

Acclimation of leaf hydraulic conductance and stomatal conductance of *Pinus taeda* (loblolly pine) to long-term growth in elevated CO₂ (free-air CO₂ enrichment) and N-fertilization

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ABSTRACT

We investigated how leaf hydraulic conductance (K_{leaf}) of loblolly pine trees is influenced by soil nitrogen amendment (N) in stands subjected to ambient or elevated CO₂ concentrations (CO₂^a and CO₂^e, respectively). We also examined how K_{leaf} varies with changes in reference leaf water potential ($\Psi_{\text{leaf-ref}}$) and stomatal conductance ($g_{\text{s-ref}}$) calculated at vapour pressure deficit, D of 1 kPa. We detected significant reductions in K_{leaf} caused by N and CO₂^e, but neither treatment affected pre-dawn or midday Ψ_{leaf} . We also detected a significant CO₂^e-induced reduction in $g_{\text{s-ref}}$ and $\Psi_{\text{leaf-ref}}$. Among treatments, the sensitivity of K_{leaf} to Ψ_{leaf} was directly related to a reference K_{leaf} ($K_{\text{leaf-ref}}$ computed at $\Psi_{\text{leaf-ref}}$). This liquid-phase response was reflected in a similar gas-phase response, with g_{s} sensitivity to D proportional to $g_{\text{s-ref}}$. Because leaves represented a substantial component of the whole-tree conductance, reduction in K_{leaf} under CO₂^e affected whole-tree water use by inducing a decline in $g_{\text{s-ref}}$. The consequences of the acclimation of leaves to the treatments were: (1) trees growing under CO₂^e controlled morning leaf water status less than CO₂^a trees resulting in a higher diurnal loss of K_{leaf} ; (2) the effect of CO₂^e on $g_{\text{s-ref}}$ was manifested only during times of high soil moisture.

Key-words: capacitance; embolism; reference conductances; reference leaf water potential; time constant; turgor loss point.

INTRODUCTION

The magnitude of plant response to elevated atmospheric CO₂ concentration (CO₂^e) often depends on the availability of other resources, such as nutrients and water (Monje & Bugbee 1998; Oren *et al.* 2001; Hyvönen *et al.* 2007). For example, although leaf level photosynthesis is generally

stimulated under CO₂^e (Medlyn *et al.* 1999; Bernacchi *et al.* 2003), gas exchange over diurnal and repeated drying cycles may be often dominated by the nitrogen availability and the hydraulic regulation of the stomata (Katul, Leuning & Oren 2003). Understanding the effects of CO₂^e on gas exchange and on tree water relations over a range of nutrient and water availabilities is therefore critical to our ability to predict forest productivity as the climate changes (Wullschleger, Tschaplinski & Norby 2002). However, at present, the interaction effects between CO₂, nitrogen (N), and water availabilities on plant hydraulics are poorly understood.

In isohydric species, stomata conservatively regulate plant water status by controlling the rate of water loss to the atmosphere such that it matches the capacity of the soil–plant hydraulic system to supply water to leaves. Should stomata fail to sense and respond to a lower capacity of the soil–plant system to supply water, xylem would embolize rapidly, increasing the risk of hydraulic dysfunction and dehydration of leaves (Maseda & Fernández 2006). However, stomatal regulation of transpiration rate imposes limits on photosynthesis. Thus, water availability is one of the most important factors limiting productivity and has likely been an important selective regime influencing the evolution of plant physiology (Schulze *et al.* 1987). Despite decades of research on the physiology of stomata, the specific mechanisms that permit coordination of stomatal conductance (g_{s}) with plant water balance and hydraulic properties remain elusive (Meinzer 2002; Buckley 2005). Nevertheless, there does seem to be a general agreement that stomata sense leaf water potential (Ψ_{leaf}) somewhere within the leaf so that similarity exists in the response of both g_{s} and leaf xylem cavitation to decreasing Ψ_{leaf} (Brodribb & Cochard 2009; Domec *et al.* 2009).

The diversity in form and longevity of leaves translates to large differences in water transport capacity and, thus, in gas exchange (Sack & Holbrook 2006). Water transport capacity can be quantified in terms of leaf hydraulic conductance

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(K_{leaf} , Aasamaa, Sober & Rahi 2001; Domec *et al.* 2009). Consistent with earlier works documenting coordination of g_s with overall plant hydraulic conductance (Meinzer *et al.* 1995; Cochard *et al.* 2002), K_{leaf} and its relationship to stomatal control has recently been investigated, revealing that maximum g_s is very sensitive to K_{leaf} within and among species (Brodribb *et al.* 2003; Woodruff *et al.* 2007). Leaves comprise the terminal portion of the liquid water transport pathway and their xylem is under greater tension than in stems, yet leaf xylem appears to be generally more vulnerable to embolism than that of the stems. This low resistance to embolism results in large decreases of K_{leaf} on a regular basis, even under non-extreme environmental conditions (Brodribb & Holbrook 2003; Bucci *et al.* 2003). Thus, although the hydraulic system of leaves represent less than 5% of the hydraulic pathway it constitutes a substantial (30–80%) and variable part of the resistance to water flow through plants (Sack & Holbrook 2006; Domec *et al.* 2009). These observations imply that the large diurnal declines of K_{leaf} may be an inherent component of the stomatal regulatory system acting as a signal rather than a catastrophe to be avoided.

Environmental changes that increase the availability of resources (either above- or below-ground) result in the long-term acclimation of a less conductive (per unit leaf area) hydraulic system because of anatomical acclimation (Mencuccini 2003). For example, loblolly pine trees growing under CO₂^e and N-fertilization exhibit some differences in wood properties such as shorter tracheids and lower wood density (Oren *et al.* 2001; McCarthy *et al.* 2006a). In addition, when soil nutrient availability is low, plants allocate more carbon to roots compared with leaves to enhance nutrient uptake (Gerbauer, Reynolds & Strain 1996; Palmroth *et al.* 2006). On the other hand, when nutrient limitation is relieved by fertilization, the pattern of biomass allocation may change, favouring the expansion of the leaf surface area to enhance CO₂ capture at the expense of below-ground plant parts (King, Thomas & Strain 1997; McCarthy *et al.* 2006b). The effect of nutrient limitation on carbon partitioning might impact leaf hydraulic architecture and, consequently, leaf water relations. For example, increase in canopy leaf area with increasing nutrient availability and CO₂^e (McCarthy *et al.* 2007) should cause K_{leaf} to decrease if not accompanied by a proportional increase in sapwood area and root area (Ewers, Oren & Sperry 2000; Addington *et al.* 2006). This reduction in K_{leaf} may then impose a series of constraints on the water economy of the plant, with ultimate impacts on the carbon economy (Sperry *et al.* 2002; Bucci *et al.* 2006).

Under CO₂^e, g_s in most species decreases (e.g. Medlyn *et al.* 2001), reducing transpiration per unit leaf area. Coniferous species generally have stomata that are less responsive to CO₂^e than broad-leaved species (Pataki, Oren & Tissue 1998; Ellsworth 1999; Maier, Palmroth & Ward 2008). However, it has also been proposed that g_s might decrease under CO₂^e only in longer-term (>1 year) experiments, and that the seasonal timing of data collection was important (Medlyn *et al.* 2001; Wang *et al.* 2005). The

magnitude of the response of stomata to CO₂^e also depends on the sensitivity of g_s to soil moisture and vapour pressure deficit (Ainsworth & Rogers 2007). In loblolly pine trees, it has been shown that g_s under CO₂^e is similar to that under ambient CO₂ concentration (CO₂^a) when subjected to water stress, but lower without moisture limitation (Murthy *et al.* 1996). Therefore seasonal monitoring of stomatal response to CO₂^e may be necessary to understand stomatal response under varying soil moisture and evaporative demand.

