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Two tropical conifers show strong growth and water-use efficiency responses to altered CO_2 concentration

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• **Background and Aims** Conifers dominated wet lowland tropical forests 100 million years ago (MYA). With a few exceptions in the Podocarpaceae and Araucariaceae, conifers are now absent from this biome. This shift to angiosperm dominance also coincided with a large decline in atmospheric CO_2 concentration (c_a). We compared growth and physiological performance of two lowland tropical angiosperms and conifers at c_a levels representing pre-industrial (280 ppm), ambient (400 ppm) and Eocene (800 ppm) conditions to explore how differences in c_a affect the growth and water-use efficiency (WUE) of seedlings from these groups.
• **Methods** Two conifers (*Araucaria heterophylla* and *Podocarpus guatemalensis*) and two angiosperm trees (*Tabebuia rosea* and *Chrysophyllum cainito*) were grown in climate-controlled glasshouses in Panama. Growth, photosynthetic rates, nutrient uptake, and nutrient use and water-use efficiencies were measured.
• **Key Results** *Podocarpus* seedlings showed a stronger (66 %) increase in relative growth rate with increasing c_a relative to *Araucaria* (19 %) and the angiosperms (no growth enhancement). The response of *Podocarpus* is consistent with expectations for species with conservative growth traits and low mesophyll diffusion conductance. While previous work has shown limited stomatal response of conifers to c_a, we found that the two conifers had significantly greater increases in leaf and whole-plant WUE than the angiosperms, reflecting increased photosynthetic rate and reduced stomatal conductance. Foliar nitrogen isotope ratios ($\delta^{15}N$) and soil nitrate concentrations indicated a preference in *Podocarpus* for ammonium over nitrate, which may impact nitrogen uptake relative to nitrate assimilators under high c_a.
• **Significance** Podocarps colonized tropical forests after angiosperms achieved dominance and are now restricted to infertile soils. Although limited to a single species, our data suggest that higher c_a may have been favourable for podocarp colonization of tropical South America 60 MYA, while plasticity in photosynthetic capacity and WUE may help account for their continued persistence under large changes in c_a since the Eocene.

Key words: *Araucaria heterophylla*, *Podocarpus guatemalensis*, *Tabebuia rosea*, *Chrysophyllum cainito*, tropical conifer, angiosperm dominance, water-use efficiency, relative growth rate, nitrate assimilation, elevated CO_2.

INTRODUCTION

Tropical lowland forests are overwhelmingly dominated by angiosperm trees, representing a major transition from the coniferous forests that began in the Cretaceous, and was largely complete by the Paleogene 66 million years ago (MYA). The current dominance of angiosperms in lowland tropical forest is generally considered to be at least in part a consequence of the inherently slow growth of conifer seedlings, which results in competitive displacement by angiosperms unless light or soil nutrient availability limits realized growth rates (Bond, 1989; Midgley and Bond, 1991; Coomes and Bellingham, 2011; Brodribb *et al*., 2012). In turn, the slow growth of conifer seedlings (but not necessarily larger individuals), can be attributed to greater leaf mass per unit area and correspondingly lower mass-based photosynthetic rates, vascular constraints on leaf size, and narrow conduit diameters in conifer wood that limit maximum conductance rates (Bond, 1989; Lusk, 2011). The importance of these constraints diminishes at high latitude and elevation, where smaller conduit diameters reduce the risk of freeze–thaw embolism, which can strongly impact stem conductivity (Feild and Brodribb, 2001; Lusk, 2011).

Despite these apparent limitations on the competitive ability of lowland tropical conifers, two families, Araucariaceae and Podocarpaceae, remain locally important components of some forests, particularly on nutrient-poor soils (Dalling *et al*., 2011; Enright and Jaffré, 2011; Kitayama *et al*., 2011). Furthermore, recent work suggests that podocarps colonized tropical latitudes only in the last 60 million years, and after the diversification of major angiosperm lineages (Jaramillo *et al*., 2011*a*, *b*; Morley, 2011; Quiroga *et al*., 2016). Ecological studies of extant tropical conifers therefore provide an opportunity to further explore the physiological characteristics of conifers that permit coexistence with angiosperms under equitable tropical climates. In addition, growth of these species under a range of atmospheric CO_2 concentrations (c_a) allows us to explore how the relative

growth advantage of angiosperms may have changed as c_a declined at least 2-fold over the past 60 million years (Beerling and Royer, 2011; Franks *et al.*, 2014), and under conditions of contemporary climate change.

Here we examine the responses of seedlings of two tropical lowland conifers and angiosperms grown under c_a representing pre-industrial, ambient and Eocene levels (280, 400 and 800 μmol mol^{-1} respectively) (Franks *et al.*, 2014). We use these data to test two hypotheses. First, we examine whether tropical conifers show a stronger photosynthetic and growth response to variation in c_a than angiosperms. This responsiveness may be mediated by mesophyll conductance. Niinemets *et al.* (2011) argued that mesophyll conductance, the capacity of CO_2 to diffuse from sub-stomatal cavities to carboxylation sites inside chloroplasts, represents a larger fraction of the total limitation of diffusion to photosynthesis in sclerophyllous plants, where thick cell walls strongly limit CO_2 conductance. The limitation imposed on photosynthesis by mesophyll conductance can be partly overcome by increased c_a irrespective of stomatal conductance. Although Niinemets *et al.* (2011) emphasized that mesophyll conductance limitation would particularly affect evergreens that are adapted to drought, similar arguments apply to coniferous species that are adapted to low-nutrient environments and that also build tough, long-lived leaves.

Second, we test whether tropical angiosperms are better able to optimize water-use efficiency than tropical conifers. Brodribb *et al.* (2009) argued that major clades of plants differ in water-use efficiency, representing a slow acquisition over 450 million years of the complex mechanisms that regulate guard cell apertures and balance mesophyll CO_2 concentrations and transpiration losses. In support of their argument, Brodribb *et al.* (2009) cite meta-analyses that suggest different CO_2 sensitivity of gas exchange parameters of angiosperms and conifers, and show experimentally that the aperture of angiosperm stomata, but not conifer stomata, are reduced significantly in response to c_a of 600 μmol mol^{-1} (but see Ward *et al.*, 2013). Differences in the stomatal responses reported by Brodribb *et al.* (2009) appear to be a consequence of the evolution of Ca^{2+}-dependent stomatal signalling (Brodribb and McAdam, 2013). Nonetheless, stomatal conductance responses can be affected by plant age and by the duration of the study (Medlyn *et al.* 2001), while instantaneous changes in c_a miss the potential for photosynthetic acclimation to CO_2, which may depend on the c_a under which leaves develop. In this experiment we were able to assess growth, allocation and physiological responses after plants were grown under the same c_a for 6–7 months.

