

RESEARCH PAPER

# Height-related trends in stomatal sensitivity to leaf-to-air vapour pressure deficit in a tall conifer

D. R. Woodruff<sup>1,\*</sup>, F. C. Meinzer<sup>1</sup> and K. A. McCulloh<sup>2</sup>

<sup>1</sup> USDA Forest Service, PNW Research Station, Forestry Sciences Laboratory, 3200 SW Jefferson Way, Corvallis, Oregon 97331, USA

<sup>2</sup> Department of Wood Science and Engineering, Oregon State University, Corvallis, Oregon 97331, USA

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## Abstract

Stomatal responses to leaf-to-air vapour pressure deficit (LVPD), leaf water potential components, and cuticular properties were characterized for Douglas-fir (*Pseudotsuga menziesii*) foliage collected from tree tops along a height gradient from 5 m to 58 m in order to explore height-related trends in stomatal sensitivity to LVPD and to investigate the role of bulk leaf turgor and leaf cuticle thickness in determining stomatal behaviour. There were three distinct phases in the response of stomatal conductance ( $g_s$ ) to changes in LVPD. At low LVPD,  $g_s$  increased with increasing LVPD (phase one). During the second phase,  $g_s$  was maximal at low to intermediate LVPD and during the third phase  $g_s$  declined steadily as LVPD increased. The responsiveness of  $g_s$  to LVPD exhibited a height-related pattern such that maximum  $g_s$  ( $g_{s-max}$ ) occurred at progressively greater LVPD with increasing height ( $r^2=0.55$ ,  $P=0.006$ ). Bulk leaf osmotic potential at full turgor decreased with height ( $r^2=0.77$ ,  $P=0.00016$ ), and LVPD at  $g_{s-max}$  and at maximum crown conductance ( $g_{c-max}$ ) in the field were significantly correlated with leaf turgor ( $r^2=0.92$ ,  $P=0.0093$ ). Cuticle thickness increased by 0.044  $\mu\text{m}$  for every metre increase in height ( $r^2=0.78$ ,  $P=0.00015$ ). The observed trends in the response of  $g_s$  to LVPD along a height gradient, and their consistency with height-related trends in foliar osmotic potential suggest that osmotic adjustment at the tops of tall trees influences the relationship between  $g_s$  and LVPD.

**Key words:** Cuticular conductance, foliar turgor, *Pseudotsuga menziesii*, stomatal conductance, tree height.

## Introduction

The direct link between stomatal conductance ( $g_s$ ) and the ability of plants to both acquire carbon and limit water loss makes stomatal behaviour a critically influential factor in tree growth, and the water and carbon cycles of terrestrial ecosystems. Although the exact mechanisms that allow for the co-ordination of  $g_s$  with plant water balance and hydraulic properties are still unknown (Meinzer, 2002; Franks, 2004; Buckley, 2005) it is clear that stomata are influenced by the water potential ( $w$ ) somewhere within the leaf and respond to decreasing leaf water potential ( $w_l$ ) by closing (Comstock and Mencuccini, 1998; Cochard *et al.*, 2002). Tall trees thus face a particular challenge because the gravitational component of water potential leads to a 0.01 MPa increase in xylem sap tension per metre increase in height (Scholander *et al.*, 1965; Hellkvist *et al.*, 1974; Domec *et al.*, 2008), and frictional resistance during

transpiration leads to an additional path-length dependent reduction in  $W_l$  (Ryan and Yoder, (1997).

Stomata function as hydraulically controlled valves within the leaf epidermis. Changes in transpiration can lead to immediate and substantial changes in epidermal turgor pressure (Shackel and Brinckmann, 1985; Mott and Franks, 2001). Stomatal aperture is positively related to the turgor pressure of the guard cells that form the pore, and negatively related to the pressure of adjacent subsidiary or epidermal cells (Buckley 2005; Mott, 2007). Stomatal conductance is therefore closely linked to guard cell and subsidiary cell solute concentrations as well as atmospheric water content. The prevailing axiom regarding the relationship between  $g_s$  and atmospheric water content is that  $g_s$  decreases as relative humidity (RH) declines or as leaf-to-air vapour pressure deficit (LVPD) increases. However, an