Here we focused on the possible effects of CO₂^e and N-fertilization on leaf water transport traits and their consequence for long distance water transport and drought tolerance of *Pinus taeda* L. (loblolly pine) trees, species very sensitive to low soil moisture (Noormets *et al.* 2009). We specifically investigated the effects on g_s regulation and possible compensatory adjustments. The 2007 severe summer drought in the Atlantic Southeast of the United States provided an opportunity to study whether: (1) N-fertilization and CO₂^e lower leaf and whole tree hydraulic conductance; (2) K_{leaf} and g_s show coordinated acclimation to long term N-fertilization and CO₂^e; and (3) N-fertilization and CO₂^e result in a reduced sensitivity of g_s to declining vapour pressure deficit and soil moisture. It was hypothesized that when compared with unfertilized trees under CO₂^a, water transport capacity would adjust to nitrogen availability and CO₂^e, with K_{leaf} and g_s being lower in fertilized trees and under CO₂^e.

MATERIALS AND METHODS

Setting

The treatments have been administered in a loblolly pine plantation established in 1983 on low fertility, acidic clay loam of the Enon series, in the Blackwood Division of Duke University Forest, in Orange County, North Carolina (35°58'N, 79°08'W). The average height of pines was ~19 m in 2007. Loblolly pine accounts for up to 90% of the basal area (McCarthy *et al.* 2007). The most prevailing co-dominant species is *Liquidambar styraciflua*, and the most common sub-canopy species are *Acer rubrum*, *Ulmus alata* and *Cornus florida*. Mean annual temperature is 15.8 °C and mean annual precipitation is 1150 mm, with usually an even distribution throughout the year. Summers are warm and humid with a growing season mean temperature of 22.1 °C. Further details about the site can be found in Oren *et al.* (1998a).

The experimental site consisted of four plots exposed to ambient CO₂ (CO₂^a) and four plots targeted at +200 μmol mol⁻¹ CO₂ (CO₂^e) above current, with half of each plot fertilized with N (Oren *et al.* 2001; Schäfer *et al.* 2002). CO₂ enrichment is implemented according to the free-air CO₂ enrichment (FACE) protocol throughout the year whenever ambient temperature is above 5 °C and wind speed is below 5 m s⁻¹ (Hendrey *et al.* 1999). In 1994, two 30-m-diameter plots were established: the FACE prototype plot (Plot 7) and its adjacent untreated reference plot (Plot 8). In 1996, six additional plots (replicated FACE; Plots 1 to 6)

were established, three of which received CO₂^e. In 1998, the prototype plot and its reference plot were halved using a ditch and a barrier, and one-half of each has received annual nitrogen fertilization (N) of 11.2 g N m⁻² (Oren *et al.* 2001); the same design was implemented in the rest of the plots in 2005.

Measurement of leaf hydraulic conductance (K_{leaf})

Aiming to ensure long-term integrity of the Duke FACE experiment, only limited sampling is permitted. Thus, one terminal branch per tree was collected from four trees per plot (two trees from each half). Branches were collected at pre-dawn and enclosed in sealed plastic bags to prevent water loss. K_{leaf} was measured on single fascicles by assessing the rehydration kinetics of needles after detachment and determined as (Brodribb & Holbrook 2003):

$$K_{\text{leaf}} = C_{\text{leaf}} \ln[\Psi_{\text{leaf(o)}}/\Psi_{\text{leaf(t)}}]/t \quad (1)$$

where K_{leaf} (mmol m⁻² MPa⁻¹ s⁻¹) is the leaf hydraulic conductance, C_{leaf} (mmol m⁻² MPa⁻¹) is the leaf capacitance, $\Psi_{\text{leaf(o)}}$ (MPa) is the leaf water potential prior to rehydration, $\Psi_{\text{leaf(t)}}$ is the leaf water potential after rehydration, and t (s) is the duration of rehydration of needles detached under water from the stem. C_{leaf} was determined from the slope of relative water content to Ψ_{leaf} obtained from pressure–volume curves. All capacitance values reported and used to calculate K_{leaf} corresponded to C_{leaf} determined before the water potential at turgor loss point (Ψ_{tp}) (Brodribb & Holbrook 2003). Values of K_{leaf} were corrected for a viscosity of water at a temperature of 20 °C.

Pressure–volume analyses (Tyree & Hammel 1972) were conducted on single fascicles taken on the same trees used to determine K_{leaf} , between the months of January and February 2007, when soil moisture and pre-dawn water potentials were high. These samples were excised early in the morning prior to significant water loss, sealed in plastic bags with moist paper to prevent desiccation, and then stored in a refrigerator within 1 h of excision. Pressure–volume curves were initiated by first determining the fresh weight of the fascicle, and then measuring Ψ_{leaf} with a pressure chamber (PMS Instrument Company, Albany, OR). Alternate determinations of fresh weight and Ψ_{leaf} were repeated during slow dehydration on the laboratory bench until values of Ψ_{leaf} ranging from –4.0 MPa to –5.0 MPa were attained. The inverse of water potential was plotted against relative water content to establish a pressure–volume curve and determine Ψ_{tp} . For normalizing C_{leaf} on a leaf area basis, needle areas were obtained geometrically from dimensions measured using a digital caliper (series 500 Mitutoyo, Aurora, IL, USA) (Rundel & Yoder 1998).

Because differences may exist between K_{leaf} measured under field condition and K_{leaf} measured in the laboratory (Tyree *et al.* 2005; Sack & Holbrook 2006), we also estimated K_{leaf} based on field measurements on some of the same trees on which stomatal conductance (g_s) and

transpiration (E ; see further discussion) were measured (Supporting Information Fig. S1). Field K_{leaf} was calculated as $K_{\text{leaf}} = v/v_0 E/(\Psi_{\text{stem}} - \Psi_{\text{leaf}})$, where v and v_0 are the kinematic viscosities of water at the measured leaf temperature and at 20 °C, respectively; E is the transpiration rate (mmol m⁻² s⁻¹) measured with a LI-6400 portable photosynthesis system (Li-Cor Inc., Lincoln, NE, USA), and Ψ_{stem} is the stem water potential estimated from non-transpiring covered shoots (Meinzer 2002). Although K_{leaf} estimated from the dehydration curves and Ψ_{leaf} was 12% lower than field K_{leaf} , the difference was not statistically significant ($P = 0.11$, paired T -test; Supporting Information Fig. S1).

Field leaf water potential (Ψ_{leaf}) and stomatal conductance (g_s)

Measurements of Ψ_{leaf} were conducted with a pressure chamber, and g_s with a LI-6400 portable photosynthesis system (Li-Cor Inc.). Measurements of Ψ_{leaf} and g_s were conducted on detached fascicles taken from the same shoot simultaneously every 90 min on one non-fertilized and one fertilized tree per ring for a total of 16 trees during each sampling period. Previous studies at the site has shown that there were no differences between excised and attached needle gas exchange when measurements were restricted to less than 15 min after excision (Maier *et al.* 2008). Vapour pressure deficit (D) and average CO₂ concentration, inside the chamber followed ambient conditions, and photosynthetically active radiation were matched with the average conditions over a 15 min period prior the measurement. Diurnal measurements commenced prior to dawn and continued until 1500 h on 22 and 23 May, 24 and 25 July, and between 19 and 23 September 2007. These dates encompassed large differences in climatic and soil conditions allowing us to study a wide range of liquid- and gas-phase values.

In two of the plots where leaf area data were available for each treatment (rings 7 and 8), mean crown canopy conductances (G_s) derived from basal sap flow were used to provide an independent assessment of stomatal control of transpiration. We used leaf area data measured in 2005 (McCarthy *et al.* 2007), which is thought to accurately represent leaf area of 2007 (McCarthy, personal communication). Sap flow was measured at breast height in at least five trees per treatment with 20 mm heat dissipation sensors installed at three depths in the sapwood. Sensor operation at the FACE site is detailed in Schäfer *et al.* (2002). The sensor signal was converted to sap flux density (J_s in g m⁻² s⁻¹) according to Granier (1987) and accounted for the effects of non-zero night-time fluxes (Kim, Oren & Hinckley 2008; Oishi, Oren & Stoy 2008). Sap flux density was scaled and converted to a tree-scale average transpiration per unit leaf area (E , in mmol m⁻² s⁻¹; Oren *et al.* 1998b; Schäfer *et al.* 2002). Basal J_s values were lagged by half an hour in order to take into account water use from capacitance and thus to more closely approximate the relationship between D and transpiration in the crown (Phillips *et al.* 1997). The half-hour lag time was derived from the observed time lag of J_s with respect to D (Ewers & Oren

2000; Chuang *et al.* 2006). The sap-flux based G_s was calculated from E and D , using the simplification of the inversion of Penman–Monteith model (Ewers & Oren 2000). The simplified calculation was permitted because in all treatments D was close to the leaf-to-air vapour pressure deficit because of the high boundary-layer conductance. Indeed, given that >90% of the daytime mean wind velocity was >0.7 m s⁻¹, and that leaf dimension never exceeded 0.0017 m, we estimated using Jones (1992) that the mean daytime boundary-layer conductance averaged 65 times G_s .

