

# Limitations of Photosynthesis in *Pinus taeda* L. (Loblolly Pine) at Low Soil Temperatures<sup>1</sup>

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## ABSTRACT

The relative importance of stomatal and nonstomatal limitations to net photosynthesis (A) and possible signals responsible for stomatal limitations were investigated in unhardened *Pinus taeda* seedlings at low soil temperatures. After 2 days at soil temperatures between 13 and 7°C, A was reduced by 20 to 50%, respectively. The reduction in A at these moderate root-chilling conditions appeared to be the result of stomatal limitations, based on the decrease in intercellular CO<sub>2</sub> concentrations (c<sub>i</sub>). This conclusion was supported by A versus c<sub>i</sub> analysis and measurements of O<sub>2</sub> evolution at saturating CO<sub>2</sub>, which suggested increases in stomatal but not biochemical limitations at these soil temperatures. Nonuniform stomatal apertures, which were demonstrated with abscisic acid, were not apparent 2 days after root chilling, and results of our A versus c<sub>i</sub> analysis appear valid. Bulk shoot water potential (Ψ) declined as soil temperature dropped below 16°C. When half the root system of seedlings was chilled, shoot Ψ and gas-exchange rates did not decline. Thus, nonhydraulic root-shoot signals were not implicated in stomatal limitations. The initial decrease in leaf conductance to water vapor after root chilling appeared to precede any detectable decrease in bulk fascicle Ψ, but may be in response to a decrease in turgor of epidermal cells. These reductions in leaf conductance to water vapor, which occurred within 30 minutes of root chilling, could be delayed and temporarily reversed by reducing the leaf-to-air vapor-pressure deficit, suggesting that hydraulic signals may be involved in initiating stomatal closure. By independently manipulating the leaf-to-air vapor-pressure deficit of individual fascicles, we could induce uptake of water vapor through stomata, suggesting that nonsaturated conditions occur in the intercellular airspaces. There was an anomaly in our results on seedlings maintained for 2 days at soil temperatures below 7°C. Lower A appeared primarily the result of nonstomatal limitations, based on large increases in calculated c<sub>i</sub> and A versus c<sub>i</sub> analysis. In contrast, measurements of O<sub>2</sub> evolution at saturating CO<sub>2</sub> concentrations implied nonstomatal limitations *per se* did not increase at these temperatures. One explanation for this paradox is that calculations of c<sub>i</sub> are unreliable at very low gas-exchange rates because of inadequate measurement resolution, and limitations of A are predominantly stomatal. An alternative interpretation is that increases in c<sub>i</sub> are real and the results from O<sub>2</sub>-evolution measurements are in error. The high CO<sub>2</sub> concentration used in O<sub>2</sub>-evolution measurements (15%) may have overcome nonstomatal limitations by enzymes that were down-regulated by a feedback mechanism. In this scenario, carbohydrate feedback

limitations may be responsible for nonstomatal reductions in A after 2 days at soil temperatures below 7°C.

In cold soils, decreased root permeability (22–24) and increased water viscosity lead to a decline in g<sub>s</sub><sup>3</sup> (16, 20). This reduction in g<sub>s</sub>, which is often associated with a decrease in shoot or leaf Ψ, presumably limits net A. However, reductions in g<sub>s</sub> at low soil temperatures (2, 5, 30), as well as apparent stomatal limitations of A (5) have also been observed without decreases in bulk leaf Ψ. In these cases, the mechanisms responsible for reduced g<sub>s</sub> are unknown but may be associated with: (a) hydraulic signals such as subtle changes in xylem flux (30) that may reduce turgor of leaf epidermal cells but go undetected at the bulk leaf or shoot level; or (b) nonhydraulic signals between the roots and shoots involving hormones (3). In addition to reductions in g<sub>s</sub>, apparent nonstomatal limitations of A may develop at low soil temperatures (6, 7, 32), possibly through carbohydrate feedback inhibition (6, 7, 14).

The objective of the present study was to assess the potential contribution of stomatal and nonstomatal limitations to A under root chilling conditions. In addition, a split-root experiment and measurements of stomatal responses to VPD under various root-temperature regimes were conducted to evaluate the potential involvement of needle water status and chemical signals from roots in initiating the stomatal response to low root temperature. Unhardened seedlings of *Pinus taeda* L. (loblolly pine) were used for these experiments. *P. taeda* is a chilling-sensitive species (17) and a dominant component of Coastal Plain forests of the southeastern United States.

## MATERIALS AND METHODS

### Growth Chamber Conditions

*Pinus taeda* seeds (Weyerhaeuser seed lot Nt 8601) were soaked in distilled H<sub>2</sub>O for 48 h and H<sub>2</sub>O<sub>2</sub> for 30 min, and

<sup>3</sup> Abbreviations: g<sub>s</sub>, leaf conductance to water vapor; Ψ, water potential; A, net photosynthesis; IRGA, infrared gas analyzer; A<sub>max</sub>, O<sub>2</sub> evolution at CO<sub>2</sub> and light saturation; A<sub>0</sub>, net photosynthesis in the absence of any diffusion resistance; c<sub>a</sub>, ambient CO<sub>2</sub> concentration; c<sub>i</sub>, intercellular CO<sub>2</sub> concentration; g<sub>c</sub>, cuticular conductance to water vapor; l<sub>s</sub>, relative stomatal limitation to A; RuBP, ribulose-1,5-bisphosphate; VP, vapor pressure; VPD, leaf-to-air vapor-pressure deficit (assuming saturated leaf); Φ, apparent quantum yield; Γ, light compensation point.

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were then cold-stratified at 10°C for 30 d before planting in a 1:1:1 mix of fine vermiculite, surface, and ground peat in torpedo pots (25 cm height). After germination, seedlings were placed in growth chambers (Convion PGW36) where they received a 14-h photoperiod. PPFD at seedling height was 600 to 700  $\mu\text{mol m}^{-2} \text{s}^{-1}$ . RH was maintained at 75%, and day/night temperatures were 24/20°C.