\* To whom correspondence should be addressed: E-mail: dwoodruff@fs.fed.us

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initial transitory increase in  $g_s$  with increasing LVPD, described as a 'wrong way' response, often occurs (Buckley, 2005). It has been proposed that the initial transitory phase of increasing  $g_s$  with increasing LVPD is due to a reduction in epidermal turgor and a subsequent reduction in the mechanical forces exerted upon guard cells by subsidiary cells (Edwards *et al.*, 1976; Spence *et al.*, 1983; Sharpe *et al.*, 1987; Franks *et al.*, 1995, 1998; Mott and Franks, 2001). Studies involving measurements of these two opposing pressures with cell pressure probes have shown that the backpressure of epidermal cells typically has a greater influence, or mechanical advantage, over the regulation of stomatal conductance than does the direct pressure resulting from the turgor of guard cells (Meidner and Edwards, 1975; Edwards *et al.*, 1976; Franks *et al.*, 1995, 1998; Buckley, 2005). The interplay of these opposing forces on stomatal aperture is likely to be at least partly responsible for what can often seem to be an inconsistent relationship between stomatal conductance and explanatory factors such as RH and  $W_1$ . Regardless of the nature of the interplay of guard cell and subsidiary cells in controlling  $g_s$ , factors that influence the turgor of these cells are likely to have a substantial impact on the mechanics that control  $g_s$ .

The turgor of leaf cells in tall trees is expected to decrease in direct proportion with  $W_1$  along a height gradient unless osmotic adjustment, the active accumulation of symplastic solutes, occurs. Osmotic adjustment has been shown to take place as an adaptive mechanism for maintaining turgor and cell volume in cases of drought and salinity stress (Hsiao *et al.*, 1976; McNulty, 1985; Rieger, 1995). Although insufficient to fully compensate for the vertical increase in tension, osmotic adjustment has also been shown to occur along a height-gradient in the foliage of tall trees (Woodruff *et al.*, 2004; Meinzer *et al.*, 2008). Given the role of epidermal turgor in the control of  $g_s$ , osmotic adjustment in foliage is likely to influence the mechanics that are involved in the regulation of  $g_s$ . In this study, the responses of  $g_s$  to LVPD are characterized in order to investigate whether the observed height-related trend in osmotic adjustment had an influence on the relationship between  $g_s$  and LVPD via the enhanced turgor of epidermal cells, including guard cells and subsidiary cells. In addition, analyses were conducted of cuticle thickness along a height gradient in order to identify any height-related trends in this anatomical characteristic which could influence the responsiveness of stomata to LVPD. Gas exchange measurements were conducted on detached foliage in order to eliminate the immediate effects of path length and gravity on the response of  $g_s$  to changes in LVPD, thereby enabling us to isolate the influence of any height-related trends in leaf solute concentrations, or cuticular properties, on gas exchange characteristics. It is hypothesized that height-related trends in foliar osmotic adjustment, and the subsequent enhancement of turgor of epidermal cells, would result in a corresponding height-related trend in the relationship between LVPD and  $g_s$ . A greater understanding of the relationships between stomatal behaviour and parameters that influence stomatal behaviour such as foliar turgor and foliar anatomical character-

istics, and how these relationships are influenced by tree height, could improve modelling capabilities for net primary productivity and net ecosystem productivity of forests.

## Materials and methods

### Field site and sampling

Four stands, each containing Douglas-fir (*Pseudotsuga menziesii* Mirb. Franco) trees of a different height class, were located within 3.1 km of each other in the Wind River Basin of south-western Washington State, USA. Access to tree tops in the tallest sampling height class was facilitated by a 75-m-tall construction tower crane at the Wind River Canopy Crane Research Facility (WRCCRF). Periodic dieback of the tops of some of the old growth trees within the WRCCRF stand suggested that these trees were close to their maximum height for this site. Tree tops in the two intermediate height classes were accessed by non-spur climbing and foliage from the lowest height class was accessed with a pole pruner. The Pacific maritime climate of the region is characterized by wet winters and dry summers. Mean annual precipitation in the region is about 2.2 m, much of which falls as snow, with a dry season from June to September. Mean annual temperature is 8.7 °C with a mean of 0 °C in January and 17.5 °C in July. The soils are well drained and of volcanic origin (Shaw *et al.*, 2004). Low precipitation between June and September (~119 mm) typically leads to drought conditions in the upper portion of the soil profile. However, soil water remains accessible to Douglas-fir roots at depths greater than about 1 m throughout the summer dry period (Warren *et al.*, 2005; Meinzer *et al.*, 2007). A fifth site was used for *in situ* measurements of crown conductance ( $g_c$ ) only. This site consisted of an even aged stand of Douglas-fir trees planted in 1978 on a clear-cut located within the Wind River Experimental forest near the WRCCRF at an elevation of 560 m.