Analysis of the response of liquid and gas conductance to changes in driving force

Stomata of isohydric plants respond to D in a manner consistent with protection of the xylem integrity for water transport. The emergent behaviour is a decreasing g_s with increasing D at a rate that is predictable and proportional to g_s at low D (Oren *et al.* 1999). Thus, g_s data from each treatment were analysed based on:

$$g_s = b - m \times \ln(D) \quad (2)$$

where b is g_s at $D = 1$ kPa (hereafter designated as reference stomatal conductance, g_{s-ref}) and m is the sensitivity of g_s to D [$-dg_s/d\ln D$, in $\text{mmol m}^{-2} \text{s}^{-1} (\ln \text{kPa})^{-1}$]. Based on the stated hydraulic consideration, $-dg_s/d\ln D$ is proportional to g_{s-ref} with the proportionality averaging ~ 0.60 , and varying predictably depending on the range of D used in the analysis (Oren *et al.* 1999; Ewers *et al.* 2007; Kim *et al.* 2008; Ward *et al.* 2008).

Similarly, we evaluated whether the sensitivity of xylem conductivity to Ψ_{leaf} is related to K_{leaf} by generating a slope of the reduction in K_{leaf} versus the natural logarithm of Ψ_{leaf} [$dK_{leaf}/d\ln \Psi_{leaf}$ in $\text{mmol m}^{-2} \text{s}^{-1} (\ln \text{MPa})^{-1}$] (Ewers *et al.* 2000). To analyse the effect of K_{leaf} on whole tree hydraulic

conductance (K_t), K_t was calculated from the slope of the relationship between E and Ψ_{leaf} (Loustau, Domec & Bosc 1998).

Statistical analysis

The effects of CO₂^e and N on K_{leaf} , C_{leaf} , Ψ_{leaf} , Ψ_{tp} and g_{s-ref} were tested through analysis of variance (ANOVA) based on a split-plot design. CO₂ concentration and N were the main and split-plot effects, respectively, and individual plots were used as replicates. Measurements made on multiple dates were analysed by repeated measure ANOVA. Statistical analyses were performed using SAS (version 9.1, Cary, NC, USA) and curve fits were performed using Sigmaplot (version 9.0, SPSS Inc., San Rafael, CA, USA).

RESULTS

Leaf hydraulic conductance (K_{leaf}) determined in the laboratory declined exponentially with declining Ψ_{leaf} (Fig. 1; R^2 ranging from 0.74 to 0.89; $P < 0.01$). We obtained the treatment means of maximum K_{leaf} and the water potentials at which 50% of maximum K_{leaf} was lost (Ψ_{50}) from curves fitted to data from each tree. CO₂^e and N significantly decreased maximum K_{leaf} by 21% and 13%, respectively (Table 1). No treatment effects were observed in Ψ_{50} .

Although CO₂^e decreased the water potential at the turgor loss point (Ψ_{tp}) by 0.12 MPa and C_{leaf} by 15%, no treatment effects were observed in the osmotic potential at full hydration, although a trend was discernable (Table 1). There was no CO₂^e × N interaction effects on any parameter measured in the laboratory (Table 1). Among the treatments, the maximum K_{leaf} and C_{leaf} decreased linearly with decreasing Ψ_{tp} (Fig. 2). Extrapolating the regression lines to zero K_{leaf} and zero C_{leaf} generated similar values of Ψ_{tp} (-2.99 MPa in Fig. 2a, and -3.08 MPa in Fig. 2b, respectively).

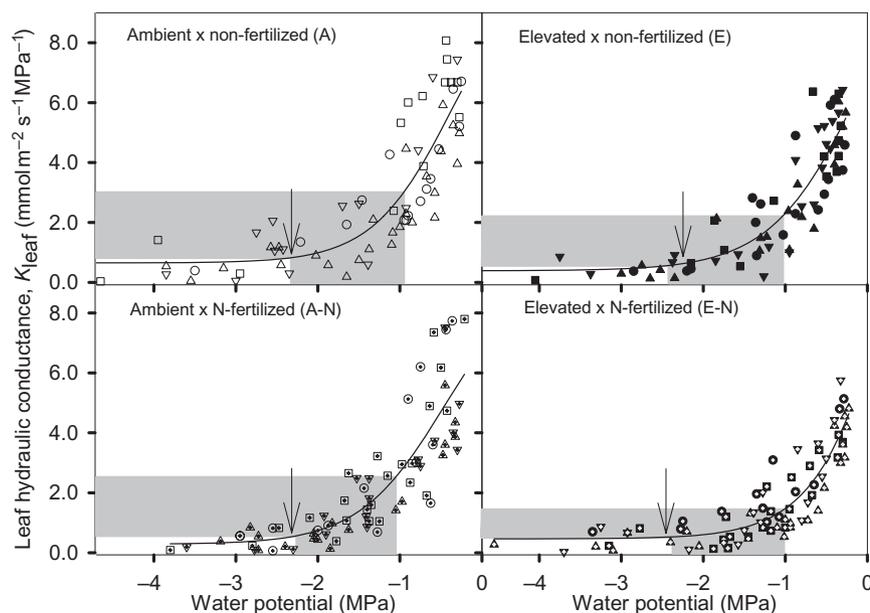


Figure 1. Leaf hydraulic conductance (K_{leaf}) in relation to leaf water potential (Ψ_{leaf}) for foliage samples obtained from the upper crowns of loblolly pine trees growing under ambient (CO₂^a), elevated (CO₂^e) and/or N-fertilized conditions (Duke free-air CO₂ enrichment site). Different symbols indicate individual trees within each treatment. Symbols with cross inside are from N plots. Closed symbols are for CO₂^e, and open symbols are for CO₂^a. Within ambient or elevated plots, same shaped symbols are from the same plots. The grey-shaded areas represent the range of K_{leaf} experienced over the seasonal range of measured Ψ_{leaf} . Arrows indicate the Ψ_{leaf} at the turgor loss point (Ψ_{tp}).

Table 1. Maximum leaf hydraulic conductance (max. K_{leaf} , $\text{mmol m}^{-2} \text{s}^{-1} \text{MPa}^{-1}$), water potential that induces 50% loss of K_{leaf} (Ψ_{50} , MPa); water potential at the turgor loss point (Ψ_{tp} , MPa), osmotic potential at full hydration (Π , MPa), leaf capacitance on a volume basis ($C_{\text{leaf-RWC}}$, %RWC MPa^{-1}) and leaf capacitance on a leaf area basis (C_{leaf} , $\text{mmol m}^{-2} \text{MPa}^{-1}$) for foliage samples obtained in the upper crowns of loblolly pine trees, growing under ambient carbon dioxide concentration, elevated and/or N-fertilized conditions

	Ambient–non-fertilized	Ambient–fertilized	Elevated–non-fertilized	Elevated–fertilized	ANOVA		
					CO ₂	N	CO ₂ × N
Max. K_{leaf}	6.38 ± 0.48	5.95 ± 0.51	5.48 ± 0.36	4.63 ± 0.27	0.035	0.014	0.104
Ψ_{50}	−0.91 ± 0.014	−0.92 ± 0.17	−1.01 ± 0.19	−0.73 ± 0.11	ns	ns	ns
Ψ_{tp}	−2.22 ± 0.02	−2.27 ± 0.03	−2.34 ± 0.03	−2.40 ± 0.03	0.016	ns	ns
Π	0.96 ± 0.06	1.13 ± 0.07	1.13 ± 0.05	1.21 ± 0.08	0.102	0.114	ns
$C_{\text{leaf-RWC}}$	10.1 ± 0.4	9.4 ± 0.7	8.5 ± 0.4	7.7 ± 0.6	0.041	ns	ns
C_{leaf}	771 ± 45	644 ± 59	641 ± 53	544 ± 35	0.030	ns	ns