### Gas-Exchange and Water Potential Response to Soil Temperature

The response of  $A$ ,  $g_s$ , and  $\Psi$  to soil temperature was measured on randomly selected groups ( $n = 5$ ) of 110-d-old seedlings maintained at different soil temperatures (1, 4, 7, 10, 13, 16, 20, and 24°C). Soil temperature was manipulated by circulating antifreeze from a water bath through an insulated copper coil surrounding each pot. A loosely fitted insulating collar was placed around the stem on the soil surface of each pot. Temperature, measured with a copper-constantan thermocouple in the geometric center of each pot, was continuously averaged with a datalogger (Campbell Scientific 21X), which also controlled the temperature of the water bath via a relay driver. This system maintained soil temperatures within 1°C of the treatment mean and within-pot variation was <0.5°C.

Seedlings were watered to saturation and placed in soil temperature treatments at 1200 h. Treatments continued for 48 h at which time gas-exchange and shoot  $\Psi$  were measured. The whole shoot was sealed in a well-mixed gas-exchange cuvette under 1200  $\mu\text{mol m}^{-2} \text{s}^{-1}$  PPFD, and the treatment soil temperature was maintained during gas-exchange measurement. Air temperature was maintained at 24°C with a water jacket, while RH of air entering the cuvette was kept at 65% (VPD about 1.0 kPa) with a Peltier-controlled condenser and a water bubbler. This irradiance, air temperature, and RH will be referred to as "standard conditions." Transpiration was measured with dew point hygrometers (General Eastern 1100DP) placed in series before and after the cuvette. Carbon dioxide depletion in the cuvette was measured with an open IRGA (ADC 225 Mk3). The  $c_a$  of air entering the cuvette was 350  $\mu\text{L/L}$ . Real-time data acquisition was provided by a datalogger that interfaced with a personal computer.  $A$ ,  $g_s$ , and  $c_i$  were calculated with the equations of von Caemmerer and Farquhar (33). Gas-exchange calculations were made on a total needle surface area basis and assuming a boundary layer conductance of 1  $\text{mol m}^{-2} \text{s}^{-1}$ . Stem respiration may have caused an error in estimates of  $c_i$ , however, relative values can be used to compare treatments. Following gas-exchange measurement, shoot  $\Psi$  was measured with a pressure chamber.

### Autoradiography and $A$ versus $c_i$ Analysis

The contribution of stomatal and nonstomatal limitations to  $A$  under root-chilling conditions was determined by  $A$  versus  $c_i$  analysis as in Farquhar and Sharkey (11). Since nonuniform stomatal apertures can result in overestimates of nonstomatal limitations to  $A$  (9, 29), we examined the effect of root chilling on heterogeneity of stomatal aperture using autoradiography after assimilation of  $^{14}\text{CO}_2$ . We fed ABA to

excised needles in an attempt to induce stomatal heterogeneity in *P. taeda*. The base of a needle fascicle was cut and maintained under distilled  $\text{H}_2\text{O}$ , placed in a gas-exchange cuvette (20  $\text{cm}^3$  volume) under standard conditions and allowed to attain steady-state  $A$ . *Cis-trans* ABA dissolved in 5% ethanol was applied to the distilled  $\text{H}_2\text{O}$  to achieve a  $10^{-4}$  M concentration. After 30 min, a decline in  $A$  was apparent and 0.5  $\mu\text{Ci}/\mu\text{mol}$  of  $^{14}\text{CO}_2$  was injected into the cuvette for 1 min. After purging the cuvette, needles were rapidly frozen between two aluminum plates and placed on x-ray film at  $-80^\circ\text{C}$  for 24 to 72 h. A similar protocol was followed with attached fascicles on seedlings exposed to soil temperatures of 24°, 7°, or 4°C for 48 h. These soil temperatures represent a control (24°C) and the temperatures at which seedlings exhibited large apparent stomatal (7°C) and nonstomatal (4°C) limitations to  $A$ , based on  $c_i$  values (see "Results"). Fascicles on at least five seedlings at each soil temperature were sampled.

Standard cuvette conditions were used for generating the  $A$  versus  $c_i$  relationship of five seedlings maintained at soil temperatures of 24°, 7°, or 1°C for 48 h. Treatment soil temperature was maintained during gas-exchange measurements, which took 2 to 3 h/seedling. The differential background sensitivity of the IRGA (ADC) was corrected by measuring  $c_a$  (which varied 1000–10  $\mu\text{L/L}$ ) with an additional IRGA (Horiba PIR-2000). The  $l_s$  was calculated as  $(A_o - A)/A_o$ , where  $A_o$  is the photosynthetic rate that would occur if resistance to  $\text{CO}_2$  diffusion were negligible (11). A fourth-order polynomial equation was fitted (least squares) to the  $A$  versus  $c_i$  relationship to estimate  $A$  and  $A_o$ . Apparent RuBP carboxylation efficiency was estimated as the slope of the initial linear part of the  $A$  versus  $c_i$  relationship ( $c_i < 200 \mu\text{L/L}$ ). We used maximum  $A$  (assumed to occur at  $c_i = 1000 \mu\text{L/L}$ ) to estimate the maximum rate of RuBP regeneration.

### $\text{O}_2$ Evolution

Measurements of  $\text{O}_2$  evolution at saturating  $c_a$  were used to identify potential biochemical limitations to  $A$ . A preliminary experiment was conducted to determine if diffusion limitations were overcome at the traditionally used 5%  $\text{CO}_2$  concentration. Measurements of the maximum rate of  $A_{\text{max}}$  were made on foliage from eight seedlings. Four seedlings had fascicle  $\Psi$  of about  $-0.8$  MPa (controls), while the other four had fascicle  $\Psi$  of about  $-2.5$  MPa (stressed) due to withholding water for 4 to 5 d. Measurements of  $A_{\text{max}}$  were made at  $\text{CO}_2$  concentrations ranging from 3 to 25%  $\text{CO}_2$  in air (v/v). In the main experiment, soil temperatures of five seedlings were maintained at 24°, 7°, or 1°C in a growth chamber. After 48 h,  $\Phi$ ,  $\Gamma$ , and  $A_{\text{max}}$  were measured with a leaf disc electrode (LD2, Hansatech) as described by DeLucia *et al.* (8). Light supplied by a metal halogen lamp (LS2, Hansatech) was passed through 4 cm of  $\text{CuSO}_4$  solution (5% in distilled  $\text{H}_2\text{O}$ ) to maintain needle temperatures at 24°C. The  $\Phi$  was determined from five irradiances between 0 and 90  $\mu\text{mol m}^{-2} \text{s}^{-1}$ , while  $A_{\text{max}}$  was measured at 1200  $\mu\text{mol m}^{-2} \text{s}^{-1}$ . Before each measurement, the chamber was flushed with humidified 15%  $\text{CO}_2$  in air.