### Gas exchange

Branch samples 30–50 cm long were collected from sun-exposed locations within 5 m of the tops of three trees per site at mean sampling heights of 5.0 (SE=0), 18.3 (SE=0.33), 33.5 (SE=1.32), and 55.0 (SE=1.1) m. Branches were collected during periods of low transpirational water loss and were placed in plastic bags with moist paper towels and stored in the dark at 5 °C. Gas exchange measurements were conducted on branches sampled exclusively from fully sun-exposed branches near the tops of trees of different height classes to rule out the potentially confounding influence of factors such as irradiance and RH upon height-related trends in leaf structural and physiological characteristics. Because branch length is relatively consistent near tree tops across a height gradient, despite differences in tree height, sampling exclusively near tree tops also substantially reduces the confounding influence of branch length on leaf physiological and structural characteristics as well. Measurements were conducted in the laboratory on detached shoots that had their bases re-cut and immersed in water to eliminate the immediate effects of path length and gravity on gas exchange parameters. Gas exchange was measured with a portable photosynthesis system equipped with a red and blue LED source and CO<sub>2</sub> injector (LI-6400, Li-Cor, Lincoln, NE). The instrument was zeroed and the chemicals were replaced prior to use each day. Before starting a gas-exchange measurement, shoots of about 15–20 cm in length were detached from the larger branch samples, taking care to submerge the shoot bases in degassed water as the cut was made. For determination of the dependence of  $g_s$  on LVPD, photosynthetic photon flux was held at 1200  $\mu\text{mol m}^{-2} \text{s}^{-1}$ , leaf temperature between 25 °C and 33 °C, and reference CO<sub>2</sub> was set at 400 ppm. Cuvette LVPD values on the first several samples were originally targeted to the following levels and in the following order: 1.0, 0.75, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0 kPa.

Measurements on subsequent samples were conducted at 0.25 increments throughout the entire curve for greater resolution. The initial measurement was not recorded until  $g_s$  had stabilized for at least 20 min. Subsequent measurements were recorded after  $g_s$  had stabilized for at least 2 min. Measurements with values of  $g_s$  near zero were abandoned due to the high level of error associated with gas exchange measurements at very low levels of  $g_s$ . A two-parameter power function ( $y=ax^b$ ) was fit to curves of assimilation (A) versus  $g_s$  in order to determine if condensation in the gas exchange system was influencing values of  $g_s$ . In cases where  $g_s$  deviated noticeably from the power function due to condensation,  $g_s$  was adjusted to fit the two-parameter regression obtained from all other points in the relationship between assimilation and  $g_s$ . Branches were 'prehydrated' by submerging the bases of the cut stems in water overnight in order to ensure that all samples were fully hydrated at the time of gas exchange measurements. Our previous work on attached and detached Douglas-fir foliage showed that Douglas-fir shoots retain the same gas-exchange characteristics for about 4 d after detachment (Woodruff *et al.*, 2009).

#### Leaf turgor and osmotic potential

Bulk foliar turgor and osmotic potential were determined from pressure-volume curves. Pressure-volume analyses (Scholander *et al.*, 1965; Tyree and Hammel, 1972) were conducted on branchlets approximately 15 cm long collected after the completion of foliar expansion. These samples were excised early in the morning prior to significant transpirational water loss, sealed in plastic bags with moist paper to prevent desiccation, and then stored in a refrigerator within 1–4 h of excision. Pressure-volume (PV) curves were initiated by first determining the fresh weight of the twig, and then measuring  $W_1$  with a pressure chamber (PMS Instrument Company, Oregon). Alternate determinations of fresh weight and  $W_1$  were repeated during slow dehydration of the twig on the laboratory bench until values of  $W_1$  exceeded the measuring range of the pressure chamber (–4.0 MPa). The inverse of the balance pressure ( $W_1$ ) was plotted against relative water deficit to create a characteristic PV curve that consists of an initial curvilinear and sharply declining series of points, followed by a more linear series with a less severe decline. The point of transition between these two portions of the graph represents the turgor loss point for the sample (Tyree and Hammel, 1972). Extrapolating the linear portion of the curve to both axes creates a line, the y-value of which provides an estimate of solute potential. Prior to the turgor loss point, the area above the extrapolated linear portion of the curve and below the initial curvilinear portion of the curve, provides an estimate of turgor. Turgor at  $g_{s\text{-max}}$  for detached branches was estimated from turgor values obtained from PV curves at  $W_1$  values equivalent to those obtained from foliage that had been stabilized at  $g_{s\text{-max}}$  during gas exchange measurements using the portable photosynthesis system. Turgor at maximum crown conductance ( $g_{c\text{-max}}$  for *in situ* canopy conductance measurements was obtained in the same manner using field  $W_1$  values.