Analysis of variance (ANOVA) probability values for carbon dioxide concentration (CO₂) and N-fertilization treatment (N) are also shown (the probability level $P < 0.15$ was considered to indicate a trend).

ns, not significant

In 2007, climate along the eastern seaboard of the United States was characterized by a long summer drought with only traces of precipitation from July through September (Fig. 3). Because the rooting zone is very shallow (~35 cm; Oren *et al.* 1998a), tree water uptake during a summer without significant rain events causes soil moisture to decline quickly. In 2007, extractable moisture from the

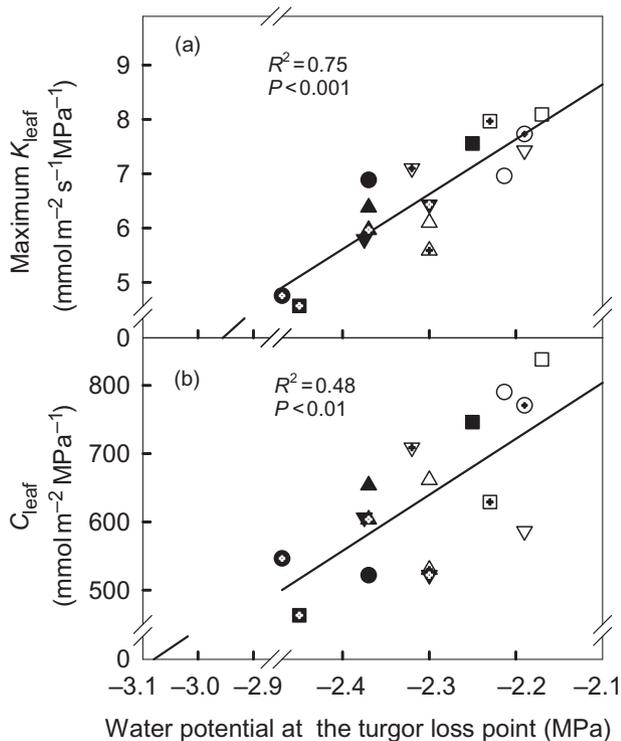


Figure 2. (a) Maximum leaf hydraulic conductance (max. K_{leaf}) and (b) leaf capacitance in relation to the water potential at the turgor loss point (Ψ_{tp}) of loblolly pine trees growing under CO₂^a, CO₂^c and/or N-fertilized conditions. Each point represents samples taken from either the non-fertilized or N-fertilized half of a plot. Symbols are as in Fig. 1. Extrapolations of the regression lines to zero K_{leaf} and zero C_{leaf} are also shown.

upper 30 cm was nearly exhausted by early August, and remained so until sizable rain events resumed in late October (Fig. 3). The seasonal pre-dawn Ψ_{leaf} decreased by 1.0 MPa from its highest in winter to its lowest following two dry months (<35 mm), averaging −1.31 MPa between May and October (Table 2). The minimum midday Ψ_{leaf} , which averaged −2.34 MPa throughout the season (Table 2, Fig. 3), never fell below −2.6 MPa. At a given sampling date neither leaf water potential variable differed between treatments ($P > 0.21$). However, there was a date effect on Ψ_{leaf} (Table 3) because from May to September, pre-dawn and midday Ψ_{leaf} decreased by −0.33 MPa and −0.18 MPa, respectively.

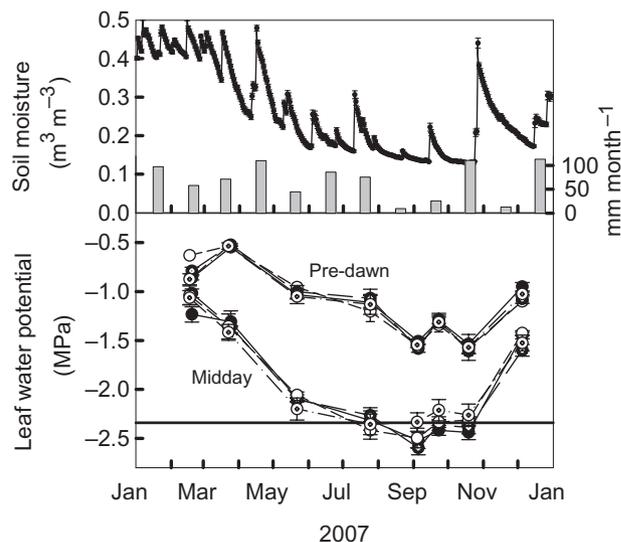


Figure 3. Seasonal course of soil moisture and monthly precipitation and seasonal variation of leaf water potentials (Ψ_{leaf}) for CO₂^a (open symbols), CO₂^a × N fertilization (open-crossed symbols), CO₂^c (closed symbols) and CO₂^c × N fertilization (closed-crossed symbols) loblolly pine trees. Soil moisture content data are shown for all treatments combined. The horizontal line in the lower panel represents the seasonal minimum Ψ_{leaf} across treatments (−2.34 MPa).

Table 2. Mean seasonal (between May and October) leaf water potential (Ψ_{leaf} , MPa) at midday (minimum) and at pre-dawn, mean seasonal leaf water potential at reference stomatal conductance ($\Psi_{\text{leaf-ref}} = \Psi_{\text{leaf}}$ at $g_{s\text{-ref}}$), daily percent loss of leaf hydraulic conductance at minimum Ψ_{leaf} (PLK_{leaf}), daily percent loss of leaf hydraulic conductance at $\Psi_{\text{leaf-ref}}$ ($\text{PLK}_{\text{leaf-ref}}$) and mean seasonal leaf time constant (τ , min) for foliage samples obtained in the upper crowns of loblolly pine trees, growing under CO₂^a (ambient), CO₂^e (elevated) and/or N-fertilized conditions

	Ambient–non-fertilized	Ambient–fertilized	Elevated–non fertilized	Elevated–fertilized
Pre-dawn Ψ_{leaf}	-1.32 ± 0.04	-1.32 ± 0.05	-1.33 ± 0.02	-1.27 ± 0.03
Minimum Ψ_{leaf}	-2.33 ± 0.05	-2.26 ± 0.08	-2.39 ± 0.05	-2.36 ± 0.06
$\Psi_{\text{leaf-ref}}$	-1.41 ± 0.09	-1.50 ± 0.07	-1.53 ± 0.07	-1.59 ± 0.04
PLK_{leaf}	70 ± 4	71 ± 3	72 ± 3	77 ± 5
$\text{PLK}_{\text{leaf-ref}}$	24 ± 2	33 ± 3	34 ± 1	42 ± 4
$\tau = C_{\text{leaf}}/K_{\text{leaf}}$	7.0 ± 0.7	8.2 ± 1.6	9.9 ± 1.15	16.5 ± 1.3

Diurnal measurements showed a typical pattern of g_s increasing in the early morning once sunlight reached the foliage and then declining from late morning (Fig. 4). Similar trends were observed in both sap flow-based crown canopy conductance (G_s) and porometry-based g_s . At the end of May, CO₂^e decreased the daily maximum g_s by 31% ($P = 0.018$, Fig. 4) but, because in July and September CO₂^e had no effect on g_s ($P > 0.55$), CO₂^e had no significant effect on g_s ($P = 0.19$) when assessed over all measurement dates. There was no CO₂^e × N interaction ($P = 0.85$) on g_s . In this study N did not significantly affect g_s ($P = 0.38$). There was no consistent relationship between g_s and Ψ_{leaf} . Unlike the peaking pattern described for g_s , Ψ_{leaf} declined continuously during the day (Fig. 4).