### Split-Root Experiment

We used a split-root experiment to evaluate whether reductions in gas-exchange rates after root cooling appeared dependent on reductions in shoot  $\Psi$ . At 20 d after germination, the soil was gently washed from seedling roots and the primary root was severed 2 cm below the stem base. The remaining root system was divided equally between two torpedo pots and seedlings were returned to the growth chamber. After 110 d, seedlings were divided into three groups and soil temperature was maintained at 24°C (five seedlings), 7°C (10 seedlings), or 1°C (10 seedlings), beginning at 1200 h. The entire root systems of five seedlings in the 7°C and the 1°C groups were chilled, while only half the root systems were chilled in the remaining five seedlings in each group. The nonchilled half of these root systems was maintained at 24°C. After 48 h, gas-exchange rates were measured at standard conditions. Treatment soil temperatures were maintained during measurements. After removal from the cuvette, shoot  $\Psi$  was measured with a pressure chamber.

### Leaf Conductance versus Water Potential

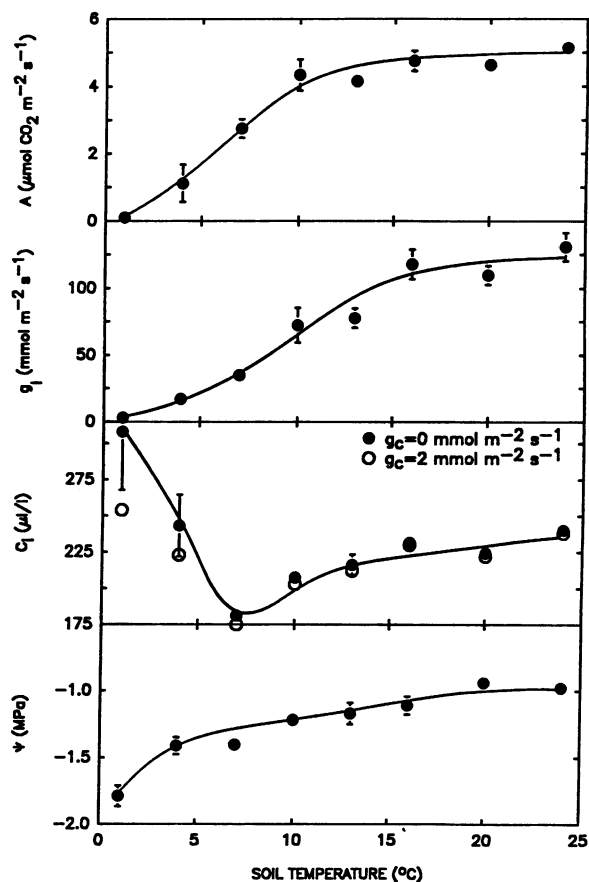
To assess the dependence of  $g_i$  on  $\Psi$  during root chilling, repeat measurements of these parameters were made on individual seedlings as soil temperature was lowered. Seedlings (110-d-old) were taken from the growth chamber to the laboratory where air temperature and RH were 24°C and 45%, respectively. Six fascicles (5–15 cm above the stem base) were propped on ring stands and needles were positioned with tape to ensure a constant PPFD (1000–1300  $\mu\text{mol m}^{-2} \text{s}^{-1}$ ) and surface area through the experiment. Leaf conductance of the fascicles was measured every 5 to 8 min with a null-balance porometer (LiCor LI-1600). After  $g_i$  was at steady state for 2 h, roots were cooled from 24° to 1°C. Soil temperatures reached 1°C within 1 h. Just before root chilling and continuing at 15- to 20-min intervals, one fascicle was severed after  $g_i$  measurement and placed in a pressure chamber for  $\Psi$  determination. Measurements continued until all six fascicles were removed. The experiment was repeated on five seedlings.