#### Sap flow and crown conductance

Sap flow measured with variable length heat dissipation sensors (James *et al.*, 2002) was used as a surrogate for transpiration. In the 55 m height class, sensors were installed in a total of seven upper branches on three individuals at a mean height of about 53 m. In a nearby stand (<5 km) with trees about 13 m tall, sensors were installed at the base of the live crown in the outer sapwood of three trees. Signals from the sap flow sensors were scanned every minute and 10 min means were recorded by a data logger (CR10X, Campbell Scientific Corp., Logan, UT) equipped with a 32-channel multiplexer (AM416; Campbell Scientific). Sap flow was expressed as sap flux ( $\text{mol m}^{-2}$  sapwood area  $\text{s}^{-1}$ ) and  $g_c$  ( $\text{mol m}^{-2} \text{s}^{-1}$ ) was estimated by dividing sap flux by the vapour pressure deficit

(expressed as a mole fraction) measured at half-hour intervals at a weather station installed at 60 m on the canopy crane tower.

#### Leaf anatomy

Branch samples 30–50 cm long were collected from sun-exposed locations within 5 m of the tops of the trees at mean sampling heights of 5.0 (SE=0), 18.3 (SE=0.33), 33.5 (SE=1.32), and 58.0 (SE=1.9) m. Cuticle thickness was measured from the peak of the guard cell beneath the cuticle, to the outer surface of the guard cell cuticle. Cross-sections of needles were made by hand-sectioning. All anatomical measurements were obtained from foliage produced during 2008. Images were obtained using a fluorescence microscope with a x40 objective lens and a total magnification of x400. Images were analysed using ImageJ version 1.27 image analysis software (Abramoff *et al.*, 2004). Data were pooled per tree and analysed using regression analysis (PROC REG; Statistical Analysis Software, version 9.1; SAS, North Carolina).

#### Data analysis

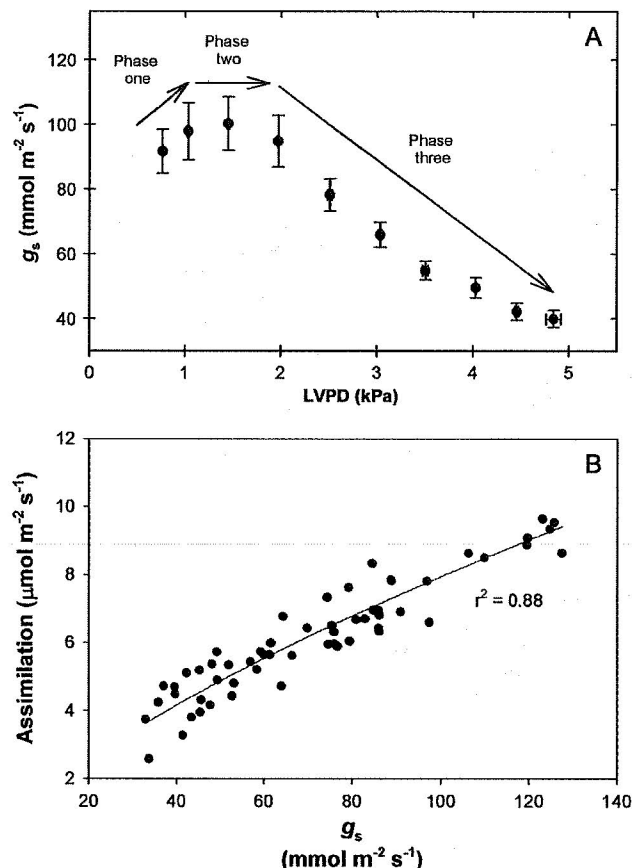
A 5-parameter wave function  $y=y_0+ae^{-\frac{1}{b}}\sin(\frac{2\pi x}{b}+c)$  was fit to curves of  $g_s$  on LVPD. LVPD at  $g_{s\text{-max}}$  was obtained by determining the value of LVPD (the curve's x-axis value) at the peak of this curve. Data were pooled per tree and analysed using regression analysis (PROC REG; Statistical Analysis Software, version 9.1; SAS, North Carolina).

## Results

There were three distinct phases in the response of  $g_s$  to changes in LVPD (Fig. 1A). Under conditions of low LVPD,  $g_s$  increased with increasing LVPD (phase one). During the second phase,  $g_s$  was maximal at intermediate LVPD and, during the third phase,  $g_s$  declined steadily as LVPD increased. The 2-parameter power function  $y=ax^b$  yielded  $r^2$  values ranging from 0.52 to 0.96 for the dependence of A on  $g_s$  for individual branches (Fig. 1B). The 5-parameter wave function  $y=Y_0+ae^{-\frac{1}{b}}\sin(\frac{2\pi x}{b}+c)$  yielded  $r^2$  values ranging from 0.76 to 0.99 for the dependence of  $g_s$  on LVPD for individual branches.  $g_{s\text{-max}}$  occurred at progressively greater LVPD with increasing height ( $P=0.006$ ; Fig. 2A). Mean osmotic potential at full turgor decreased by  $1.25 \times 10^{-2}$  MPa  $\text{m}^{-1}$  increase in height ( $P=0.00016$ ; Fig. 2B), indicating a height-related increase in foliar symplastic solute concentration. *in situ* and laboratory measurements of LVPD at maximum conductance showed a significant linear increase with increasing turgor ( $P=0.0093$ ; Fig. 3), suggesting that the height-related trend in LVPD at  $g_{s\text{-max}}$  was related to solute-mediated trends in turgor with increasing height.