Compared with trees growing under CO₂^a, trees growing under CO₂^e tended to have lower K_{leaf} at high Ψ_{leaf} and lower g_s at low D and showed reduced hydraulic sensitivity to Ψ_{leaf} and reduced g_s sensitivity to D (Fig. 5a,b). Using the diurnal relationships between Ψ_{leaf} and D , we were able to calculate Ψ_{leaf} at $g_{s\text{-ref}}$ taken as the Ψ_{leaf} at $D = 1$ kPa (hereafter, $\Psi_{\text{leaf-ref}}$). Similarly, $K_{\text{leaf-ref}}$ (K_{leaf} at $D = 1$ kPa) was calculated from $\Psi_{\text{leaf-ref}}$ and the relationships between K_{leaf} and Ψ_{leaf} (Fig. 1). There was a date effect on $\Psi_{\text{leaf-ref}}$ (Table 3), generated by a -0.31 MPa drop from May to September. There was a marginal decrease in the $\Psi_{\text{leaf-ref}}$ under CO₂^e (Table 3). Between treatments, the sensitivity of K_{leaf} to Ψ_{leaf}

increased linearly with $K_{\text{leaf-ref}}$ (Fig. 5c). We also tested whether the coefficients from Equation 2, used on g_s in all measurement days, conformed to the theoretical expectations of the response of g_s to D (Oren *et al.* 1999). In all treatments g_s showed an expected sensitivity to D that was proportional to $g_{s\text{-ref}}$. The slope was equal to 0.64, similar to the general slope of 0.60 ($P = 0.13$; Fig. 5d).

With rapidly decreasing pre-dawn Ψ_{leaf} and soil moisture over the growing season, a date effect was apparent with a sharp decrease in $g_{s\text{-ref}}$ (Table 3; Fig. 6). There was no N effect on $g_{s\text{-ref}}$ (Table 3). The decline in $g_{s\text{-ref}}$ associated with a decline in soil moisture was more pronounced ($P = 0.02$, slope analysis) in the CO₂^a plots (50%) than in the CO₂^e plots (33%).

Values of field K_{leaf} were estimated using the relationships between Ψ_{leaf} and K_{leaf} established from laboratory measurements (Fig. 1) and Ψ_{leaf} measured in the field (Fig. 4). The percent loss of needle hydraulic conductance at the lowest measured Ψ_{leaf} (PLK_{leaf}) and at $\Psi_{\text{leaf-ref}}$ ($\text{PLK}_{\text{leaf-ref}}$) were calculated by comparing K_{leaf} at pre-dawn to either K_{leaf} at midday or to $K_{\text{leaf-ref}}$, respectively. Averaged across all days, $\text{PLK}_{\text{leaf-ref}}$ increased by ~10% under CO₂^e, whereas PLK_{leaf} at minimum Ψ_{leaf} increased marginally by ~5% (Tables 2 & 3). N marginally increased $\text{PLK}_{\text{leaf-ref}}$ (~8%), and there was no CO₂^e × N interaction on PLK_{leaf} or on $\text{PLK}_{\text{leaf-ref}}$. Based on these field K_{leaf} and the calculated capacitance (Table 1), we

Table 3. Analysis of variance probability values for carbon dioxide concentration (CO₂), N-fertilization treatment (N) and date of measurements (date) on pre-dawn and minimum (midday) leaf water potential (Ψ_{leaf}), reference stomatal conductance ($g_{s\text{-ref}} = g_s$ at $D = 1$ kPa), leaf water potential at reference stomatal conductance ($\Psi_{\text{leaf-ref}} = \Psi_{\text{leaf}}$ at $g_{s\text{-ref}}$), daily percent loss of leaf hydraulic conductance at minimum leaf water potential (PLK_{leaf}), daily percent loss of leaf hydraulic conductance at $\Psi_{\text{leaf-ref}}$ ($\text{PLK}_{\text{leaf-ref}}$), and the leaf time constant (τ)

Effect	d.f.	Pre-dawn Ψ_{leaf}	Minimum Ψ_{leaf}	$g_{s\text{-ref}}$	$\Psi_{\text{leaf-ref}}$	PLK_{leaf}	$\text{PLK}_{\text{leaf-ref}}$	τ
CO ₂	1	ns	ns	ns	0.063	0.051	0.012	0.036
N	1	ns	ns	ns	ns	ns	0.075	<0.001
CO ₂ × N	1	ns	ns	ns	ns	ns	ns	0.013
Date	2	0.001	0.004	<0.001	0.03	0.022	0.017	0.059
CO ₂ × date	2	ns	ns	0.002	ns	ns	ns	ns
N × date	2	ns	0.134	ns	ns	ns	ns	ns
CO ₂ × N × date	2	ns	ns	ns	ns	ns	ns	ns

The probability level $P < 0.15$ was considered to indicate a trend. ns, not significant; d.f., degrees of freedom.

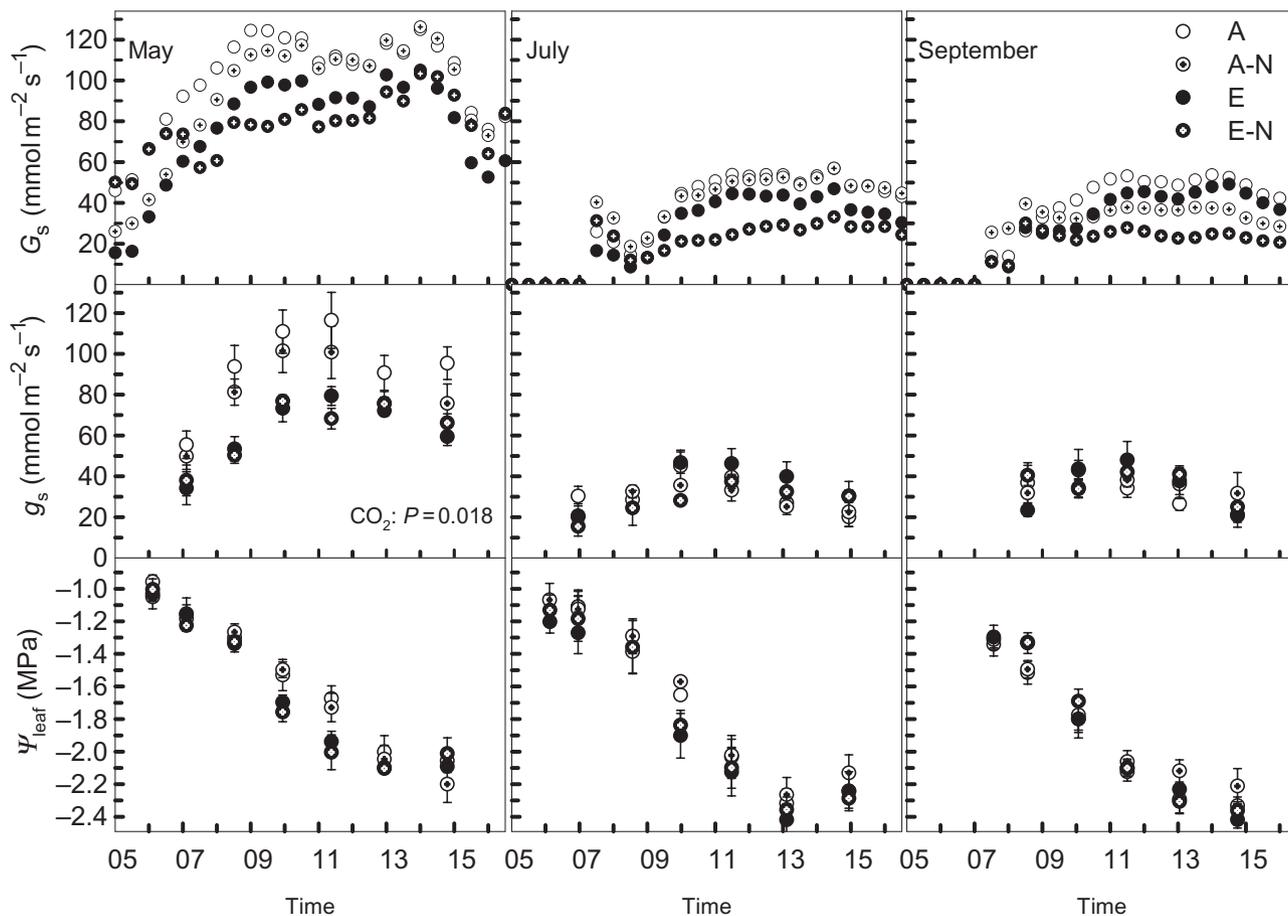


Figure 4. Diurnal canopy stomatal conductance (G_s), stomatal conductance (g_s) and needle water potentials (Ψ_{leaf}) at the beginning (May), in the middle (July) and at the end of the growing season (September) in CO_2^a (A), $\text{CO}_2^a \times \text{N}$ -fertilization (A-N), CO_2^c (E) and $\text{CO}_2^c \times \text{N}$ -fertilization (E-N) trees.