### Gas-Exchange Response to Vapor-Pressure Deficit

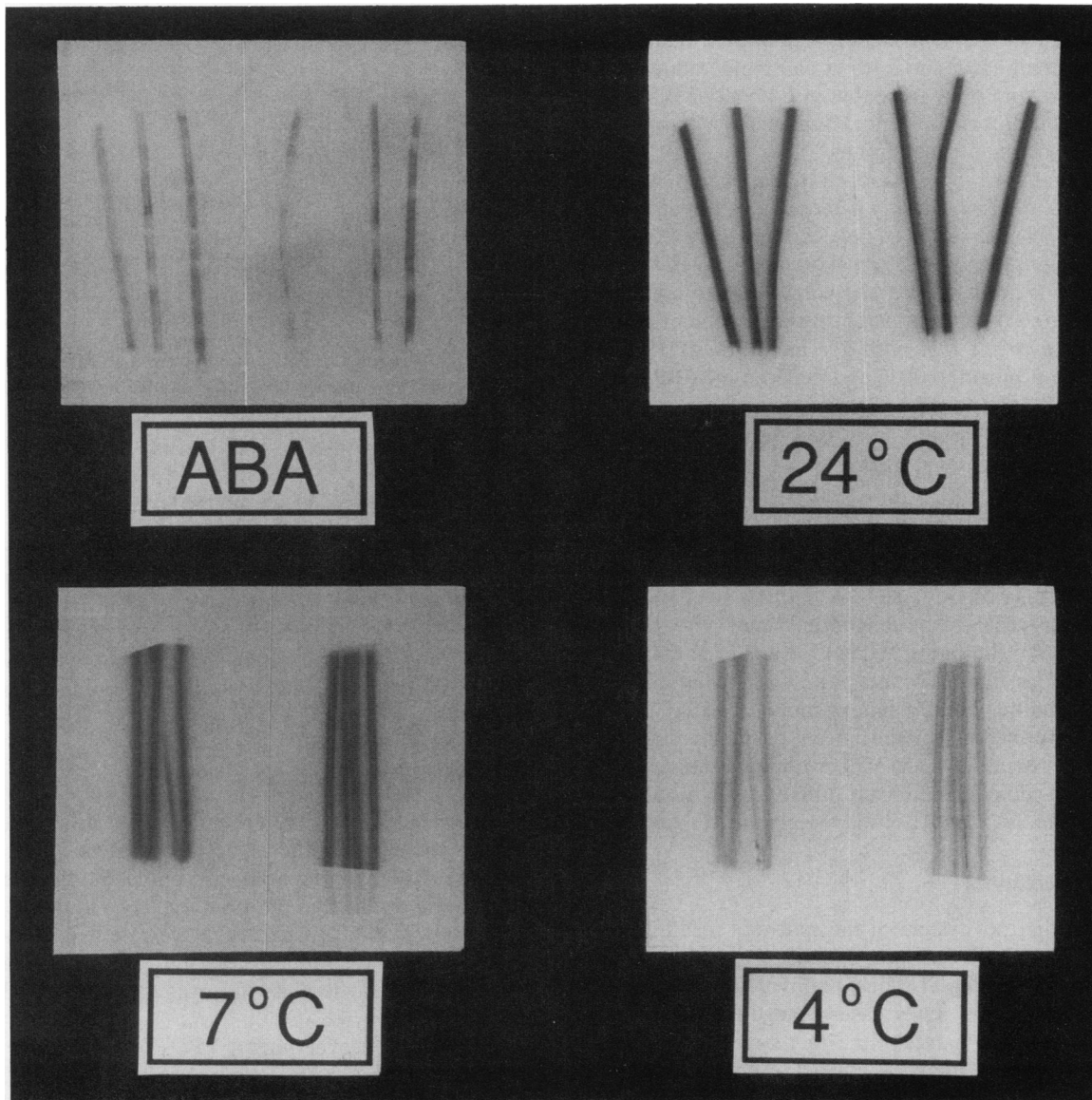
The potential involvement of hydraulic signals in the stomatal response to root chilling was examined further by investigating the interaction of VPD and root chilling on gas exchange. Seedlings (110-d-old) were taken from the growth chamber to the laboratory. Needles of one fascicle at mid-height on the stem were placed in an open-system cuvette, and gas-exchange rates were monitored under standard conditions, except for RH of air entering the cuvette (see below). Leaf conductance of two fascicles exposed to the laboratory environment and attached to the stem above the cuvette and two fascicles attached below the cuvette was also measured every 5 to 8 min with a null-balance porometer. These fascicles were propped on ring stands and positioned as described previously. Air temperature and RH in the laboratory were 24°C and 45%, respectively, corresponding to a VPD of about 1.5 kPa. After gas-exchange rates were at steady state for 2 h,

roots were cooled from 24 to 1°C. Soil temperatures reached 1°C within 1 h.

Three experiments were conducted to investigate VPD/cold soil interactions, and each experiment was repeated on four seedlings. In the first experiment, we examined the effect of VPD on root-chilling induced decreases in  $g_i$  by maintaining air entering the cuvette at a VPD of 0.3 kPa compared with 1.5 kPa for fascicles outside the cuvette. The possibility that low VPD could offset root-chilling-induced decreases in  $A$  and  $g_i$  was examined in a second experiment. Air entering the cuvette was 1.5 kPa VPD, and when root-chilling induced a decrease in gas-exchange rates, VPD in the cuvette was decreased to 0.3 kPa. Fascicles outside the cuvette remained at 1.5 kPa VPD. Results of this experiment were surprising in that when VPD in the cuvette was decreased,  $g_i$  of the fascicle in the cuvette became negative, suggesting that  $\text{H}_2\text{O}$  vapor was absorbed through stomata. This phenomenon was further investigated in a third experiment in which fascicles outside of the cuvette were misted with distilled  $\text{H}_2\text{O}$  to reduce VPD while the fascicle inside the cuvette had negative values of  $g_i$ .



**Figure 1.** Response of  $A$ ,  $g_i$ ,  $c_i$ , and  $\Psi$  to soil temperature. Measurements were made after 2 d at a respective temperature. Intercellular  $\text{CO}_2$  concentration was calculated by assuming a  $g_s$  of 0 (●) or 2 (○)  $\text{mmol m}^{-2} \text{s}^{-1}$ . Values are means  $\pm$  1 SE ( $n = 5$ ).



**Figure 2.** Autoradiograms of needles after 30 min of feeding ABA, or after 2 d at a soil temperature of 24°, 7°, or 4°C. Needles were exposed to 0.5  $\mu\text{Ci}/\mu\text{mol}$  of  $^{14}\text{CO}_2$  for 1 min.

## RESULTS

### Gas-Exchange and Water Potential Response to Soil Temperature

Net photosynthetic rates of *P. taeda* seedlings gradually declined as soil temperatures dropped from 24° to 10°C (Fig. 1). A sharp decline in A was evident below soil temperatures of 10°C, with rates dropping to essentially 0  $\mu\text{mol m}^{-2} \text{s}^{-1}$  at 0°C. Leaf conductance appeared slightly more sensitive to soil temperatures than A, as a sharp decline in  $g_i$  was apparent below 13°C. Shoot  $\Psi$  gradually declined at soil temperatures <20° and dropped sharply at temperatures <4°C.

Intercellular  $\text{CO}_2$  concentrations decreased as soil temperature dropped to 7°C, suggesting an increase in stomatal

limitations of A. At lower soil temperatures,  $c_i$  increased sharply, implying an increase in nonstomatal limitations of A. However, very low  $g_i$  cuticular transpiration, as well as nonuniform stomatal apertures, may lead to an overestimation of  $c_i$ . To estimate the potential error resulting from cuticular transpiration, we recalculated  $c_i$  assuming a  $g_c$  of 2  $\text{mmol m}^{-2} \text{s}^{-1}$ , well within the suspected range of  $g_c$  reported by Nobel (21) and Kirschbaum and Percy (18). When  $g_c$  is accounted for,  $c_i$  at low  $g_i$  decreases relative to initial estimates, but an increase in  $c_i$  below 7°C is still apparent (Fig. 1).