Estimates of  $g_c$  from sap flow measurements indicated  $g_{c\text{-max}}$  occurred at nearly the same level of LVPD in both the 55 m trees and in a nearby stand with 13 m trees (1.0 kPa for the 55 m trees, Fig. 4A; 0.99 kPa for the 13 m trees, Fig. 4B), indicated by the dashed lines in Fig. 4. The lack of a height-related trend in LVPD at maximum conductance at the crown level contrasted with the pattern seen in the laboratory gas-exchange measurements (Fig. 2A).

Mean cuticle thickness increased with increasing height by  $0.044 \text{ } \mu\text{m m}^{-1}$  increase in height, representing a significant height-related trend in cuticle thickness ( $P=0.00015$ ; Fig. 5A).



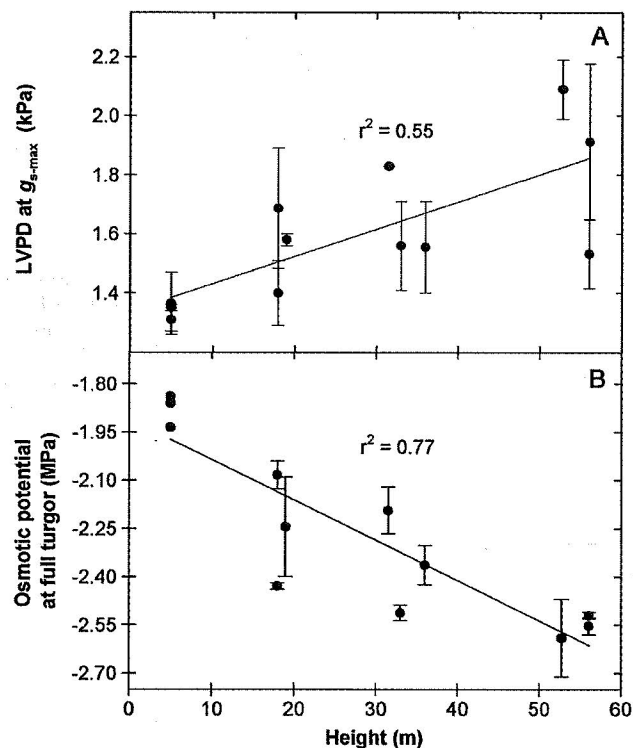
**Fig. 1.** (A) Mean of all stomatal conductance ( $g_s$ ) versus leaf to air vapour pressure deficit (LVPD) response curves for the 5 m height class. Under conditions of low LVPD,  $g_s$  is minimal and increases rapidly with increasing LVPD (phase one) followed by a peak in  $g_s$  at intermediate LVPD (phase two), then a steady decline in  $g_s$  with further increases in LVPD (phase three). Error bars represent standard errors of  $g_s$  at set values of LVPD from six different curves. (B) Assimilation ( $A$ ) plotted against  $g_s$  from all 5 m height class response curves.

There was a significant correlation between LVPD at  $g_{s-\max}$  and cuticle thickness ( $P=0.0011$ ; Fig. 5B) suggesting a potential role for cuticle thickness in the height-related trend in LVPD at  $g_{s-\max}$ .

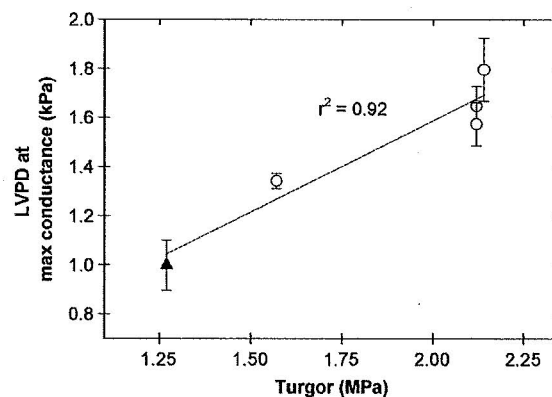
## Discussion

### Stomatal response to LVPD

Although there is a widespread assumption that  $g_s$  generally decreases as RH declines or LVPD increases, measurements showing an initial increase in  $g_s$  with increasing LVPD have been presented in studies on a number of tree species (Osonubi and Davies, 1980; Eamus and Cole, 1997; Prior *et al.*, 1997; Yang *et al.*, 1998; Day, 2000; Chang and Lin, 2007; Eamus *et al.*, 2008). Given that increasing guard cell turgor enhances stomatal aperture and increasing subsidiary cell turgor decreases stomatal aperture (Buckley, 2005, and