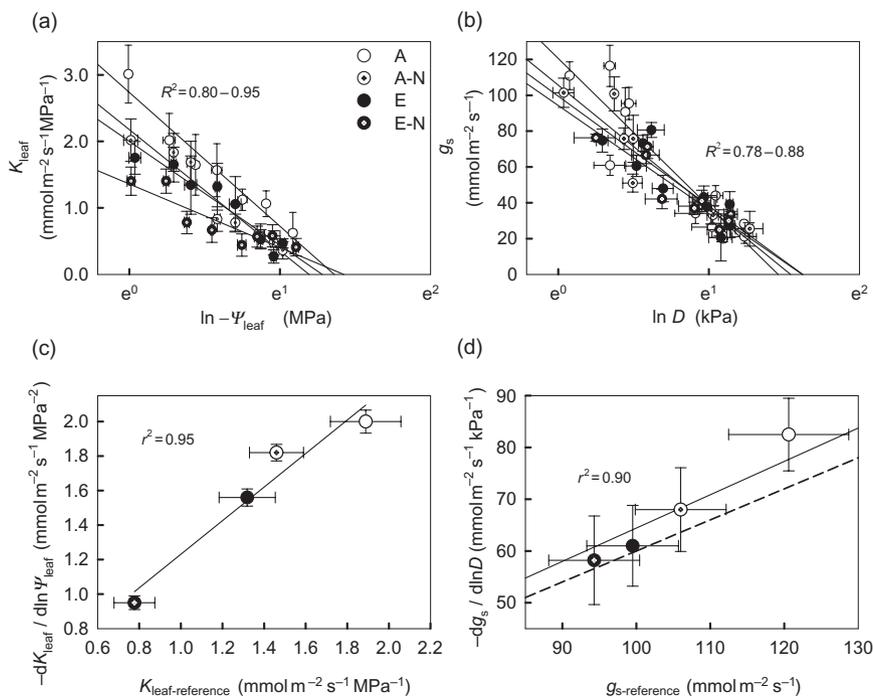


Figure 5. (a) Leaf hydraulic conductance (K_{leaf}) versus the natural logarithm of leaf water potential (Ψ_{leaf}), and (b) stomatal conductance (g_s) versus the natural logarithm of air vapour pressure deficit (D). (c) Slope of the response of K_{leaf} to the natural logarithm of Ψ_{leaf} versus $K_{\text{leaf-reference}}$ (K_{leaf} at $D = 1$ kPa) and (d) slope of the response of g_s to the natural logarithm of D versus g_s reference (g_s at $D = 1$ kPa). Dotted line in (d) represents the theoretical slope of 0.6 (Oren *et al.* 1999).

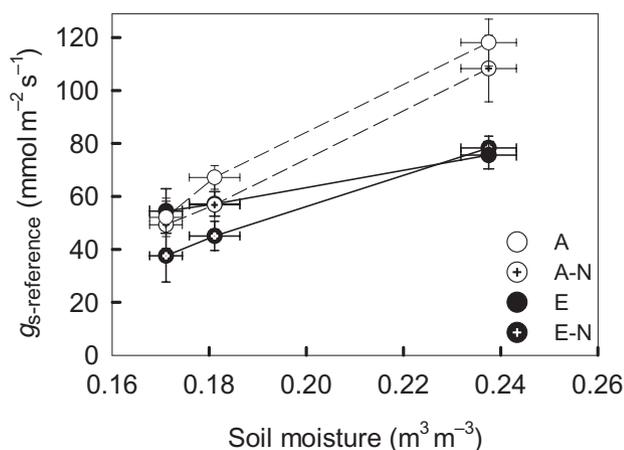


Figure 6. Effect of soil moisture on reference stomatal conductance ($g_{s\text{-reference}}$) in CO₂^a (A), CO₂^a × N-fertilization (A-N), CO₂^e (E) and CO₂^e × N-fertilization (E-N) trees.

estimated the time constant ($\tau = C_{\text{leaf}}/K_{\text{leaf}}$) of water flow in the leaf. The time constant represents the time required for Ψ_{leaf} to reach 63% of its steady state value after a step change in transpiration. Mean τ varied from 7 min in non-fertilized trees growing under CO₂^a to 16 min in fertilized trees growing under CO₂^e (Tables 2 & 3) and there was a significant CO₂^e × N interaction on τ (Table 3).

The $g_{s\text{-ref}}$ obtained from the four treatments in the three measurement periods increased linearly and proportionally with $K_{\text{leaf-ref}}$ (Fig. 7a). Similarly, $g_{s\text{-ref}}$ increased with treatment and seasonally based increases in whole tree hydraulic conductance (K_t , calculated from the relationship between leaf transpiration and Ψ_{leaf} ; Fig. 7b), but the reduction in $g_{s\text{-ref}}$ was less than proportional to the reduction in K_t (i.e. changes in $g_{s\text{-ref}}$ were more sensitive to changes in $K_{\text{leaf-ref}}$). Between May and September, K_t declined by 56% in trees growing under CO₂^a, and by 41% in trees growing under CO₂^e. There was a linear relationship between leaf hydraulic resistance ($1/K_{\text{leaf-ref}}$) and whole tree hydraulic resistance ($1/K_t$) (Fig. 7c). The contribution of $1/K_{\text{leaf-ref}}$ to $1/K_t$ decreased with $1/K_{\text{leaf-ref}}$, which was affected by treatments and changed seasonally (Fig. 7c). Therefore, $K_{\text{leaf-ref}}$ exerted a greater constraint on whole-plant water transport under CO₂^a than under CO₂^e, and mostly in May, when $K_{\text{leaf-ref}}$ was high (or $1/K_{\text{leaf-ref}}$ was low), than in July or September. As a consequence, the contribution of the woody parts (root-to-branch hydraulic resistance) to the whole tree hydraulic resistance increased from ~50% in May to ~70% in July–September ($P = 0.007$).

Across treatments, $\Psi_{\text{leaf-ref}}$ was higher (less negative) but strongly correlated with Ψ_{tp} ($P < 0.001$; Fig. 8), indicating that stomata tended to begin closing before the leaf reached its turgor loss point. The minimum measured field Ψ_{leaf} on the other hand were similar to Ψ_{tp} ($P = 0.21$).

DISCUSSION

There have been no studies on the effects of CO₂^e and N availability on leaf hydraulics and their downstream effect

on gas exchange. Our study revealed that CO₂^e and N had a significant effect on maximum K_{leaf} , and that both treatment-induced seasonal variation in $K_{\text{leaf-ref}}$ translated to similar variation in $g_{s\text{-ref}}$ and, thus, gas exchange. The similarity of trends in liquid- and gas-phase conductances

