for cabinets #106 and #110) tended to be smaller than in Phase I for the same cabinets (4.4 and 5.8 mm, respectively). Thus, control diameters in Phase III were about one half that in Phase I, but smaller diameters (and heights) of the Phase III control seedlings need not contribute to the replication disparity. In fact, smallest diameters in both phases tended to be in cabinet #106, but temperature replicates were similar. Mean environmental values were generally within *ca.* 3% of their predesignated values, but perhaps differences between phases in the timing of extreme values associated with environmental variance may have affected growth at more critical developmental times. The growth pattern seen for the control cabinet in Phase III is closer to *a priori* expectations for growth with constant environmental conditions, *i.e.* stable environmental conditions should promote relatively constant xylem growth. However, ontogenetic or maturation effects may be playing a large role in cell development beyond the environmental effects.

Plants from all cabinets in Phase I show a similar pattern of increasing cell size (repeated measures ANOVA F-test, $p < 0.001$ in each cabinet) throughout the growth period (Figure 4A) despite the variety of environmental changes. Comparisons for each period in Phase I indicate significant differences among treatments for Period 1 (ANOVA F-test, $p = 0.03$) and a tendency to be different in Periods 4 and 5 ($p = 0.10$ and $p = 0.11$, respectively). The control cabinet (#110) pattern was not distinguished from the other treatments, with the exception of cabinet #112 from which it was different in Periods 3 (t-test, $p < 0.01$) and 4 ($p < 0.05$). In Phase III, the patterns were less synchronous (Figure 4B). Only the progressive changes in the temperature treatment (cabinet

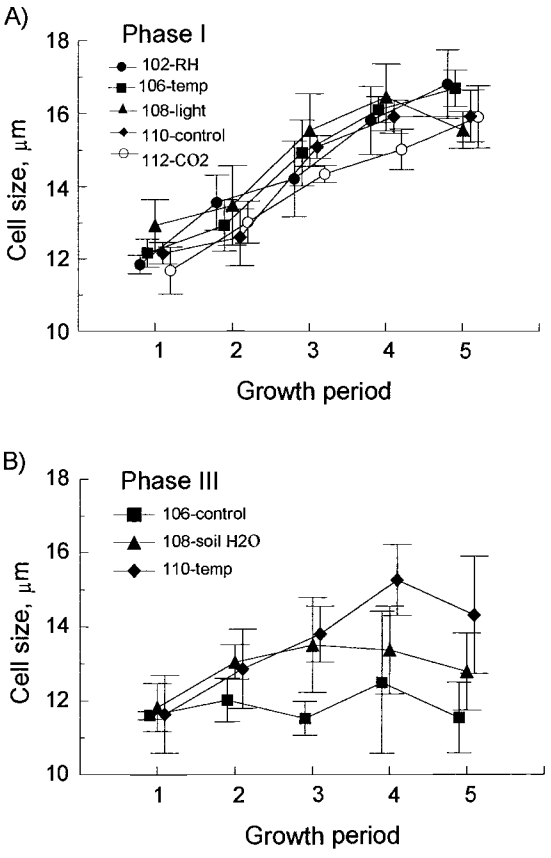


**Figure 4.** Mean radial tracheid cell size in all treatments during (A) Phase I, and (B) Phase III. Vertical bars represent 1 standard deviation.

#110) were significantly different (repeated measures ANOVA F-test, $p < 0.001$), and the 3 treatments were significantly different in Periods 3 (one-way ANOVA F-test, $p = 0.01$), 4 ($p = 0.058$) and 5 ($p = 0.03$).

Decreased soil moisture (Figures 2 and 4B) had the greatest tendency to limit cell size toward the end of the growing season after Period 3. Although increasing or decreasing environmental parameters step-wise monotonically was intended to induce xylem development changes, none of the treatments, even that of decreasing soil moisture, was sufficient to promote development of true latewood in which the combined width of adjacent cell walls exceeds the lumen diameter. Thus, any growth limitations imposed by the range over which the environmental parameters were varied