### Autoradiography and A versus $c_i$ Analysis

Feeding ABA to fascicles of *P. taeda* caused a decrease in A in about 30 min and induced nonuniform stomatal aper-

tures as indicated by the banded pattern of  $^{14}\text{CO}_2$  uptake (Fig. 2). However, there was no evidence of nonuniform stomatal apertures on seedlings maintained at soil temperatures of 7° or 4°C for 48 h, and we assume that interpretations of the  $A$  versus  $c_i$  relationships are not biased by stomatal heterogeneity under these experimental conditions.

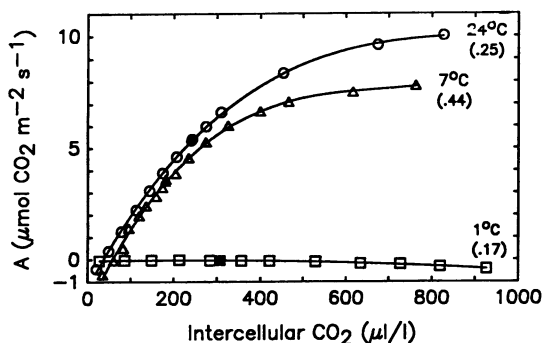
Based on the  $A$  versus  $c_i$  relationship, the relative stomatal limitation to  $A$  was significantly higher at a soil temperature of 7°C ( $l_s = 0.44$ ) than 24°C ( $l_s = 0.25$ ,  $t$  test  $P < 0.05$ , Fig. 3). The maximum rate of RuBP regeneration was significantly lower at 7°C ( $7.5 \mu\text{mol m}^{-2} \text{s}^{-1}$ ) than at 24°C ( $10.6 \mu\text{mol m}^{-2} \text{s}^{-1}$ ,  $t$  test  $P < 0.05$ ), while apparent carboxylation efficiency was  $0.028 \text{ mol m}^{-2} \text{s}^{-1}$  at both 24° and 7°C. At 1°C soil temperature,  $l_s$  declined to 0.17, and maximum RuBP regeneration rate and carboxylation efficiency were essentially zero.

### O<sub>2</sub> Evolution

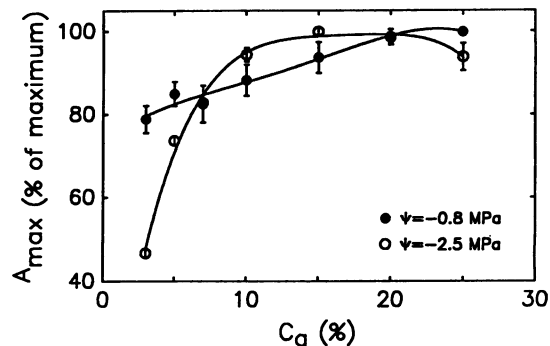
To ensure that O<sub>2</sub>-evolution measurements were made at a CO<sub>2</sub> concentration that overcame diffusion limitations, we measured  $A_{\text{max}}$  at concentrations ranging from 3 to 25% CO<sub>2</sub>. At the traditionally used 5% CO<sub>2</sub>,  $A_{\text{max}}$  of fascicles at a  $\Psi$  of -0.8 MPa was <85% of its maximum value (Fig. 4). This apparent diffusion limitation at 5% CO<sub>2</sub> was even greater in fascicles at a  $\Psi$  of -2.5 MPa;  $A_{\text{max}}$  at 5% CO<sub>2</sub> was <75% of values at 15 to 20%. Thus, a concentration of 15% CO<sub>2</sub> was used for measurements on the effects of root chilling on photosynthesis. Measurements of O<sub>2</sub> evolution at this concentration did not indicate significant differences in  $\Phi$ ,  $A_{\text{max}}$ , or  $\Gamma$  of seedlings at 24°, 7°, or 1°C soil temperature (Table I).

### Split-Root Experiment

When half the root system of the split-root seedlings was chilled to 7° or 1°C, no decreases in shoot  $\Psi$  or gas-exchange rates were apparent (Fig. 5). Thus, nonhydraulic root signals were not implicated in lower gas-exchange rates after root chilling. Chilling both halves of the root system of seedlings caused reductions in shoot  $\Psi$  and gas-exchange variables comparable to when intact root systems were chilled (Fig. 1).



**Figure 3.** Typical relationship between  $A$  and  $c_i$  after 2 d at 24°, 7°, or 1°C soil temperature. ●, ▲, ■, Actual  $A$  at ambient CO<sub>2</sub> concentration (350  $\mu\text{L/L}$ ). The  $l_s$  at each soil temperature is shown in parentheses. Results from four other seedlings at each soil temperature were similar.



**Figure 4.** Relationship between the maximum rate of O<sub>2</sub> evolution ( $A_{\text{max}}$ ) and  $c_i$  for needles at -0.8 or -2.5 MPa. Values are means  $\pm$  1 SE ( $n = 5$ ).

### Leaf Conductance versus Water Potential

Repeated measurements of  $g_i$  and  $\Psi$  on the same seedling during root chilling suggest a decrease in  $g_i$  before any change in  $\Psi$  (Fig. 6). After chilling roots,  $g_i$  of all fascicles declined within 30 min. A comparison of  $g_i$  and  $\Psi$  of these fascicles with those sampled just before chilling suggests relatively small reductions in  $g_i$  preceded any detectable reductions in bulk fascicle  $\Psi$ .

### Gas-Exchange Response to VPD

In the initial VPD experiment, VPDs inside the cuvette and in the surrounding laboratory were 0.3 and 1.5 kPa, respectively. Upon root chilling,  $g_i$  of the four fascicles outside the cuvette declined in 30 min (Fig. 7). Within 1.5 h,  $g_i$  was reduced by >30%. In contrast, a decrease in  $g_i$  of the fascicle in the cuvette was not apparent until 2.5 to 3.0 h after root chilling. A >30% decrease in  $g_i$  was apparent after 3.5 to 4.0 h.