MATERIALS AND METHODS

Study species

In February 2010, seeds of the two angiosperm species, *Tabebuia rosea* (Bignoniaceae) and *Chrysophyllum cainito* (Sapotaceae), were collected from lowland forests in the Panama Canal area, and germinated in a shadehouse at the Smithsonian Tropical Research Institute in Gamboa, Panama (9°07′ N, 79°42′ W). Seedlings of *Araucaria heterophylla* (Araucariaceae) were purchased from a commercial nursery in Cerro Punta, Chiriqui, Panama, in early March 2010 and transferred to Gamboa. Seedlings were grown for 3 months under 30 % full sun prior to starting the experiment. Recently germinated seedlings of *Podocarpus guatemalensis* (Podocarpaceae) were collected from lowland forest at Playa Hermosa, Coiba Island, Panama (7°10′ N, 81°32′ W) and transplanted to pots in Gamboa in January 2010. They were grown under the same shadehouse conditions for 5 months before the experiment began. Henceforth, species are referred to by genus name. Species were selected to match, as far as possible, seed mass and shade tolerance. *Chrysophyllum* and *Podocarpus* are both relatively shade-tolerant, maintaining seedling banks beneath closed canopy forest (J. W. Dalling, pers. obs.). Seedlings of *Tabebuia* are relatively light-demanding (Kitajima, 2002), while *Araucaria* seedlings are likely to be at least moderately shade-tolerant based on the ecology of congeneric species (Enright, 1982; Duarte *et al.*, 2002). *Chrysophyllum*, *Podocarpus* and *Tabebuia* also co-occur in forests of northern Coiba Island (Perez *et al.*, 1996), and are widely distributed through Central America. No *Araucaria* species are native to Central America; *A. heterophylla* is native to Norfolk Island in the tropical South Pacific. It differs from the other species in having imbricate scale-like leaves, resulting in less efficient light capture in forest understories (Coomes and Bellingham, 2011). Both *Tabebuia* and *Chrysophyllum* have been used extensively in previous ecophysiological work in Panama (e.g. Cernusak *et al.*, 2008, 2011). All four genera form arbuscular mycorrhizal associations (Dickie and Holdaway, 2010).

Seedling growth experiment

In May 2010, 24 seedlings of each species were transplanted into 19-L pots containing a 1:1 by volume mix of soil and rice husks (added to improve drainage). The soil was collected from an area formerly under lowland tropical forest and now in a private orchard, allowing the removal of a large quantity of soil for experimentation. Soil was air-dried (~27 °C) and passed through a 10-mm sieve. Details of the soil used in the experiment and its analysis are described in Dalling *et al.* (2013). Briefly, the soil has a high concentration of exchangeable phosphate (17·8 mg P kg^{-1}), based on extraction from anion exchange membranes, and a high effective cation exchange capacity (53·2 $cmol_c$ kg^{-1}) based on extraction in 0·1 M $BaCl_2$. Total KCl-extractable inorganic N availability is 62·5 mg N kg^{-1}, but was reduced by the addition of the rice husks (Dalling *et al.*, 2013), counteracting the observation of excess N mineralization observed in un-amended soils (e.g. Johnson *et al.*, 1995; Ross and Hales, 2003). At the time of seedling transplant, three additional seedlings of each species were harvested to determine initial dry mass and leaf area.

Eight pots per species were randomly assigned to one of three glasshouses maintained at pre-industrial (280 ppm), ambient (400 ppm) and twice-ambient (800 ppm) atmospheric CO_2 concentration (Fig. 1). Transplanted seedlings were grown for 189–202 d under the treatment CO_2 concentrations before harvest. The air in the treatment chambers was well mixed using four 18-inch floor fans (McMaster-Carr, Robbinsville) in each chamber. Control of CO_2 concentrations was based on GMW21D carbon dioxide transmitters (Vaisala, Helsinki) and

a CR-5000 measurement and control system (Campbell Scientific, Logan, UT). For the 800-ppm c_a treatment, injection of pure CO_2 was initiated when the CO_2 concentration in the chamber fell below 795 ppm and was terminated when CO_2 concentration reached 800 ppm. The CO_2 concentration was prevented from overshooting by providing CO_2 in multiple pulses of 2 s interrupted by 5 s without CO_2 injection. To prevent CO_2 concentration from decreasing significantly below 400 ppm due to the photosynthetic activity of plants in the current-ambient CO_2 treatment, during daytime, pulsed CO_2 injections were initiated when CO_2 concentration fell below 380 ppm and terminated when CO_2 concentration reached 400 ppm. For the below-current-ambient CO_2 treatment, whenever CO_2 exceeded 280 ppm, air inside the chamber was sucked through an acrylic glass column (10 cm diameter, 100 cm height) filled with CO_2 absorber up to a height of 40 cm (Sofnolime 812 Mesh, Molecular Products, Boulder) using the air pump and dustbag of an industrial vacuum cleaner (Shop-Vac 125 Gal Peak HP, Novey, Panama). The air pump was switched off when CO_2 concentration reached 275 ppm.

Light conditions were maintained at 50 % full sun using Aluminet Shade Cloth to cover the top of the chambers. The glasshouses were air-conditioned, with the air conditioners programmed to turn on when air temperature exceeded 30 °C. Mean air temperature and humidity, recorded every 15 min during the experiment with a data logger (CR5000; Campbell

FIG. 1. Glasshouse showing soda lime column and vacuum pump in foreground (A), seedling of *Araucaria heterophylla* (B) and (C) seedling of *Podocarpus guatemalensis*.

Scientific, Logan, UT, USA), remained very similar across the three glasshouses (mean daytime temperature range 27·9–28·3 °C; night-time temperature 25·3–26·7 °C; daytime relative humidity 60·9–61·3 %; night-time relative humidity 86·8–89·3 %).

Five additional pots without seedlings were included in each glasshouse to determine soil evaporative water loss for calculations of whole-plant water-use efficiency (WUE_{wp}, g dry mass kg^{-1} water transpired). All experimental pots were watered to field capacity and allowed to drain overnight at the initiation of the experiment. The drain holes at the bases of the pots were then sealed for the duration of the experiment, and the pots were weighed and re-watered to near their initial weight each week, or at shorter intervals, as necessary. Pots were weighed to the nearest 5 g with a 64-kg-capacity balance (Sartorius QS64B; Thomas, Swedesboro, NJ, USA). Cumulative plant water use (CWU) over the course of the experiment was calculated as the sum of pot water loss minus the average sum of water loss from the control pots that did not hold plants. Mean transpiration rate (MTR), calculated as kg m^{-2} leaf area d^{-1}, was calculated following Sheshshayee *et al*. (2005):

$$MTR = \frac{CWU}{(LA_2 + LA_1) \times 0{\cdot}5t} \quad (1)$$

where LA_1 and LA_2 are the initial and final leaf areas respectively, and t is the duration of the experiment.

The WUE_{wp} over the duration of the experiment was calculated for each plant as final dry mass minus initial dry mass plus leaf litter produced divided by its CWU.

During the week preceding harvest, leaf gas exchange was measured between 08:00 and noon on one leaf per plant with a portable photosynthesis system (Li-Cor 6400, Li-Cor Inc., Lincoln, NE, USA). These measurements were made at an irradiance of 1200 µmol photons m^{-2} s^{-1}, supplied by an artificial light source attached to the leaf cuvette (6400-02B LED, Li-Cor Inc.). The environment within the leaf cuvette was controlled to be similar to ambient glasshouse conditions. Leaf temperatures during the measurements were between 28 and 35 °C. Hereafter, we refer to these area-based net photosynthesis rates as A_{max} and to the ratio of intercellular to ambient CO_2 concentration as c_i/c_a. These gas exchange data were also used to calculate leaf-level WUE as net photosynthesis divided by transpiration (WUE_{leaf}, µmol CO_2 $mmol^{-1}$ H_2O). For measurements of *Araucaria*, with its scale-like leaves, a section of shoot was placed inside the 2×3 cm cuvette for the measurement, and then immediately harvested for determination of the amount of leaf area that had been contained within the cuvette. This was accomplished using an automated leaf area meter (LI-3000A, Li-Cor Inc.). The same leaf area meter was used to measure the total leaf area of all seedlings following harvest. In the case of *Araucaria*, however, whole-plant leaf area was underestimated as the minute and recurved leaves meant we could only measure the projected area of individual branches. Calculated values of specific leaf area and net assimilation rate (see below) are nonetheless included for within-species comparison of c_a treatment effects.