were not sufficient to induce late-wood formation. In the case of the non-water-stressed seedlings, lack of latewood may have been promoted by the abundant moisture. Jagels and Telewski (1990) found that true latewood failed to form in some growth rings of wet-site fertilized red spruce trees. Lack of latewood formation may also result from a constant, 16-hour daylight photoperiod (Denne and Dodd 1981). Photoperiod exerts a strong influence on cambial activity (Thomas and Vince-Prue 1996; Wareing 1951), although this influence may be indirectly related to the photoperiodic response of the foliar organs (Larson 1962, 1994). Larson (1960) notes that the "atypical" anatomy of juvenile wood makes it difficult to identify latewood cells by most definitions used for growth rings of mature wood. Conditions beyond the range of those in this experiment may be necessary to induce common latewood. Continued shoot elongation was found to maintain large cells in red pine seedlings (Larson 1960), and elongation was continuing in all periods of the treatments (not shown) although the rates of elongation were decreasing in most treatments in Phase I by Period 4. In Phase III the elongation rates had not yet begun to decrease by the fifth growth period.

Decreasing temperature, light levels and soil moisture are parameters that seemingly could most strongly influence general tracheid development. There was some experimental evidence for a soil moisture effect and there is a large body of literature on water supply effects on xylem development (summarized in Creber and Chaloner 1990). The common ecological affiliation of red pine with dry (extremely well-drained), sandy soils (Rudolf 1990) suggests that it may take exceptionally stressful drought conditions to affect xylem cell development. In spite of declining light level, a favorable (at least for *Pinus sylvestris*, Wareing 1951) 16-hour photoperiod throughout the experiment may have prevented expression of any light-related effects. It is surprising, however, that there was no effect of decreasing light levels because of the ecological classification of red pine as strongly shade intolerant (Rudolf 1990). Previous controlled-growth experiments with *Pinus sylvestris* seedlings (Denne 1971) found minor (*ca.* 10%) temperature effects on tracheid diameter over a *ca.*

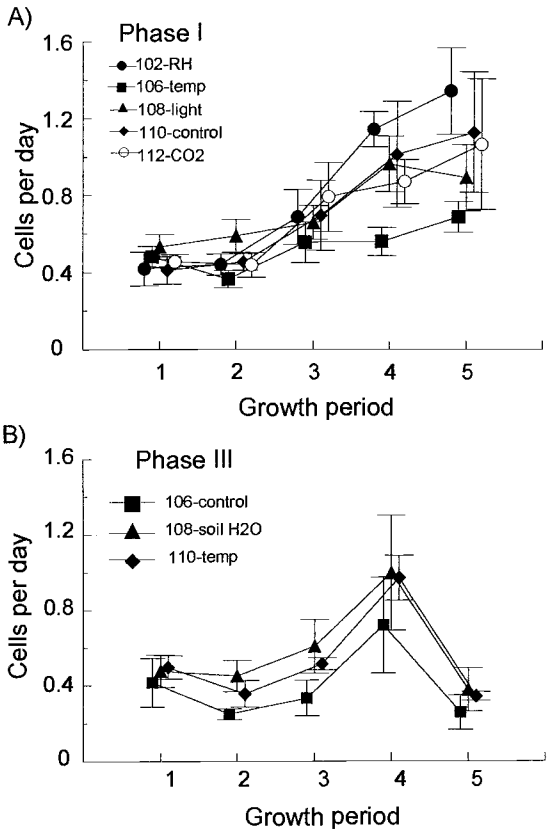


**Figure 5.** Mean number of cells per day in each growth period of all treatments during (A) Phase I, and (B) Phase III. Vertical bars represent 1 standard deviation.

10°C temperature range, with temperature influence on rates of cell enlargement and wall thickening apparently being counterbalanced by temperature effects on the duration of time in these cell-development stages. Additionally, dark-period temperatures less than 20°C may have suppressed respiration losses, thereby allowing retention of more carbon resources contributing to cell development.