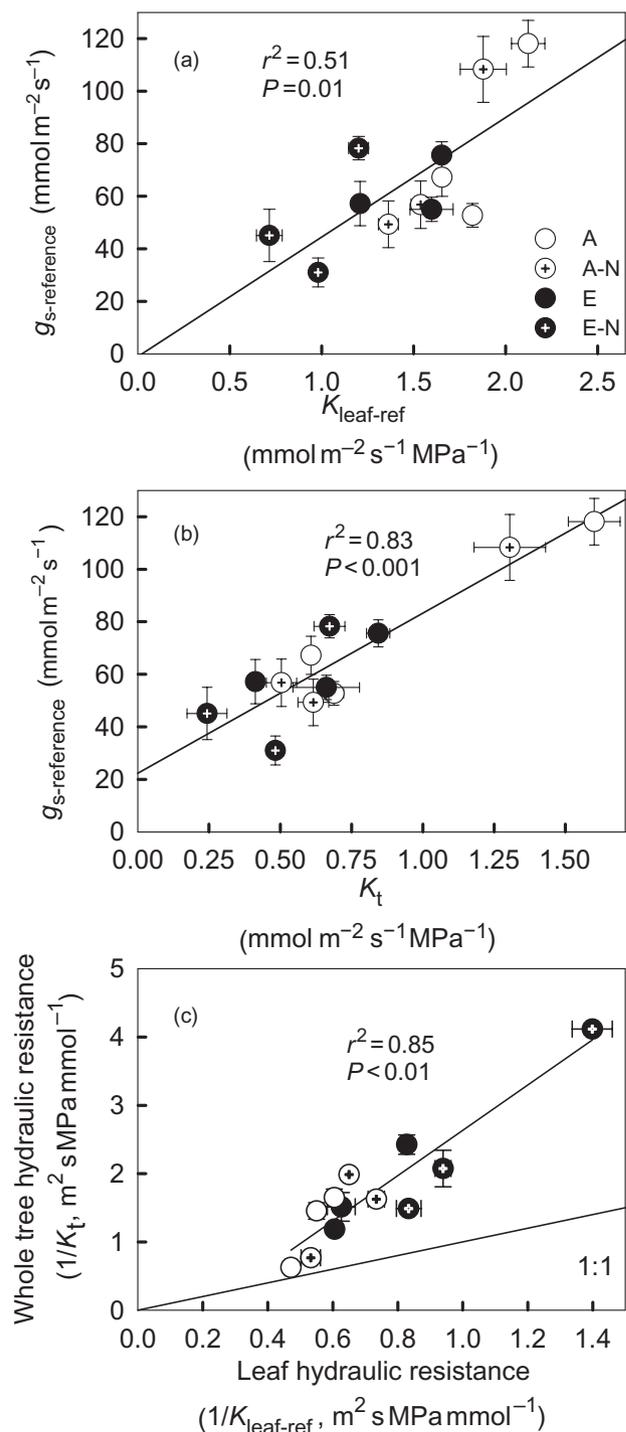


Figure 7. Reference stomatal conductance ($g_{s\text{-ref}}$) as a function of (a) reference leaf hydraulic conductance ($K_{\text{leaf-ref}}$) and (b) whole tree hydraulic conductance (K_t). (c) Whole tree hydraulic resistance ($1/K_t$) as a function of reference leaf hydraulic resistance ($1/K_{\text{leaf-ref}}$).

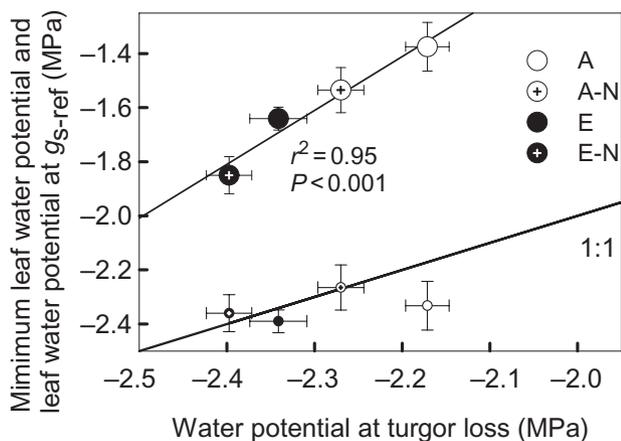


Figure 8. Mean seasonal leaf water potential at maximum stomatal conductance (Ψ_{leaf}) (small symbols) and mean seasonal leaf water potential at reference stomatal conductance ($\Psi_{\text{leaf-ref}} = \Psi_{\text{leaf}}$ at $g_{\text{s-ref}}$) (big symbols) as a function of water potential at the turgor loss point (Ψ_{tp}) in CO_2^{a} (A), $\text{CO}_2^{\text{a}} \times \text{N}$ -fertilization (A-N), CO_2^{e} (E) and $\text{CO}_2^{\text{e}} \times \text{N}$ -fertilization (E-N) trees.

implies that functional adjustments under CO_2^{e} and N contributed to homeostasis in the operation of the hydraulic regulatory systems that were driven by $K_{\text{leaf-ref}}$ and the point of turgor loss. The results of this study show that, in loblolly pine, it required a structural change of the hydraulic pathway to produce stomatal closure under CO_2^{e} . This contrasts with the current view that CO_2^{e} directly causes stomatal closure (Ainsworth & Rogers 2007).

Effect of elevated CO_2 and N-fertilization on K_{leaf} , C_{leaf} and Ψ_{tp}

As shown in a study on needle ‘vulnerability curves’ of *Pseudotsuga menziesii* (Woodruff *et al.* 2007), K_{leaf} was highly sensitive to Ψ_{leaf} (Fig. 1). However, compared with trees growing under CO_2^{e} , trees growing under CO_2^{a} showed higher maximum K_{leaf} and higher sensitivity of K_{leaf} to Ψ_{leaf} . The differences observed in maximum K_{leaf} among treatments was not associated with differences in Ψ_{50} , in contrast to the pattern observed in other tree organs (Tyree & Zimmermann 2002; Domec *et al.* 2008). This lack of trade-off between hydraulic efficiency and hydraulic safety points to the more complex role K_{leaf} plays in water transport, and to the refilling dynamics of leaves. The mechanism explaining the reduction in K_{leaf} with Ψ_{leaf} likely involves cavitation-induced embolism (Johnson *et al.* 2009), although we can not rule out that needle xylem might have collapsed at lower Ψ_{leaf} (Cochard *et al.* 2004; Brodribb & Holbrook 2005). The decrease in maximum K_{leaf} , C_{leaf} , and their lower sensitivities to Ψ_{leaf} in trees growing under CO_2^{e} and higher soil fertility, as hypothesized (first hypothesis), may be partly related to the development of conducting tissue with different hydraulic characteristics (Centritto *et al.* 1999). Treatment-induced structural and anatomical adjustments, such as a decrease in the length and diameter

of leaf tracheids (Prior *et al.* 1997; Woodruff, Meinzer & Lachenbruch 2008), and probably in the size of connecting pit membranes between adjacent conduits, would reduce K_{leaf} . Such structural modifications may also explain the lower Ψ_{tp} because thick and stiff cell walls have been shown to reduce turgor loss (Marshall & Dumbroff 1999).

Coordination between liquid- and gas-phase leaf conductances

Following our second hypothesis, treatment-induced reductions in $g_{\text{s-ref}}$ were consistent with those in $K_{\text{leaf-ref}}$ (Table 3). However, and in contrast to another study on loblolly pine (Ewers *et al.* 2000), N did not affect $g_{\text{s-ref}}$, although a tendency for reduced g_{s} was observed in May (Fig. 4). The reason for lack of statistical difference may simply be the power of the experiment, owing to the low number of replicates ($n = 4$) and the split-plot N factor. Furthermore, stomata of trees growing under CO_2^{e} and high soil fertility were less sensitive to D than those of trees growing under CO_2^{a} , unfertilized conditions (Fig. 5). These results also showed that the sensitivity of g_{s} to D was related to the variation in $g_{\text{s-ref}}$, meaning that CO_2^{e} and N-fertilization did not affect the relative sensitivity of g_{s} to D , a behaviour consistent with an isohydric regulation of water potential (McNaughton & Jarvis 1991; Oren *et al.* 1999). These results are consistent with the third hypothesis.

Mirroring the stomatal behaviour, the sensitivity of K_{leaf} to Ψ_{leaf} was related to $K_{\text{leaf-ref}}$, as has been found in roots of the same species (Ewers *et al.* 2000). Furthermore, the common patterns in the sensitivity of K_{leaf} to Ψ_{leaf} and g_{s} to D were additional evidence of the strong coordination between liquid- and gas-phase conductances (Fig. 5). Because of this coordinated sensitivity between the liquid- and gas-phase fluxes, treatment-induced differences in $K_{\text{leaf-ref}}$ and $g_{\text{s-ref}}$ have implications to gas exchange on both the short (diurnal) and long (drying cycle) time scales (Domec *et al.* 2009).