Mass of foliage, stems and roots of harvested seedlings was measured after drying for 72 h at 70 °C. A sample of dried mature leaf material from each seedling was ground to a fine powder for chemical and isotopic analyses using a Cyclotec 1093 mill with a 0·5-mm screen (FOSS, Eden Prarie, MN, USA). Foliar N concentrations were determined by automated combustion and thermal conductivity detection on a Thermo Flash EA112 analyser (CE Elantech, NJ, USA) and foliar P by ashing at 550 °C, followed by dissolution in 1 M H_2SO_4, with phosphate detection by automated molybdate colorimetry using a Lachat Quikchem 8500 (Hach Ltd, Loveland, CO, USA). In addition, at harvest time, soil was sampled from each pot containing a seedling, and KCl-extractable nitrate and ammonium concentrations were measured after extraction in 2 M KCl by automated colorimetry using a Lachat Quikchem 8500 (Hach Ltd, Loveland, CO, USA), and available P was determined by extraction in Bray solution (Bray and Kurtz, 1945).

Foliar dry matter was analysed for its stable carbon isotope ($\delta^{13}C$) and stable nitrogen isotope ($\delta^{15}N$) ratios. Analyses were carried out in the Stable Isotope Laboratory of the Smithsonian Tropical Research Institute with an elemental analyser (CE Instruments, Milan, Italy) coupled to an isotope ratio mass spectrometer (Delta V; Thermo Fisher Scientific, Bremen, Germany). The $\delta^{13}C$ and $\delta^{15}N$ values are expressed relative to standards of PeeDee Belemnite and air, respectively. The carbon isotope discrimination ($\Delta^{13}C$) of leaf dry matter was calculated as $\Delta^{13}C = (\delta_a - \delta_p)/(1 + \delta_p)$, where δ_a is the $\delta^{13}C$ of CO_2 in air in each glasshouse and δ_p is the $\delta^{13}C$ of leaf carbon. The δ_a was estimated by growing two species of C_4 plant, *Saccharum spontaneum* and *Portulaca oleracea*, in each glasshouse and outside in the open air. The $\delta^{13}C$ of source CO_2 for plants grown outside was assumed to be –8‰, which was used to calculate the $\Delta^{13}C$ for each C_4 species. This $\Delta^{13}C$ of the C_4 species was assumed to be the same in the glasshouses and was used to back-calculate δ_a in each glasshouse. Thus, δ_a was calculated as:

$$\delta_a = \Delta^{13}C_{C4}(1 + \delta_{pC4}) + \delta_{pC4} \quad (2)$$

where subscript C4 refers to either *S. spontaneum* or *P. oleracea*. By this method, we estimated a δ_a of –8·2 ‰ in the 280 ppm CO_2 glasshouse, –7·9 ‰ in the 400 ppm CO_2 glasshouse and –6·1 ‰ in the 800 ppm CO_2 glasshouse.

Relative growth rate (RGR, mg g^{-1} d^{-1}) was calculated as the difference between ln(*final dry mass*) and ln(*initial dry mass*) divided by the number of days in the experiment. Net assimilation rate (NAR; biomass increment per unit leaf area, g m^{-2} d^{-1}) was calculated for individual plants according to the following equation:

$$NAR = \frac{(W_f - W_i)(\ln A_f - \ln A_i)}{t(A_f - A_i)} \quad (3)$$

where W_f and W_i are the final and initial dry mass (g), respectively, A_f and A_i are the final and initial leaf area (m^2) respectively, and t is the duration of the experiment (d). Leaf mass fraction (LMF; leaf mass per unit whole plant mass, g g^{-1}), stem mass fraction (SMF; stem and petiole mass per unit whole

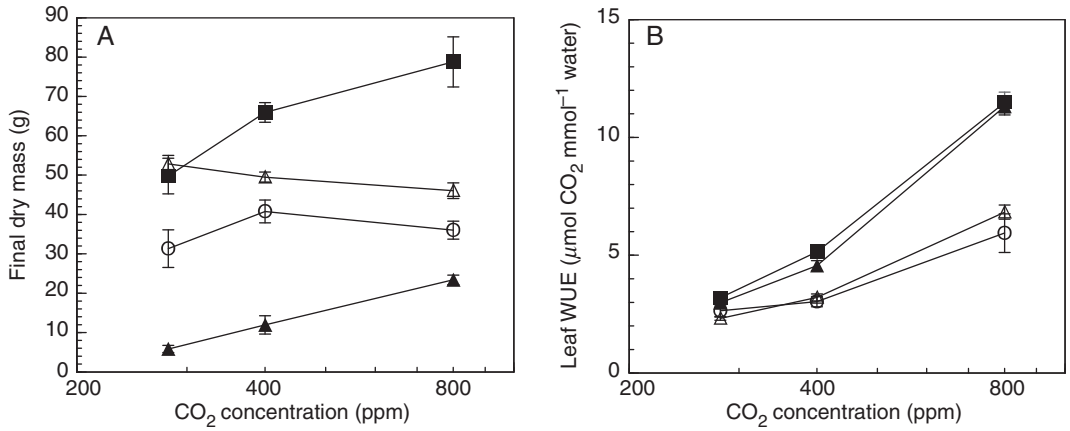

FIG. 2. Response of (A) final biomass and (B) water-use efficiency (WUE) of two angiosperm species (*Chrysophyllum*, open circles; *Tabebuia*, open triangles) and two conifer species (*Araucaria*, black squares; *Podocarpus*, black triangles) to growth at three levels of atmospheric CO_2 concentration (280, 400, 800 ppm, log scale). Values are means ± 1 s.e.

plant mass, units g g^{-1}), root mass fraction (RMF; root mass per unit whole plant mass, g g^{-1}), leaf area ratio (LAR; leaf area per unit whole plant mass, cm^{-2} g^{-1}) and specific leaf area (SLA; leaf area per unit leaf mass, cm^{-2} g^{-1}) were calculated from the final harvest data. Photosynthetic nitrogen use efficiency (PNUE; net carbon assimilation per unit leaf nitrogen, μmol CO_2 mmol N^{-1} s^{-1}) was calculated from A_{max} measurements and foliar nitrogen concentration.

The youngest fully expanded leaves were sampled in the weeks preceding harvest for measurements of stomatal density. Leaf area was measured (as above), and an impression of each leaf was taken in the interveinal area of the lamina using clear fingernail polish. Impressions were taken only from the abaxial surface of the leaves. The impressions were mounted on slides and photographed with a light microscope (Nikon Eclipse 200) at 200× to 400× magnification. Three fields were observed for each leaf. *Podocarpus* stomatal density was estimated in two sections at the widest region of the blade; each measurement covered an area from the mid vein to the leaf margin (averaging 8·7 mm^2). In *Araucaria*, leaves have narrow bands of stomata separated by wide veins. Stomatal densities were calculated excluding non-stomatal areas.

Statistical analysis

Seedlings receiving the same c_a were all grown in the same glasshouse and therefore shared the same growth environment. Consequently, individual seedlings are not true experimental replicates and only mean response values for each species × c_a were used in analyses. To evaluate species performance differences across treatments, c_a was treated as a continuous variable after log transformation. Response variables were also log-transformed, as necessary, after checking for a linear relationship with c_a. Two *a priori* contrasts were made of the species × c_a interaction. First, we compared the two angiosperm and two conifer species. Second, the broad-leaved conifer, *Podocarpus*, was compared with the two angiosperms. Analyses were performed in R version 2·15·3 (R Core Team, 2013).