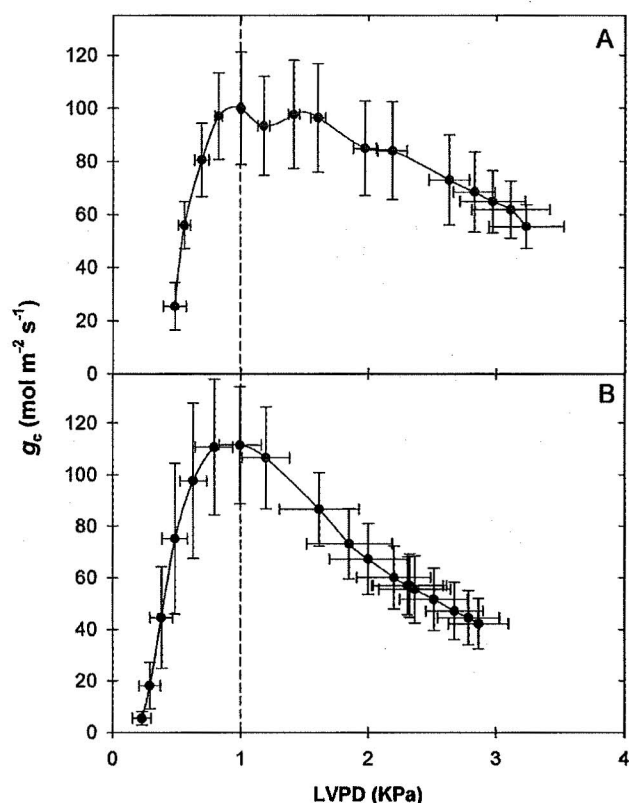


**Fig. 2.** (A) Leaf to air vapour pressure deficit (LVPD) at maximum stomatal conductance ( $g_{s-\max}$ ) and (B) osmotic potential at full turgor plotted against sample height.



**Fig. 3.** Leaf to air vapour pressure deficit (LVPD) at maximum conductance plotted against bulk leaf turgor. Open circles represent mean values obtained from gas exchange measurements on cut branches in the laboratory. Error bars represent standard errors of between two and four branches per tree. The filled triangles and associated error bars represent the mean and standard errors for the peak value of crown conductance for three trees in the 55 m stand. The crown conductance data were obtained from branch sap flow measurements averaged over four separate days for which LVPD reached at least 3.75 kPa.

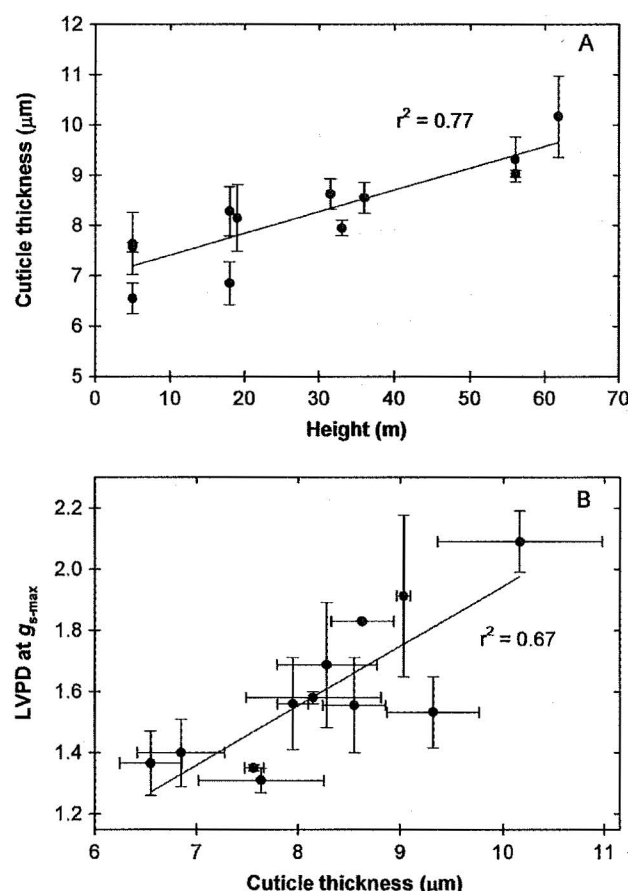
references within), it has been suggested that the initial increase in  $g_s$  with increasing LVPD during phase one is due to reduced epidermal turgor resulting from increased LVPD