In the second experiment, VPDs in the cuvette and the laboratory were 1.5 kPa. Within 1.5 h of root-chilling,  $g_i$  of all fascicles declined by >30%, at which time VPD in the cuvette was decreased to 0.3 kPa (data not shown). This decrease in VPD was paralleled by a slight increase in  $A$  of the fascicle in the cuvette and a sharp drop in calculated  $g_i$  below  $0 \text{ mmol m}^{-2} \text{s}^{-1}$ . This protocol was repeated in the third experiment. About 10 min after VPD in the cuvette was reduced and  $g_i$  of the fascicle in the cuvette became negative, the fascicles outside the cuvette were misted. When the gas-exchange rate in the cuvette was remeasured 15 s after misting fascicles outside the cuvette,  $g_i$  was near pre-chill values while  $A$  remained constant (Fig. 8). Misting was repeated twice. Following each misting,  $g_i$  rose sharply and then declined after 10 to 30 min.

### DISCUSSION

Relative to conifers from colder climates, gas-exchange rates in *P. taeda* seedlings appear quite sensitive to low soil temperatures. While  $A$  and  $g_i$  in *P. taeda* are almost completely inhibited at a soil temperature of 1°C, gas-exchange rates in unhardened seedlings of *Picea sitchensis* (32), *Picea engel-*

**Table I.**  $\Phi$ ,  $A_{max}$ , and  $\Gamma$  of Needles of *P. taeda* after 2 d at 24°, 7°, or 1°C Soil Temperature

Measurements were made at 15% CO<sub>2</sub> in air. There were no significant treatment effects ( $n = 5$ ,  $P > 0.10$ ).

Soil Temperature	$\Phi$	$A_{max}$	$\Gamma$
°C	mol/mol	$\mu\text{mol m}^{-1} \text{s}^{-1}$	$\mu\text{mol m}^{-2} \text{s}^{-1}$
24	0.087	21.6	17.6
7	0.083	20.9	17.7
1	0.091	19.6	15.7

*mannii* (7), *Picea abies*, and *Pinus sylvestris* (19) are reduced <50%. These differences are not surprising since cold soil sensitivity varies considerably among species, with greater sensitivity in species native to warmer soil regimes (6, 17).

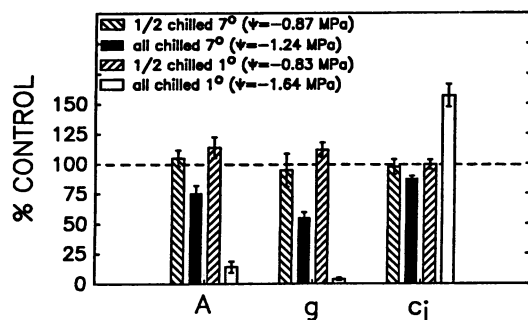
At soil temperatures down to 7°C, reductions in  $A$  result primarily from stomatal limitations since  $c_i$  declines (Fig. 1) and  $l_s$  increases (Fig. 3). Similar increases in stomatal limitations of  $A$  have been reported in *Pinus contorta* (5) at low soil temperatures, as well as in *P. taeda* after soil drying (31). Consistent with these results, our measurements of O<sub>2</sub> evolution at saturating CO<sub>2</sub> concentrations suggest that increases in biochemical limitations may be insignificant, as  $\Phi$ ,  $A_{max}$ , and  $\Gamma$  are similar at 24° and 7°C (Table I). While increases in biochemical limitations appear minor, if they do exist,  $A$  versus  $c_i$  analysis suggests that they result from limiting RuBP regeneration, not ribulose-1,5-bisphosphate carboxylase/oxygenase activity, since regeneration rate declines while carboxylation efficiencies are unchanged from 24° to 7°C (31).

As soil temperatures decrease from 7° to 1°C, the rise in calculated  $c_i$  values suggests an increase in the nonstomatal limitations of  $A$  (Fig. 1). Similar apparent increases in nonstomatal limitations have been documented in *P. engelmannii* and *P. sylvestris* at soil temperatures of 1°C (7, 8). Surprisingly, measurements at saturating CO<sub>2</sub> failed to show any declines in  $\Phi$ ,  $A_{max}$ , or  $\Gamma$  at 1°C, suggesting nonstomatal limitations *per se* did not increase.

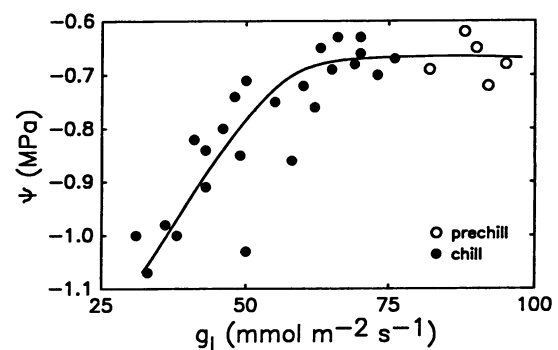
This anomaly in results presents two alternative interpretations with respect to the dominant limitation to  $A$  at soil temperatures below 7°C. If we assume that the O<sub>2</sub>-electrode results at 1°C are real, stomatal limitations are responsible for

lower  $A$ . The increase in calculated  $c_i$  at this soil temperature (Fig. 1) must therefore be artifact. While overestimates of  $c_i$  may result from nonuniform stomatal apertures and failure to consider  $g_c$ , our data suggest that stomatal apertures were uniform (Fig. 2), and  $g_c$  effects cannot fully account (Fig. 1) for the large increase in  $c_i$  at soil temperatures below 7°C. At very low  $A$  and  $g_i$ , the resolution of measurement may be inadequate and result in artificially high  $c_i$  values (1).