RESULTS

Overall, we observed a stronger enhancement of both growth (Fig. 2A) and WUE (Fig. 2B) in the conifers than in the angiosperm taxa in response to increasing c_a.

Growth and allocation

Species showed contrasting growth responses to CO_2 concentration (Figs 2A and 3A, Table 1). The angiosperms showed no net increase in RGR between 280 and 800 ppm, and did not differ statistically in response from *Araucaria*, which showed a stimulation of 19 % in RGR. However, *Podocarpus* differed significantly in growth response from the angiosperms ($t = 3·5$, $P = 0·024$), with 66 % stimulation in RGR from the lowest to the highest c_a treatment (Fig. 3A). This represented a change in the mean plant biomass at harvest from 5·8 g in the lowest c_a treatment to 23·4 g in the highest c_a treatment (Fig. 2A). Differences in growth performance were not related to differences in allocation. There were no significant species × c_a interactions for leaf mass ratio (LMR), root mass ratio (RMR) or SLA (Table 1, Fig. 3B–D). Similarly, there were no significant differences among species in the response of NAR to c_a (Fig. 3E), although the increase in mean NAR from the lowest to the highest c_a was greater for *Araucaria* (41 %) and *Podocarpus* (118 %) than for *Chrysophyllum* (28 %) and *Tabebuia* (26 %). Increases in A_{max} per unit leaf area in response to c_a did not differ across species for *Araucaria*, *Chrysophyllum* and *Podocarpus*, but appeared to saturate at ambient c_a in *Tabebuia*, the species with the highest SLA (Fig. 3F). In contrast, *Podocarpus* showed a very strong enhancement of A_{max} across c_a treatments, with a >4-fold increase from 280 to 800 ppm, compared with a 2-fold increase in the remaining species.

Nutrient relations

Foliar N and P (Table 1, Supplementary Data Fig. 1) differed significantly among species, but did not vary consistently

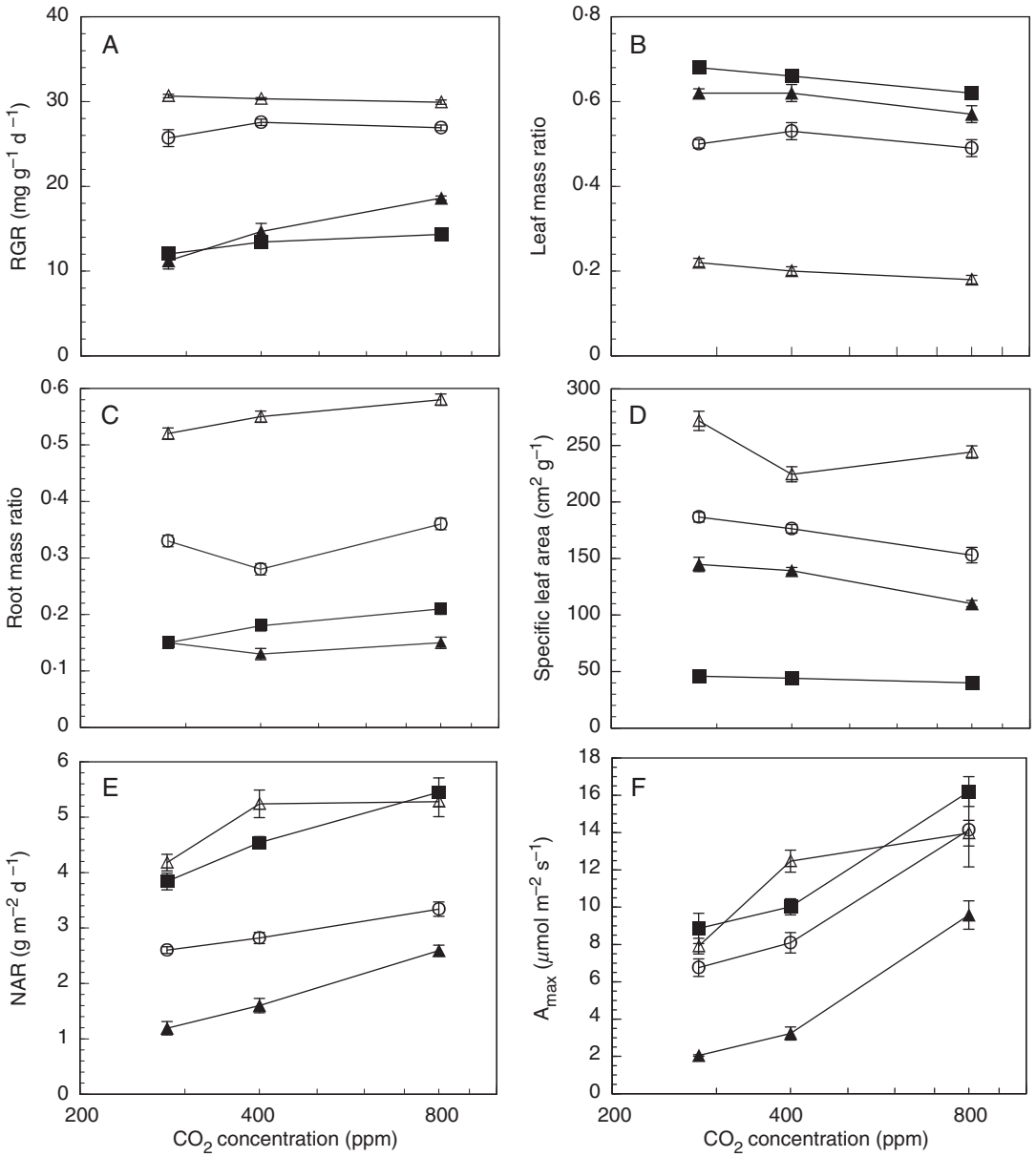

FIG. 3. Response of (A) relative growth rate (RGR), (B) leaf mass ratio, (C) root mass ratio, (D) specific leaf area, (E) net assimilation rate (NAR) and (F) maximal photosynthetic rate (A_{max}) of two angiosperm species (*Chrysophyllum*, open circles; *Tabebuia*, open triangles) and two conifer species (*Araucaria*, black squares; *Podocarpus*, black triangles) to growth at three levels of atmospheric CO_2 concentration (280, 400, 800 ppm, log scale). Values are means ± 1 s.e.

between the two species groups. *Podocarpus* and *Tabebuia* had the highest foliar N concentration, but conversely the lowest foliar P concentration. For all species, foliar N concentration declined significantly with increasing c_a, whereas foliar P either remained unchanged with c_a (*Podocarpus* and *Tabebuia*) or increased significantly (*Araucaria* and *Chrysophyllum*). Foliar N:P suggested that all species became progressively more N-limited at higher c_a (Fig. 4A), although species did not show differential responses of N:P to c_a (Table 1). Consistent with N limitation, soil-extractable P remained high in all treatments and species (range 6–23 mg P kg^{-1}; Fig. 4B), while soil-extractable ammonium and nitrate were reduced to low levels, particularly at 800 ppm c_a (Fig. 4C, D). There was also a significant species × c_a interaction for extractable nitrate (Table 1), with higher nitrate concentrations in pots containing the conifers (Fig. 4C). In addition, pots that contained *Podocarpus* seedlings maintained a higher foliar δ^{15}N across c_a treatments (Fig. 4E). Finally, the angiosperm taxa had higher PNUE and showed greater increases in PNUE with c_a (Fig. 4F) despite having foliar N:P values that were intermediate between *Araucaria* and *Podocarpus* (Fig. 4A).