**Fig. 4.** Crown conductance ( $g_c$ ) in relation to leaf to air vapour pressure deficit (LVPD) for (A) the 55 m stand and (B) a nearby 13 m stand during periods of high LVPD. Data for the 55 m stand are from days 216, 217, 226, and 227 of 2005. Data for the 13 m stand are from days 190, 191, 192, and 198 of 2003. Error bars represent standard errors of three trees. Values of  $g_c$  are expressed on a unit sapwood area basis. The dashed line indicates the approximate LVPD at which maximum crown conductance ( $g_{c-max}$ ) occurs.

and a subsequent decrease in the pressure exerted upon guard cells by adjacent subsidiary cells (Edwards *et al.*, 1976; Spence *et al.*, 1983; Wu *et al.*, 1985; Franks *et al.*, 1995; Mott and Franks, 2001). The height-related increase in LVPD at maximum conductance (Fig. 2A) suggests that increased height is associated with a decreased sensitivity of guard cells to the factors that lead to stomatal closure, or that it is associated with an impact on subsidiary cell properties that has the equivalent effect. Mean osmotic potential at full turgor decreased by  $0.0125 \text{ MPa m}^{-1}$  increase in height. This is steeper than the gravitational gradient of  $-0.01 \text{ MPa m}^{-1}$  but less steep than the observed midday gradient of  $0.017\text{--}0.018 \text{ MPa m}^{-1}$  in transpiring Douglas-fir trees (Woodruff *et al.*, 2004; Domec *et al.*, 2008), suggesting that there are constraints on osmotic adjustment that prevent complete compensation of the height-related trends in  $W_1$  associated with gravity and path length resistance during transpiration.

In the three-phase response curve of  $g_s$  to LVPD,  $g_s$  both increases and then decreases in response to increasing LVPD (Fig. 1A). The trends along a height gradient in



**Fig. 5.** (A) Cuticle thickness plotted against height and (B) leaf to air vapour pressure deficit (LVPD) at maximum stomatal conductance ( $g_{s-max}$ ) plotted against cuticle thickness (B). Error bars represent standard errors from between three and five branches per tree.

LVPD at  $g_{s-max}$  were consistent with height-related trends in foliar osmotic potential (Fig. 2B) and with the mechanical advantage of subsidiary cells over guard cells in their control of stomatal aperture (Edwards *et al.*, 1976; Spence *et al.*, 1983; Franks *et al.*, 1995). That is, in the case of fully hydrated detached branches, the height-related reduction in foliar osmotic potential should lead to an increase in foliar turgor in excised branches with their bases in water. Under conditions of enhanced foliar turgor, the mechanical advantage of epidermal cells over guard cells is likely to lead to an offset in the response of stomata to increasing LVPD. The apparent effect of enhanced subsidiary cell turgor during phase one was to shift maximal  $g_s$  to a greater LVPD resulting in a height-related increase in LVPD at  $g_{s-max}$  of  $9.7 \times 10^{-3} \text{ kPa m}^{-1}$  increase in height (Fig. 2A). The observed shift in maximal  $g_s$  towards greater LVPD in fully hydrated detached samples alludes to an enhanced role of subsidiary cells with increasing height in determining stomatal aperture under conditions of low LVPD and it suggests that enhanced turgor through osmotic adjustment may function to maintain  $g_s$  under conditions of greater evaporative demand.

Diurnal courses of conductance from *in situ* crown sap flow measurements indicate a consistency in LVPD at maximum  $g_s$  between the two distinctly different height classes (Fig. 4). Despite the strong height-related trend in the relation between  $g_s$  and LVPD for fully hydrated detached foliage (Fig. 3), *in situ* measurements of crown conductance in 55 m trees under similar meteorological conditions showed a similar LVPD at maximum conductance across height classes (Fig. 4). This suggests that *in situ*, the intrinsic capacity to maintain  $g_s$  under greater LVPD for foliage in tall trees is offset by hydraulic constraints associated with gravity and frictional resistance during transpiration, both of which act to increase xylem tension and water stress of foliage (Scholander *et al.*, 1965). The consistent relationship between LVPD at  $g_{s-max}$  and turgor in both the laboratory and the field data (Fig. 3) highlights the apparent role of foliar turgor in modulating this relationship between conductance and LVPD and it suggests that height-related foliar osmotic adjustment results in substantial homeostasis in the response of stomata to LVPD across a height gradient.