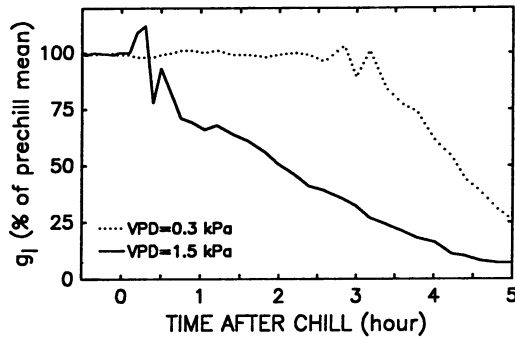
An alternative explanation is that such increases in  $c_i$  are real and limitations to  $A$  are primarily nonstomatal below 7°C. This interpretation conflicts with our O<sub>2</sub>-electrode data. One possible cause of error in these data may be that the very high  $c_a$  (15%) used during O<sub>2</sub>-evolution measurements overcame nonstomatal metabolic limitations to  $A$  caused by root chilling. Graan and Boyer (12) observed that metabolic limitations in water stressed sunflower were overcome at a  $c_a$  of 3%. They speculated that high  $c_a$  may stimulate photosynthesis by altering stromal pH, providing substrate concentrations high enough to overwhelm decreased enzyme activity or activate inhibited enzymes. In our study, high  $c_a$  may have overcome limitations by enzymes that were down-regulated by a carbohydrate feedback mechanism (13). DeLucia (7) found that low root temperature caused an increase in nonstomatal limitations of  $A$  in *P. engelmannii*, as well as increases in starch content of needles and stems, and Day *et al.* (6) observed increases in nonstomatal limitations in late afternoon, suggesting carbohydrate feedback inhibition of  $A$  (13). Increases in leaf and stem carbohydrate concentrations (27) and reductions in translocation of photoassimilates from leaves (14) have been reported in several other species at low soil temperatures.



**Figure 5.**  $A$ ,  $g_i$ , and  $c_i$  after total root systems or half the root system were maintained for 2 d at 7° or 1°C. Values are expressed as a percentage of control (e.g. 24°C soil temperature). Shoot  $\Psi$  are shown in parentheses. Mean shoot  $\Psi$  of controls was  $-0.88$  MPa. Values are means  $\pm 1$  SE ( $n = 5$ ).



**Figure 6.** Relationship between fascicle  $\Psi$  and  $g_i$  just before and after soil temperature was dropped from 24° to 1°C.



**Figure 7.** Typical response of  $g_l$  to a decline in soil temperature from 24° to 1°C. Soil temperatures reached 1°C within 1 h. Fascicles outside the gas-exchange cuvette were at a VPD of 1.5 kPa (—), while the fascicle in the cuvette was at a VPD of 0.3 kPa (·····). Leaf conductance is expressed as a percentage of the mean prechill value. The values for the fascicles outside the cuvette are means ( $n = 4$ ). Results from three other seedlings were similar.

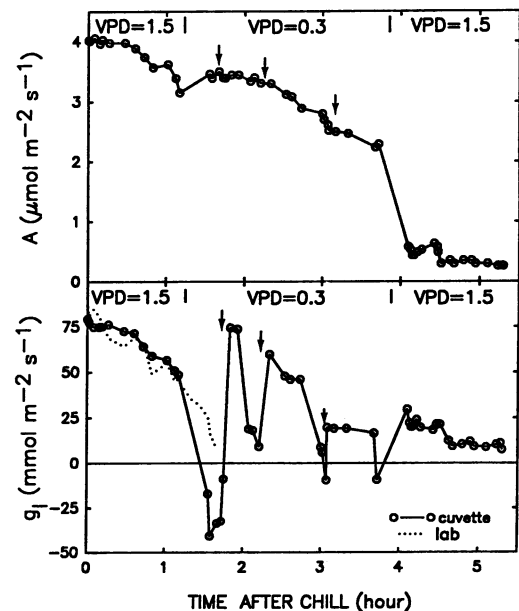
One explanation for the decline in  $g_l$  after root chilling involves a response to reductions in shoot  $\Psi$ . We observed reductions in  $g_l$  and  $\Psi$  as soil temperature decreased (Fig. 1), and found when shoot  $\Psi$  was maintained after chilling only half the root system  $g_l$  failed to decline (Fig. 5). However, declines in bulk leaf or fascicle  $\Psi$  do not appear obligatory for reductions in  $g_l$ , since reductions in  $g_l$  appear to precede reductions in  $\Psi$  (Fig. 6). Similar reductions in  $g_l$  have been observed before declines in  $\Psi$  after root chilling (2, 30) and soil drying (4, 35).

Several split-root experiments have implicated nonhydraulic root-shoot signals that may cue stomatal responses to changing soil conditions. The results of our split-root experiment do not support the involvement of hormones in the cold-soil induced reduction of  $g_l$ . Saab and Sharp (25) were also unable to demonstrate declines in  $g_l$  after drying half the root system of maize. They suggested that previous implications of nonhydraulic root controls on  $g_l$  may be an artifact of using pressurized root systems in former split-root studies, and also noted that stomatal sensitivity to root-shoot chemical signals may vary with respect to genotype, xylem pH, or nutrient balance. As Teskey *et al.* (30) showed, declines in  $g_l$  can occur within 30 min of root chilling (Figs. 7 and 8); whether chemical root-shoot communication systems can alter stomatal behavior in such short time periods remains in question. Smith and Dale (28) observed large increases in leaf ABA content in *Phaseolus vulgaris* within 2 h of root chilling, but it was not clear whether this ABA originated in the roots. Although results of our split-root experiment do not suggest any nonhydraulic control of  $g_l$ , we cannot dismiss such a mechanism.