Water-use efficiency

Species differed significantly in MTR over the course of the experiment, with a large decline for *Araucaria* with increasing

TABLE 1. F-*values for species (d.f. = 3),* c_a *(d.f. = 1) and species* × c_a *(d.f. = 3) effects on dependent variables for four tree species and three levels of* c_a. *Where the species* × c_a *interaction is significant, contrasts between angiosperm and conifer taxa are reported in the text*

Variable	Species	c_a	Species × c_a
Growth and allocation			
RGR	407·9***	22·3***	11·4*
NAR	79·8***	35·4***	0·96
LMR	531·9***	15·9*	0·8
RMR	150·4***	5·0	0·5
SLA	91·0***	5·0	0·4
Nutrient relations			
Foliar [N]	27·5**	15·8*	0·6
PNUE	160·9***	134·1***	5·7
$\delta^{15}N$	6·6*	15·8*	0·6
Foliar [P]	68·6***	30·2**	6·5*
Foliar N:P	279·4***	92·8***	2·9
Bray extractable soil P	25·4**	2·4	1·0
KCl-extractable soil NO_3-N	308·0***	79·8***	62·2***
KC-extractable soil NH_4-N	6·4	6·7	6·2
Photosynthesis and water-use efficiency			
Mean transpiration rate	14·0*	5·77	0·82
WUE_{leaf}	11·6*	155·1***	3·59*
WUE_{wp}	274·3***	1009·7***	99·4***
A_{max}	16·1**	58·0**	0·3
Stomatal conductance	21·0**	10·0*	2·9
c_i/c_a	175·9***	70·1**	17·1**
C isotope discrimination	37·6**	0·3	1·0
Stomatal density	1866·6***	12·0*	16·8*

*$P<0·05$; **$P<0·01$; ***$P<0·001$.

c_a and for *Tabebuia* between 400 and 800 ppm CO_2 (Fig. 5A, Table 1). The response of WUE_{wp} to c_a differed significantly between the species groups (Fig. 5B, Table 1), with a stronger positive response of WUE_{wp} to c_a in the conifers than in the angiosperms ($t = 8·36$, $P < 0·001$). A similar pattern was seen in the instantaneous measurements of WUE_{leaf} taken in the several days before harvest (Fig. 2B, Table 1). There was a strong correlation between WUE_{wp} and WUE_{leaf} across the dataset ($r = 0·75$, $P < 0·001$, $n = 93$), indicating consistency between the time-integrated measure of water-use efficiency, based on cumulative water use and biomass production, and the instantaneous measurement, based on CO_2 and water vapour fluxes measured on single leaves.

The steeper increases in WUE_{leaf} with increasing c_a in the conifer species compared with the angiosperm species could be explained by decreases in the ratio of intercellular to ambient CO_2 concentrations (c_i/c_a) with increasing c_a (Fig. 5C, Table 1). In *Araucaria*, the decline in c_i/c_a was associated with both decreasing stomatal conductance and increasing photosynthesis, whereas in *Podocarpus* the decline in c_i/c_a mainly resulted from increasing photosynthesis from 280 to 800 ppm c_a. In contrast, c_i/c_a remained similar or showed a smaller decline with increasing c_a in the two angiosperm species. The leaf dry matter $\Delta^{13}C$ results, which provide a time-integrated measure of c_i/c_a, generally supported this pattern (Fig. 5D), although $\Delta^{13}C$ did not decrease as much as expected based on the change in instantaneous c_i/c_a in the 800 ppm c_a glasshouse. However, across the full dataset, instantaneous c_i/c_a and leaf dry matter $\Delta^{13}C$ were reasonably well correlated ($r = 0·79$, $P < 0·001$, $n = 96$).

The slope of the relationship between $\log(c_a - c_i)$ and WUE_{wp} was significantly greater for the two conifer species than the two angiosperm species (d.f. = 1,89, $F = 13·6$, $P < 0·001$; Fig. 6), indicating that a mechanism additional to $c_a - c_i$ contributed to the stronger responsiveness of WUE_{wp} to c_a in the conifers. Leaf dry matter $\Delta^{13}C$ and stomatal conductance did not show a species × c_a interaction (Fig. 5D, E, Table 1), although both $\Delta^{13}C$ (d.f. = 1,8, $F = 84·1$, $P < 0·001$) and conductance (d.f. = 1,8, $F = 16·5$, $P = 0·003$) were significantly lower for conifers than angiosperms. Finally, the stomatal density response showed a significant species × c_a interaction (Fig. 5F, Table 1), attributable to a significant reduction in density with increasing c_a in *Podocarpus* compared with the angiosperms ($t = 6·3$, $P = 0·008$). Reduced stomatal density at high c_a in *Podocarpus* was also associated with a significant increase in leaf size, from 5·9 ±1·3 cm^2 (mean ± 1 s.d.) at 280 ppm to 9·9 ±1·7 cm^2 at 800 ppm.

DISCUSSION

Photosynthetic and growth responses to variation in c_a

Consistent with our first hypothesis that tropical conifers would show a stronger photosynthetic and growth response to variation in c_a than angiosperms, we saw a significantly stronger growth stimulation across treatments in *Podocarpus* (a 66 % increase in RGR) and a weaker, but not statistically significant, enhancement in *Araucaria* (a 19 % increase). There was no positive response to increasing c_a in either of the two angiosperm species. The lack of a growth response of *Tabebuia* and *Chrysophyllum* to c_a is consistent with previous experiments in which seedlings of tropical tree species were grown in unfertilized soil (Lovelock *et al*., 1998; Winter *et al*., 2000; Cernusak *et al*., 2011).

The causes of differential responses of plants to elevated c_a in greenhouse experiments have recently been revisited by Ali *et al*. (2013). They contrasted evidence from short-term pot experiments, where species with high RGR are found to be most responsive to elevated c_a (Poorter and Navas, 2003; Körner, 2006), with model predictions that species with low C allocation to leaf production (LMR), and low stomatal conductance (g_s), instantaneous PNUE and SLA will be most responsive. Differences between longer-term, model-predicted responses to c_a and those observed in greenhouses were suggested to result from a short-term advantage to fast-growing species able to rapidly increase in leaf area during the quasi-exponential phase of seedling growth (Ali *et al*., 2013).

Although in our experiment plants grew during this rapid initial seedling growth phase, we nonetheless observed stronger growth enhancement in the two conifers with less than half the RGR of the angiosperm taxa. In agreement with the predictions of Ali *et al*. (2013), the conifers had markedly lower g_s, PNUE and SLA, but had the highest LMR. Low LMR in *Tabebuia*, although this species had the highest SLA, may therefore in part explain its low responsiveness species to c_a.