#### Anatomical characteristics

A great deal of structural diversity exists in stomatal characteristics and several features are considered to be influential for stomatal conductance and stomatal sensitivity to LVPD (Willmer and Fricker, 1996; Franks and Farquhar, 2007; Roth-Nebelsek, 2007). Some of these characteristics include cuticle thickness, presence or lack of an internal cuticle, location of the pore (sunken versus non-sunken), stomatal shape ('dumb-bell' versus 'kidney'), chamber length, and maximum stomatal aperture size. Which anatomical characteristics may be most influential to stomatal function depends on the mechanisms by which stomata are able to respond to LVPD and despite abundant research there is little consensus on the nature of these mechanisms. Cuticle thickness was analysed along a height gradient due to its influence on cuticular permeance and its potential impact on epidermal turgor and stomatal mechanics. Cuticle thickness increased linearly and significantly with tree height ( $1.2=0.78$ ,  $P=0.00015$ ). Mean cuticle thickness ranged from approximately 7.25  $\mu\text{m}$  for the 5 m height class to approximately 9.5  $\mu\text{m}$  for 58 m height class, representing over 30% increase in cuticle thickness along a height gradient of 53 m. The significant height-related increase in cuticle thickness of foliage from tree tops (Fig. 5A) in conjunction with the height-related trends in LVPD at  $g_{s-max}$  is consistent with the potential role of cuticular transpiration in influencing stomatal mechanics and in the control of stomatal response to changes in LVPD.

A number of studies have shown evidence that stomatal response to changes in LVPD occurs as a response to either bulk leaf or epidermal transpiration (Meinzer and Grantz, 1991; Mott and Parkhurst, 1991; Monteith, 1995; Meinzer *et al.*, 1997). Farquhar (1978) showed that the feed-forward response of stomata to changes in LVPD (declining transpiration with increasing LVPD, Grantz, 1990; Franks

*et al.*, 1997) could be explained by  $g_s$  responding to cuticular transpiration, independent of  $W^1$ . In a study investigating stomatal sensitivity to LVPD, Meinzer (1982) was able to increase the water permeability of the cuticle and increase the sensitivity of the foliage to changes in LVPD by partially removing the cuticle of Douglas-fir foliage with a hexane wash. Kerstiens (1996) also emphasized the importance of cuticular conductance in controlling the response of  $g_s$  to LVPD. A model by Eamus and Shanahan (2002) of the relationship between  $g_s$  and transpiration highlights the importance of cuticular conductance ( $g_{cut}$ ) in determining  $g_s$  and Eamus *et al.* (2008) provide experimental and modelling data to support the argument that cuticular transpiration plays a substantial role in the feedback between  $g_s$  and LVPD. Regardless of whether  $g_s$  is predominantly controlled by transpiration through the cuticle, cuticular transpiration clearly occurs and it directly influences epidermal turgor which is widely understood to have substantial influence on stomatal mechanics.

The relationship between cuticle thickness and permeance can be derived from Fick's law of diffusion which states that the rate of diffusion is directly related to the thickness of the membrane through which transport is occurring. Thus, for membranes of a given permeance, water flux will be inversely proportional to the thickness (Becker *et al.*, 1986) as illustrated in the following equation describing the permeance to water ( $P$ ) of a waxy membrane (from Kirsch *et al.*, 1997):

$$P = D \times \frac{K_{ww}}{\delta x} \quad (1)$$

where  $D$  is the diffusion coefficient of the membrane material ( $\text{m}^2 \text{S}^{-1}$ ),  $K_{ww}$  is the wax water partition coefficient, and  $\delta x$  is the thickness of the membrane ( $\text{m}$ ). Although cuticle thickness is not always a good predictor of cuticle permeance across multiple species (Kamp, 1930), plants that are adapted to drought stress often have thicker wax coatings than those from more moist environments (Shepperd and Griffiths, 2006) and deposition of cuticular wax is a common response to water stress that can occur within just a few days (Bengston *et al.*, 1978; Premachandra *et al.*, 1991). A number of forms of stress can influence cuticular waxes and they commonly involve effects that are closely associated with biosynthesis, such as the induction of changes in the amount and composition of wax (Shepperd and Griffiths, 2006). Suppression of wax production is a common occurrence for tissue cultures developed in high humidity (Sutter and Langhans, 1979, 1982) and plants grown *in vitro* are often susceptible to desiccation due to the lack of waxy cuticle development (Baker, 1982; Sutter and Langhans, 1982; Koch *et al.*, 2006).

Our data support the hypothesis that the response of  $g_s$  to changes in LVPD is influenced by foliar turgor pressure and that the pattern of this response would follow a height-related trend similar to those observed in foliar osmotic adjustment in the same species. Our anatomical analyses provide evidence of a height-related trend in cuticular

anatomy that is consistent with observed trends in LVPD at  $g_s$ -max. Future research involving experimental manipulation of the leaf cuticle could provide greater insight into the role of leaf cuticle thickness in guard cell sensitivity to changes in LVPD.

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