Cell number counts in each period were expressed as average number of cells per day in each period because of the different lengths of the growth periods. In Phase I (Figure 5A) there was a general increase in mean number of cells produced per period, particularly after the increase in photoperiod in the second growth period. Repeated measures ANOVA indicates that for each treatment, values among the 5 periods are significantly

different (F-test, $p < 0.003$). Within periods, the treatments were different in Period 1 (one-way ANOVA F-test, $p = 0.10$), 2 ($p < 0.002$), 4 ($p < 0.002$) and 5 ($p < 0.02$). In Phase III (Figure 5B), however, number of cells per day for the 3 treatments in Periods 1–3 was fairly constant, but cells per day were high in Period 4 and low in Period 5, with all 3 treatments showing significant differences among periods (repeated measures ANOVA F-test, $p < 0.005$). Within periods, treatments were different in Period 2 and 3 (one-way ANOVA F-test, $p < 0.006$ and 0.012, respectively). The large shift in mean cell numbers per day in period 4 to period 5 was common among treatments, even in the soil moisture treatment which would have been very dry by the time Period 4 was reached. Only the Phase III temperature treatment showed both the peak in mean cell size and mean cells per day in Period 4. Overall, the smallest mean number of cells per day tended to be in the temperature treatment in Phase I and the control treatment in Phase III. With respect to the replicated temperature treatments, the mean number of cells per day was not different in each period with the exception of Period 5 (one-way ANOVA F-test, $p < 0.001$). However, mean cells per day in control replicates were significantly different in Periods 2 ($p < 0.001$), 3 ($p < 0.02$) and 5 ($p < 0.002$).

## SUMMARY AND CONCLUSIONS

Xylem tracheid cell-size measurements revealed fairly consistent variability (a) among the cells formed within any growth period (1 standard error [SE] $\approx$ 1 $\mu$m), (b) among radii from the same seedling for each growth period (1 SE generally $<1$ $\mu$m), and (c) among seedlings within a treatment for any growth period (1 SE nearly always $<1$ $\mu$m). This reflects positively on the accurate methodology and hardware of measurement and the consistent behavior of the individuals with respect to whatever cues, environmental or otherwise, to which their tracheid cell size is responding. Furthermore, the low inter-tree variability within treatments is favorable to detecting differences among treatments given that the mean cell sizes over all periods and treatments ranged from *ca.* 12 to 17 $\mu$m.

The outcome of replication was inconsistent. The temperature treatment replicates (cabinets #106 and #110 in Phase I and III, respectively) were not significantly different except for the 5th growth period, and during that period the variability among seedlings in cabinet #110 was unusually high. The replication of control treatments (cabinets #110 and #106 in Phase I and III, respectively), however, resulted in mean cell-size trends that were very different in 4 of 5 periods, and in all periods the mean cell-size was greater in Phase I. Although the temperature replication gives us confidence in the reproducibility of the systematics of the experiment, the control replicate suggests that overall there are factors operating for which we are not accounting.

The mean cell size tended to increase throughout the experiment in six of the cabinets, including one control treatment. The exceptions were the other control treatment in which cell size remained fairly constant as might be expected with uniform environmental, and the soil moisture treatment where cell size tended to decline as moisture stress increased late in the growing season. Of the treatments with cell size increasing, the $CO_2$ treatment commonly had the smallest cells in Phase I, but this included the first growth period when all seedlings experienced the same environment.

Mean number of cells per day ranged from *ca.* 0.2 to 1.3 among treatments and periods, with similar rates of *ca.* 0.4 cells per day in the first 2 periods of all treatments. Cells per day increased over growth periods in both Phases, except for a sharp decline from Period 4 to 5 in all Phase III treatments and a similar but smaller decline in the Phase I light treatment.

The patterns of increasing cell size and cells per day in many of the treatments (including control), regardless of the environmental prescription, may be the key to understanding these results. This suggests the programmed environmental changes actually had little effect on the seedling xylem tracheid growth pattern. The continued stem elongation with associated auxin production may have been responsible for the continuous formation of large tracheids (Larson 1960), effectively "disconnecting" cell-size parameters from environment except as they might influence stem elongation.

Additionally, some element of genetic programming for rapid growth in seedlings for competitive advantage may not be excluded. The environmental range over which variables were altered was not sufficient to override the programming and induce distinctive, environmentally-driven cell-size changes. With minor exceptions, red pine seedling cell size seems unresponsive to the changing environmental conditions.

Controlled growth studies in general provide a powerful tool by which to study cell growth because one can isolate influences of individual environmental parameters. With seedlings and their juvenile wood, however, genetic programming to maximize growth, or stem elongation over very broad ranges of environmental conditions may render all but extreme environmental conditions "invisible" with respect to stem tracheid cell-size response. Applying this to mature trees, at least for red pine the inner juvenile rings are less likely to reveal environmental information retrievable from cell-size measurements within the environment ranges of this experiment.

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