An additional mechanism to account for the high responsiveness of the conifers has been proposed by Niinemets *et al*. (2011). While limited stomatal conductance is widely recognized to limit photosynthetic rate, Niinemets *et al*. (2011) reviewed the effects of mesophyll conductance in plants and

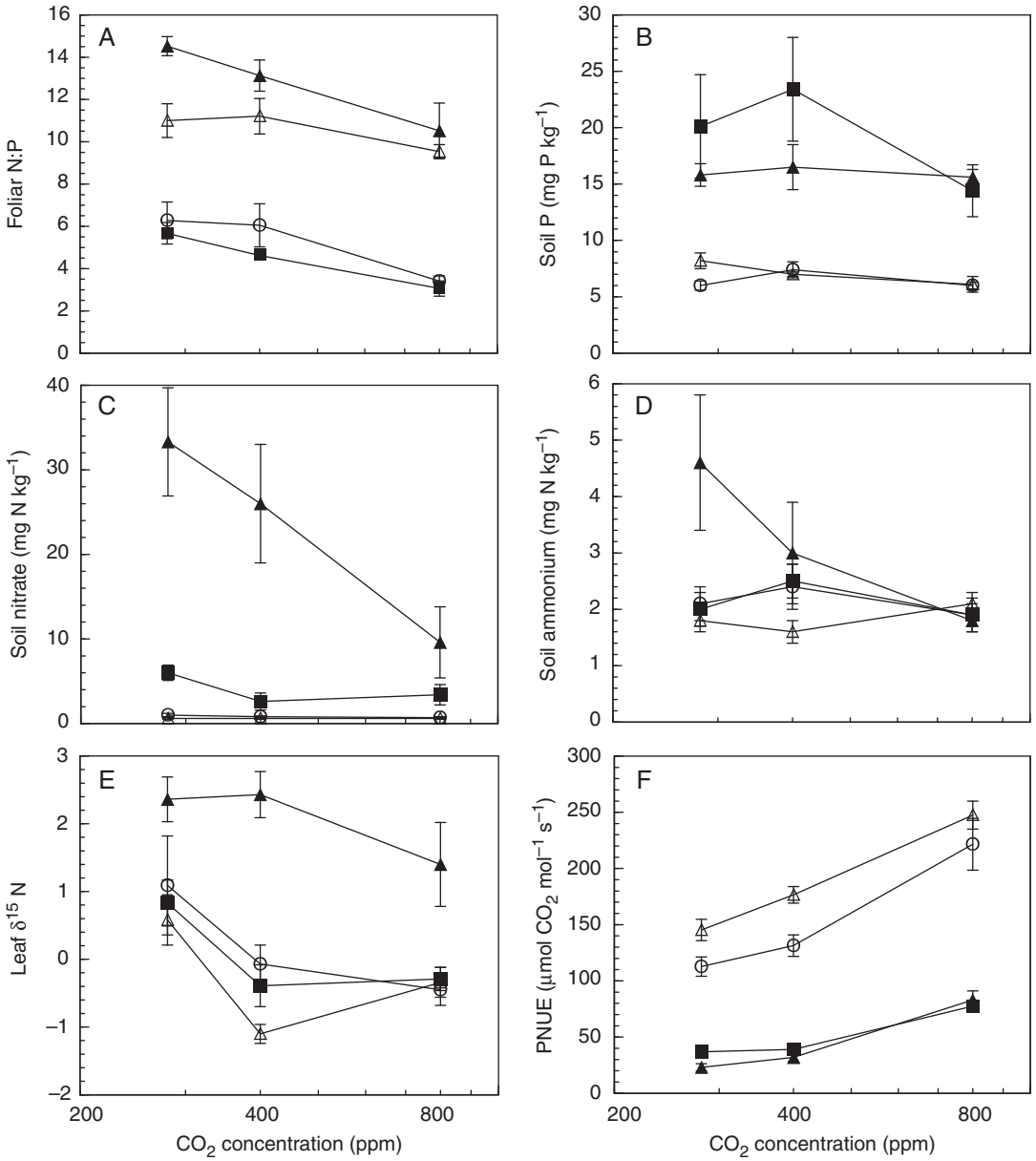

FIG. 4. (A) Seedling foliar N:P ratio, (B) soil-extractable P, (C) soil-extractable nitrate, (D) soil-extractable ammonium, (E) foliar δ^{15}N and (F) photosynthetic nitrogen use efficiency of two angiosperm species (*Chrysophyllum*, open circles; *Tabebuia*, open triangles) and two conifer species (*Araucaria*, black squares; *Podocarpus*, black triangles) in response to growth at three levels of atmospheric CO_2 concentration (280, 400 and 800 ppm, log scale). Values are means ± 1 s.e.

highlighted the limited internal diffusion conductance of sclerophyllous plants, including conifers, as providing a constraint to photosynthetic rate that can be as large as that due to stomatal conductance. Reduced mesophyll conductance in sclerophyllous plants is in turn attributable to greater investment in support tissue, particularly thicker cell walls, associated with greater leaf longevity (Niinemets *et al*., 2005). Low mesophyll conductance has been observed in the New Zealand podocarp *Dacrydium cupressinum*, to the extent that the diffusive constraint on photosynthesis imposed by mesophyll exceeded that imposed by stomata (DeLucia *et al*., 2003).

Despite the strong growth response of *Podocarpus* to increasing c_a, there was no significant species × c_a interaction effect for allocational traits (LMR and SLA), suggesting a primarily physiological response to c_a. Contrary to expectation, however, photosynthetic responses to increasing c_a did not differ across species, although the magnitude of increase in A_{max} in *Podocarpus* was twice that in *Chrysophyllum* and *Tabebuia* (Table 1, Fig. 3). Likewise, there was no significant interaction for net assimilation rate, although NAR enhancement was much larger in *Araucaria* and *Podocarpus* (Fig. 3).

Differences in nutrient uptake and use efficiency

Pot environments often result in concentrations of inorganic N that are orders of magnitude higher than those in field soil