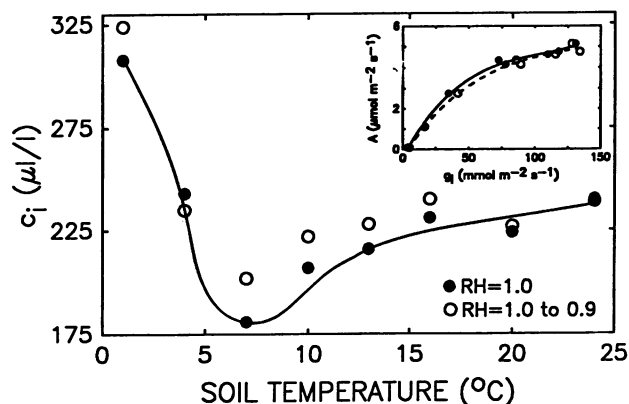
While direct stomatal responses to VPD are well documented, our demonstration of negative  $g_l$  (*e.g.*, leaf uptake of water vapor) in response to a reduction in VPD is surprising. Using a dual-surface cuvette, Ward and Bunce (34) found that exposing an upper leaf surface to high VPD resulted in a negative transpiration rate or uptake of water vapor by the lower leaf surface. Negative  $g_l$  in our experiment could have resulted from chamber adsorption of water vapor; however,

we did not observe condensation in the cuvette. In addition, these negative values do not appear to be spurious underestimations of  $g_l$  after stomatal closure, since considerable gas exchange was occurring as evidenced by high photosynthetic rates (Fig. 7). Finally, negative  $g_l$  could be rapidly increased by misting fascicles outside the cuvette. Apparently, water uptake by fascicles outside the gas-exchange cuvette after misting led to a rapid increase in VP of intercellular airspaces in the fascicles in the cuvette, which resulted in a positive transpiration rate.

For water vapor uptake to occur through stomata, VP in the intercellular airspaces must be less than that in air surrounding the fascicle. As Ward and Bunce (34) noted, such a deviation from saturation cannot be explained by low bulk leaf  $\Psi$ ; needle  $\Psi$  would have to be unrealistically low (about -13 MPa) for such a deviation. Thus, VP of air in the cuvette would be a maximum estimate of the VP in the intercellular airspaces. This translates into a maximum RH of 91% in intercellular airspaces during these periods, being similar to previously reported values of nonsaturated intercellular air (10, 15, 34). That intercellular air can be substantially below saturation may result in significant errors in the calculation of  $g_l$  and  $c_l$ . As a first approximation of these errors, we recalculated gas-exchange rates from our initial soil temperature response data (Fig. 1), assuming that the decline in RH in the intercellular airspaces follows the same general pattern as the decline in bulk shoot  $\Psi$  (*e.g.*, RH decreases linearly



**Figure 8.** Response of  $A$  and  $g_l$  of a fascicle in the gas-exchange cuvette to a decrease in soil temperature from 24° to 1°C. Soil temperature reached 1°C within 1 h. VPD in the cuvette, indicated on the top of the graphs, was reduced from 1.5 to 0.3 kPa about 1 h after chilling, and increased back to 1.5 kPa about 2.5 h later. ·····, Mean  $g_l$  of four fascicles outside the cuvette. These fascicles were maintained at a VPD of 1.5 kPa through the experiment. Arrows indicate when fascicles outside the gas-exchange cuvette were misted with distilled H<sub>2</sub>O. Similar gas-exchange patterns were found in experiments on three other seedlings.



**Figure 9.** Comparison of  $c_i$  versus soil temperature when RH of intercellular airspaces is assumed to vary from 100 to 90% (○) or to remain at 100% (●). For the former, we assumed RH of the intercellular air space declined with soil temperature in a pattern similar to shoot water potential (Fig. 1): RH declined linearly from 100% at 24°C to 95% at 4°C, and dropped to 90% at 1°C. Insert: relationship between  $A$  and  $g$ , when RH of the airspaces is 100% (●) or varies from 100 to 90% as mentioned (○). Gas-exchange data were recalculated from Figure 1.

from 100% at 24°C to 95% at 4°C, and then drops to 90% at 1°C). While this changes absolute values of  $g_s$  and  $c_i$ , the patterns in  $c_i$  with respect to soil temperature remain the same, and our conclusions on stomatal versus nonstomatal limitations are unchanged (Fig. 9). Nonsaturated air within leaves could also have large effects on the  $A$  versus  $g_s$  relationship, since calculations of  $A$ , but not  $g_s$ , are relatively insensitive to these changes. Under our assumed pattern of decline of RH in intercellular airspaces, changes in the relationship between  $A$  and  $g_s$  appear small (Fig. 8). More information on RH in intercellular airspaces with respect to  $H_2O$  vapor and  $CO_2$  flux are needed before the significance of this phenomenon can be fully evaluated.

With respect to mechanisms controlling stomatal apertures following root chilling, it has been demonstrated that a rapid decline in VPD can result in a rapid increase in turgor of epidermal cells, followed by an increase in  $g_s$  (26). Short-term reductions in  $g_s$  after root chilling may be in response to such decreases in turgor of epidermal cells, which go undetected by measurement of bulk leaf  $\Psi$ . This decline in  $g_s$  in response to root chilling appears related to hydraulic signals in that it could be delayed if VPD were maintained at 0.3 rather than 1.5 kPa (Fig. 7). Additional evidence comes from an apparent partial reversal of cold-soil induced stomatal closure when we reduced VPD. Although there is some variability in the relationship between  $A$  and stomatal aperture, when VPD in the cuvette was decreased to 0.3 kPa, the increase in  $A$  suggests that stomatal apertures were no longer declining and may have increased. (While  $g_s$  is negative, this is due to a reversal of water flux, not stomatal closure.) Thus, it appears that reductions in stomatal aperture after root chilling can be delayed as well as partially reversed by hydraulic signals associated with VPD.

Soil perturbations such as root chilling can result in a rapid decrease in root permeability (22, 23) and xylem water flux

to leaves. A close coupling between xylem water flux, epidermal cell turgor, and stomatal aperture could explain the rapid decline in  $g_s$  and subsequent stomatal limitation to  $A$  after root chilling down to 7°C. While our results failed to suggest any chemical signals from roots that control  $g_s$ , both hydraulic and nonhydraulic root messages may act in concert. At soil temperatures <7°C, the disparity between our  $c_i$  values and  $O_2$ -evolution results makes an analysis of the potential contribution of stomatal versus nonstomatal limitations difficult. It appears that these techniques need further evaluation before a definitive analysis of limitations to  $A$  can be made.

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