Diurnally, the reduction in g_{s} with D is proportional to $g_{\text{s-ref}}$, meaning that on an absolute basis, treatments with higher $g_{\text{s-ref}}$ experienced a greater loss of CO_2 uptake than those with lower $g_{\text{s-ref}}$. The diurnal patterns in K_{leaf} , Ψ_{leaf} and g_{s} provide insight to a possible mechanism involved in the coordination between the liquid- and gas-phase water flows. The patterns indicate that K_{leaf} does not directly control g_{s} . For example, K_{leaf} begins to decline immediately after dawn whereas g_{s} is increasing with light, generating a fast decrease in Ψ_{leaf} even though D is not very high at this time. This sets up the leaf hydraulic system to a state in which g_{s} will respond quickly to increasing D later in the morning, thus regulating minimum Ψ_{leaf} . Indeed, later, g_{s} decreases sharply in response to D whereas K_{leaf} decreases slowly, mirroring the slow decrease in Ψ_{leaf} . The balance between xylem tension and the loss of conductivity has caused several authors to suggest a functional role for cavitation as part of a feedback mechanism linking stomatal regulation to hydraulic conductance and plant water status (Sperry 2000; Meinzer 2002). The diurnal patterns described earlier

suggest that hypersensitive leaf xylem serves to protect the integrity of the upstream woody portion of the hydraulic pathway by causing a rapid stomatal closure before water potential drops in the woody xylem, consistent with the hydraulic segmentation hypothesis (Sperry 1986).

During drying cycles, or seasons in areas where soil moisture becomes progressively limiting, the coordinated sensitivity between the liquid- and gas-phase fluxes coupled with treatment-induced differences in $K_{\text{leaf-ref}}$ and $g_{\text{s-ref}}$ have different implications to gas exchange. Lower values of $g_{\text{s-ref}}$ at the beginning of the growing season, and the lower seasonal reduction in $g_{\text{s-ref}}$ in trees growing under CO₂^e and N were related to lower reductions in $K_{\text{leaf-ref}}$ and K_t (Fig. 7). Reduced K_t has seldom been explicitly reported in studies comparing trees growing under CO₂^a and CO₂^e (Bunce & Ziska 1998; Kupper *et al.* 2006). In our study, treatment-induced differences in K_t were to a great extent explained by differences in $K_{\text{leaf-ref}}$ because the short portion of the pathway through the needles constituted a major part (30–50%) of the whole tree hydraulic resistance to water flow ($1/K_t$). Seasonally, the effect of $K_{\text{leaf-ref}}$ on K_t decreased in all treatments (Fig. 7c). In May, $K_{\text{leaf-ref}}$ dominated K_t , whereas in July and September, K_t became less limited by $K_{\text{leaf-ref}}$, probably because of a large decrease in root and stem hydraulic conductances as soil moisture declined (Hacke *et al.* 2000; Domec *et al.* 2009).

The treatment-induced differences in the sensitivity of g_s to D remained proportional to $g_{\text{s-ref}}$ on both diurnal and seasonal time scales. However, treatment effects on $g_{\text{s-ref}}$ changed over the season as the soil dried (Fig. 6). As a result of a greater sensitivity of $g_{\text{s-ref}}$ to soil moisture under CO₂^a, the differences in g_s observed early in the season among treatments disappeared by the end of the season even though soil moisture was similar in all treatments. Indicative of a severe soil water depletion, values pre-dawn Ψ_{leaf} fell below -1.5 MPa, which has already been reported for this site during a previous dry year (Ellsworth 1999). Although pre-dawn Ψ_{leaf} was similar in all treatments during the growing season (Fig. 3), this might not reflect access to the same amount of water as the soil dries (Donovan, Richards & Linton 2003). The lower sensitivity to soil drying of $g_{\text{s-ref}}$ under CO₂^e might reflect deeper penetration of fine roots (King *et al.* 2001; Pritchard *et al.* 2008), accessing soil moisture beyond the measurement depth, as reflected in a lesser seasonal decrease in K_t of CO₂^e trees (Fig. 7b). Regardless of whether CO₂^e trees had access to more moisture at the end of the year, the differences in g_s observed early in the season but not late, might also explain differences among studies on the effect of CO₂^e on g_s .

Consequences of adjustments in turgor loss, K_{leaf} and time constant on tree water economy

The minimum Ψ_{leaf} observed in field measurements corresponded across treatments to the Ψ_{leaf} at turgor loss (Fig. 8). Leaves of trees in treatments (ambient and unfertilized) with higher (less negative) Ψ_{tp} began regulating stomata at

a higher Ψ_{leaf} . It is possible that the strong linear correlation across treatment between Ψ_{tp} and $\Psi_{\text{leaf-ref}}$ means that declining K_{leaf} was related to declining cell turgor in the leaf prior to the onset of cavitation in the needle xylem (Brodrribb & Holbrook 2005; Woodruff *et al.* 2007). As result, the rate of decrease in Ψ_{leaf} is greater under CO₂^a and without fertilization between the time in which stomata begin to respond to D and the time in which Ψ_{leaf} reaches its minimum value. Moreover, in trees growing under CO₂^e, more negative Ψ_{tp} allowed stomata to remain open at lower Ψ_{leaf} , which decreased the effect of drought on $g_{\text{s-ref}}$. However, lower K_{leaf} and lower sensitivity to Ψ_{leaf} under CO₂^e induced a steeper drop in Ψ_{leaf} at $g_{\text{s-ref}}$ relative to the decrease in turgor loss points, and therefore increased the percent loss of needle hydraulic conductance (Table 2).

Another consequence of the differences in C_{leaf} and maximum K_{leaf} among treatments was reflected in the time constants (Table 2). The time constant of the needles growing under ambient conditions represented around half the time constant of the soil–trunk–leaf compartment calculated at the same site (Phillips *et al.* 1997), implying that woody tissue and leafy tissue both exhibit a similar time constant. An important consequence of the increase in time constant in trees growing under CO₂^e and N is that, under dry conditions, these trees would have less time to restore their equilibrium water content and could experience a greater degree of leaf dehydration.

The results from this study are useful in a number of ways. Firstly, we show that in species such as loblolly pine, CO₂^e and N-fertilization alter the hydraulic pathway, most likely structurally, affecting the liquid phase transport, thus reducing stomatal conductance. This contrasts with previous studies focusing on a direct stomatal response to CO₂^e. We also show evidence that the hydraulic changes allow plants growing under CO₂^e to reduce stomatal conductance less under drought than plants growing under current CO₂. These conclusions will inform models employed to predict ecosystem responses to climate change, and biosphere-atmosphere interactions under current and future climates. Secondly, the results show that clear effects of CO₂ and N-fertilization treatments on g_s can be observed only under certain conditions. This may explain some of the contradictions in published responses. Furthermore, stomatal response to elevated CO₂^e has been investigated in two types of studies. One type exposes leaves grown under CO₂^a to CO₂^e, detecting clear stomatal closure in most species but conifers (Murthy *et al.* 1996; Saxe, Ellsworth & Heath 1998). The other type grows plants under CO₂^e and compares their g_s with that of plants grown under CO₂^a. In broadleaved species, studies of this type find similar responses to the first type, but the responses in conifers are more variable, with a tendency for the longer studies to find an effect (Medlyn *et al.* 2001; Wang *et al.* 2005). If the response we describe is particularly important in conifers, the length of exposure to CO₂^e necessary to produce a reduction in g_s would depend on the time it takes the species to replace a large proportion of pretreatment foliage with foliage produced under CO₂^e.

ACKNOWLEDGMENTS

The Duke FACE research site was supported by the United States Department of Energy (DOE) through the Office of Biological and Environmental Research (BER) Terrestrial Carbon Processes (TCP) programme (DE-FG02-95ER62083). Support for S. Palmroth also came from the National Science Foundation (NSF-EAR 0628342).

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Received 17 March 2009; received in revised form 25 May 2009; accepted for publication 26 May 2009

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Figure S1. Leaf hydraulic conductance (K_{leaf}) in relation to leaf water potential (Ψ_{leaf}) for foliage samples obtained from the upper crowns of loblolly pine trees growing under ambient conditions. Closed symbols are from dehydration curves obtained in the laboratory (see Eq. 1). Open symbols are from field measurements using the evapourative flux method and calculated as $K_{\text{leaf}} = v/v_o E/(\Psi_{\text{stem}} - \Psi_{\text{leaf}})$, where v and v_o are respectively the kinematic viscosities of water at the measured leaf temperature and at 20 °C, E is the transpiration rate ($\text{mmol m}^{-2} \text{s}^{-1}$) measured with a LI-6400 portable photosynthesis system, and Ψ_{stem} is the stem water potential estimated from non-transpiring covered shoots.

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