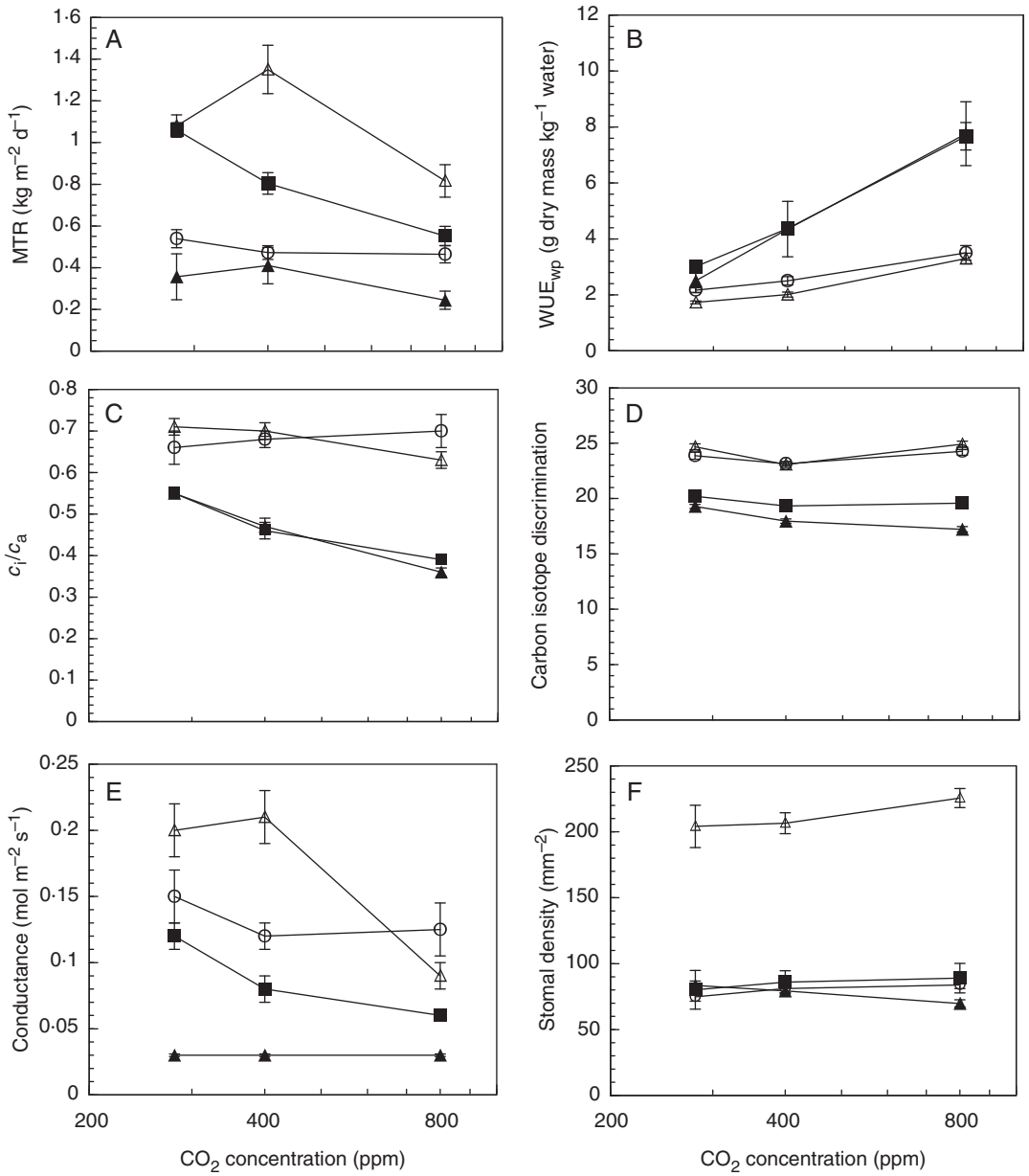

FIG. 5. (A) Mean transpiration rate (MTR), (B) whole-plant water-use efficiency (WUE_{wp}), (C) ratio of intercellular to ambient CO_2 concentration (c_i/c_a), (D) carbon isotope discrimination ($\Delta^{13}C$), (E) stomatal conductance (g_s) and (F) stomatal density in response to growth at three levels of atmospheric CO_2 concentration (280, 400 and 800 ppm, log scale). Values are means ± 1 s.e. Symbols are as in Fig. 2.

due to *in situ* mineralization (Johnson *et al*., 1995; Dalling *et al*., 2013). Here we added rice husks as a soil conditioner to stimulate microbial uptake of N. In a previous pot experiment, rice husk addition reduced soil-extractable nitrate availability 10-fold over 7 weeks (Dalling *et al*., 2013), providing soil conditions more in keeping with the low (<5 μg N g^{-1}) instantaneously extractable inorganic N measured in tropical lowland forest soils (Turner and Romero, 2009).

Analysis of soil and foliar N concentrations at the end of the experiment indicated that the conifers likely differ in N uptake patterns from the angiosperm taxa. *Podocarpus* had a significantly higher foliar $\delta^{15}N$ than the remaining species across all levels of c_a, potentially suggesting a greater preference of organic N or ammonium over nitrate as an N source (Högberg, 1997; Mayor *et al*., 2014) when compared with *Araucaria* and the angiosperm taxa. Consistent with the $\delta^{15}N$ data, soil nitrate concentrations were much higher in pots in which *Podocarpus* seedlings were grown. Differences in nitrate pool size across species may in part be attributable to differences in N demand driven by plant size. At harvest, *Podocarpus* seedlings were much smaller than those of the other species (mean dry mass 5·8 versus 31·3–52·9 g at 280 ppm; 23·4 versus 36·0–78·8 g at 800 ppm), and may have resulted in lower N limitation in *Podocarpus* relative to the other taxa. Nitrate concentrations,

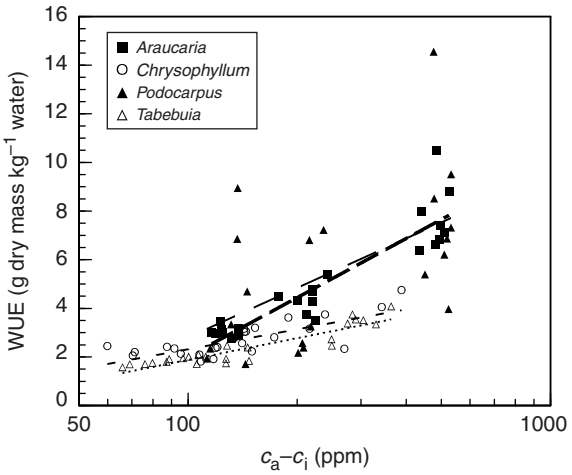

FIG. 6. Relationship between the draw-down in CO_2 concentration from ambient air to the intercellular air spaces (c_a-c_i) and water-use efficiency for two conifer species (*Araucaria* and *Podocarpus*) and two angiosperm species (*Chrysophyllum* and *Tabebuia*).

however, were also somewhat elevated in the pots containing *Araucaria*, even though *Araucaria* dry mass at harvest was similar to or larger than that of *Chrysophyllum* and *Tabebuia* (Fig. 2A). Temperate conifers have frequently been reported to show a strong preference for ammonium over nitrate uptake (Lavoie *et al.*, 1992; Kronzucker *et al.*, 1997; but see Rothstein and Cregg, 2005). More recently, *Araucaria* has been reported to grow faster when provided with ammonium rather than nitrate (Garbin *et al.*, 2006), while *Phyllocladus* (Podocarpaceae) has been shown to preferentially take up ammonium over nitrate in excised roots (Warren and Adams, 2007). Although we lack $\delta^{15}N$ data for field-grown trees, the congener of our study species, *Podocarpus oleifolius*, does have high foliar $\delta^{15}N$ compared with co-occurring tree species in montane forest in Panama, consistent with the results found here (K. Heineman, University of Illinois at Urbana-Champaign, USA, unpubl. res.).

It has recently been shown that assimilation of nitrate into organic N compounds in leaves of C_3 plants can be inhibited by elevated c_a, leading to limitations on growth responses to c_a associated with declining N status as c_a increases (Bloom *et al.*, 2010, 2012). This inhibition of nitrate assimilation in C_3 leaves by elevated c_a appears to be linked to inhibition of photorespiration (Rachmilevitch *et al.*, 2004). Plants that preferentially take up ammonium are unlikely to experience the same inhibition, because ammonium is mainly assimilated into organic compounds in roots (Peoples and Gifford, 1997), which are presumably unaffected by any change in photorespiration in leaves associated with elevated c_a. Thus, if *Podocarpus*, and to a lesser extent *Araucaria*, relied more on ammonium uptake than did the angiosperm taxa, this could provide an additional explanation for the stronger growth responses in these two conifers.

Despite potential differences in uptake, neither foliar N nor P allocation differed significantly between the two species groups. Foliar N:P declined significantly in all species with c_a, indicating greater N limitation as plant size increased. Nonetheless, N:P varied substantially among species, with both the highest and lowest values in the conifers (Fig. 4A). Despite large differences in foliar N and P concentrations among species, PNUE was 3- to 4-fold lower in the conifer taxa. Furthermore, the angiosperms showed a stronger increase in PNUE with c_a. Conifer leaves, however, are likely to be longer-lived than the angiosperm leaves, and PNUE differences may be smaller, or reversed when integrated over leaf lifespans.

Optimization of water-use efficiency in conifers and angiosperms

Brodribb *et al.* (2009) argued that major clades of plants differ in their ability to optimize WUE, with only angiosperms capable of responding to above-ambient c_a through reduced stomatal aperture. Evidence for these differences in CO_2 sensitivity are suggested from meta-analyses that show considerable variation in responses of stomatal conductance to elevated c_a among species (Curtis and Wang, 1998), and a stronger reduction in conductance in temperate angiosperm than coniferous trees (Medlyn *et al.*, 2001). Furthermore, when Brodribb *et al.* (2009) exposed leaves of plants grown at ambient c_a to elevated (600 ppm) and low (100 ppm) CO_2, they found that stomata of four coniferous species were significantly less responsive to c_a than those of five angiosperms. These differences in the stomatal closure response translated into a significantly larger increase in WUE at elevated c_a in angiosperms.

Short-term exposure to altered c_a, however, may not reflect the longer-term capacity of plants to regulate stomatal conductance and WUE (Medlyn *et al.*, 2001). Although our study was relatively short in duration (6 months), there were strong responses of WUE in the conifer species, with much larger increases in WUE observed at high c_a than in the two angiosperm taxa. Moreover, the responses were confirmed with both instantaneous gas exchange measurements (WUE_{leaf}) and experiment-long measurements of biomass production relative to cumulative water use (WUE_{wp}). Interestingly, a lack of stomatal closure in response to growth at elevated c_a was indeed observed in *Podocarpus* in our experiment (Fig. 5E), in agreement with the results of Brodribb *et al.* (2009). However, a higher WUE was achieved in this species through larger proportional increases in photosynthesis with increasing c_a than in the other taxa (Fig. 3F). These data highlight the combined role of stomatal conductance and photosynthetic capacity in defining WUE, and demonstrate a pitfall associated with predicting WUE responses to c_a based only on short-term stomatal responsiveness. Stomatal densities showed contrasting patterns between *Podocarpus* and the remaining taxa. Consistent with higher WUE, stomatal density was reduced at high c_a in *Podocarpus* but not in the angiosperms. Reduced stomatal density may reflect greater leaf size at high c_a in this species (Carins Murphy *et al.*, 2014).

It is intriguing that both *Podocarpus* and *Araucaria* achieved very similar increases in WUE_{leaf} in response to c_a, but by different means. *Podocarpus* mainly increased photosynthesis, whereas *Araucaria* both increased photosynthesis and reduced stomatal conductance. The overall consequence for c_i/c_a (Fig. 5C) and WUE_{leaf} (Fig. 2B) was much the same in either case.

The plot of WUE_{wp} against c_a-c_i (Fig. 6) shows that there may be additional mechanisms to photosynthesis and stomatal

conductance that allowed the conifers to achieve larger increases in WUE_{wp} with increasing c_a than did the angiosperms. The WUE_{wp} can be decomposed into components as follows (Farquhar and Richards, 1984):

$$WUE_{wp} = \frac{(1-\varphi_c)(c_a - c_i)}{1 \cdot 6v} \times \frac{2}{3k} \quad (4)$$

where ϕ_c is the proportion of carbon gained through net photosynthesis that is subsequently lost from the plant by respiration, 1·6 is the ratio of diffusivities of water vapour and CO_2 in air, v is the leaf-to-air vapour pressure difference, 2/3 is the ratio of molecular weights of carbon and water, and k is the whole-plant carbon mass fraction. The results shown in Fig. 6 in conjunction with eqn (4) suggest that the combined term $(1 - \phi_c)/vk$ differed across the range of c_a between the conifers and angiosperms, because the slope of the relationship between WUE_{wp} and $c_a - c_i$ differed. A larger $(1 - \phi_c)$ in the conifers could indicate increasing carbon-use efficiency as c_a increased. Further research is needed to tease these effects apart, but it is apparent that multiple physiological mechanisms contributed to the stronger responsiveness of WUE_{wp} to c_a in the conifer compared with angiosperm taxa.

An interesting result in our experiment was that the apparent change in $\Delta^{13}C$ from low to high CO_2 was less in the conifer taxa than expected from the instantaneous measurements of c_i/c_a [compare panels (C) and (D) in Fig. 5]. We did not have direct measurements of the $\delta^{13}C$ of CO_2 in air in the glasshouses, and so relied on the C_4 method to assign these values (Marino and McElroy, 1991). If there were a bias in this method at high CO_2, it might have caused us to overestimate the $\delta^{13}C$ of CO_2 in air in the 800 ppm c_a glasshouse by 1–2‰. This could at least partly reconcile the variation in trends in panels (C) and (D) in Fig. 5. On the other hand, if our estimates of $\Delta^{13}C$ were accurate, when combined with the instantaneous measurements of c_i/c_a they would suggest that changes in stomatal conductance and mesophyll conductance were not proportional in response to increasing CO_2 in the conifer taxa. It is not known whether the response of mesophyll conductance to varying c_a differs between conifers and angiosperms, but it has recently been speculated that this could be an important consideration for predicting responses of these two groups of plants to rising c_a (Flexas *et al*., 2014).

Implications for understanding forest composition

In this study, the conifer *Podocarpus guatemalensis*, in particular, showed a strong growth response to c_a, accumulating twice as much biomass at 400 μmol mol^{-1} CO_2 and more than four times as much at 800 μmol mol^{-1} CO_2 compared with pre-industrial c_a (280 μmol mol^{-1} CO_2). This result suggests that podocarps, and perhaps other low-latitude conifers, are likely to have experienced a significant enhancement of competitive ability as c_a has risen over the past century (and over the lifespan of individual trees). Although the physiological responses of seedlings described here may not scale to larger size classes, or to evolutionary timescales, differences in seedling growth rates between angiosperms and conifers may play an important role in recruitment success, and are thought to at least partly underlie angiosperm dominance at low latitude (Bond, 1989). Replication of similar studies in unfertilized soils across a broader group of tropical tree taxa will help resolve whether c_a responses of low-latitude conifers are consistent, and whether they can therefore help explain how podocarps colonized angiosperm-dominated tropical northern South America 60 MYA (Quiroga *et al*., 2016).

Results from this study are consistent with the expected growth enhancement for evergreen species with low mesophyll conductance (Niinemets *et al*., 2011), and more generally for slow-growing species with low carbon-use efficiency (Ali *et al*. 2013). Alternatively, the growth response of the conifers may reflect an advantage in nutrient (N) acquisition. Evergreen species have increased in abundance in a range of ecosystems, although the drivers of these changes are not always clear (Niinemets *et al*., 2011, and references therein). Podocarps are currently patchily distributed across the lowland and montane tropics, and for the most part are restricted to low-fertility habitats, where soil nutrient availability constrains realized growth rate (Adie and Lawes, 2011; Dalling *et al*., 2011; Enright and Jaffré, 2011; Punyasena *et al*., 2011). Contemporary increases in c_a that improve the growth performance of tropical forest conifers relative to angiosperm trees might therefore be expected to influence their abundance and distribution.

Although higher c_a may have provided greater opportunities for podocarps to expand their distributions when they first colonized South America and tropical South-East Asia in the Palaeocene and late Eocene (Jaramillo *et al*., 2011*a*, *b*; Morley, 2011), high c_a was not apparently a requisite for range expansion. Podocarps and the araucarian *Cyclusphaera* continued to expand in range during the Oligocene (Jaramillo *et al*., 2013), when c_a was closer to current levels (Franks *et al*., 2014). Furthermore, podocarps show evidence of distributional shifts, potentially including the colonization of lowland forests, during glacial maxima (e.g. Bush *et al*., 1990; Colinvaux *et al*., 1996) when c_a was <200 ppm (Monnin *et al*., 2001). Evidence presented here shows that extant tropical conifers exhibit considerable plasticity in photosynthetic capacity and WUE to accommodate these changes in c_a.

SUPPLEMENTARY DATA

Supplementary data are available online at www.aob.oxfordjournals.org and consist of Figure S1: (A) foliar nitrogen concentration and (B) foliar phosphorus concentration of seedlings of the conifers *Araucaria* (black squares) and *Podocarpus* (black triangles) and the angiosperms *Chrysophyllum* (open circles) and *Tabebuia* (open triangles) grown at 280, 400 and 800 ppm CO_2.

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