Responses of Legume Versus Nonlegume Tropical Tree Seedlings to Elevated CO₂ Concentration1[OA]

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We investigated responses of growth, leaf gas exchange, carbon-isotope discrimination, and whole-plant water-use efficiency (Wₑ) to elevated CO₂ concentration ([CO₂]) in seedlings of five leguminous and five nonleguminous tropical tree species. Plants were grown at CO₂ partial pressures of 40 and 70 Pa. As a group, legumes did not differ from nonlegumes in growth response to elevated [CO₂]. The mean ratio of final plant dry mass at elevated to ambient [CO₂] (Mₑ/Mₐ) was 1.32 and 1.24 for legumes and nonlegumes, respectively. However, there was large variation in Mₑ/Mₐ among legume species (0.92–2.35), whereas nonlegumes varied much less (1.21–1.29). Variation among legume species in [CO₂] rises and that the water-use efficiency of tropical tree species will increase under elevated [CO₂].

Tropical forests play an important role in the global carbon cycle, accounting for about one-third of global primary productivity (Saugier et al., 2001; Beer et al., 2010). Thus, responses of tropical tree growth and water use to steadily increasing atmospheric [CO₂] over the next century will partly control the response of the global carbon cycle to continuing human industrialization. In general, physiological responses of tropical trees to elevated [CO₂] have been poorly studied compared with the responses of temperate trees (Stork et al., 2007; Körner, 2009), and there is debate about how tropical tree physiology will respond to continuing increases in atmospheric [CO₂].

Leguminous tree species, in the family Fabaceae, are both abundant and diverse in tropical forests, especially in the Neotropics and Africa (Gentry, 1988; Losos and Leigh, 2004). Some, but not all, leguminous tree species have the ability to form N₂-fixing nodules on their roots, in symbiosis with rhizobial bacteria (de Faria et al., 1989; Moreira et al., 1992; Sprent, 2009). Because nodule-forming legumes have the ability to acquire N₂ from the atmosphere, they may be able to respond more strongly to elevated [CO₂] than species that lack this capacity, particularly in nitrogen-poor soils. Thus, N₂-fixing legumes may be able to overcome nutritional constraints that could otherwise limit growth responses to elevated [CO₂] (Tissue et al., 1997; Schortemeyer et al., 2002; Reich et al., 2006; Rogers et al., 2009). An ability to up-regulate N₂ fixation may also be beneficial for phosphorus (P) acquisition, because N-rich phosphatase enzymes can be deployed on root surfaces and released into the soil to hydrolyze organically bound P, making it available for plant uptake (Richardson et al., 2005; Houlton et al., 2008; Turner, 2008).

Changes in plant water use may additionally play an important role in controlling the responses of tropical forest trees to rising atmospheric [CO₂] (Holtum and Winter, 2010). In general, water-use efficiency is expected to increase as atmospheric [CO₂] increases (Drake et al., 1997). If pᵢ/pₑ, the ratio of intercellular to ambient CO₂ partial pressures, remains constant, leaf-level water-use efficiency (Wₑ) is expected to increase linearly as atmospheric [CO₂] increases, all else being equal (Farquhar and Richards, 1984). Under such con-
ditions, a doubling of \( p_a \) would cause a doubling of \( W_t \). Therefore, it is important to determine whether \( p_r/p_a \) varies in response to growth at elevated [CO\(_2\)] in tropical forest trees. For C\(_3\) plants, an integrated estimate of \( p_r/p_a \) can further be obtained from the carbon isotope discrimination of plant tissues (Farquhar et al., 1982).

Our principle objective in this study was to investigate whether growth responses to elevated [CO\(_2\)] would be stronger in leguminous than in nonleguminous tropical tree seedlings. A second objective was to investigate responses of water-use efficiency to elevated [CO\(_2\)] in tropical tree seedlings, both in the presence and absence of constraints on growth caused by low nutrient availability and/or drought. We aimed to test the following two hypotheses: (1) growth responds more strongly to elevated [CO\(_2\)] in legumes than in nonlegumes for tropical tree seedlings grown in unfertilized soil; and (2) \( p_r/p_a \) remains approximately constant in tropical tree seedlings grown at ambient and elevated [CO\(_2\)], even under conditions of variable nutrient and water supply, such that elevated [CO\(_2\)] causes proportional increases in whole-plant water-use efficiency (\( W_p \)). To test these hypotheses, we conducted two experiments. In the first experiment, one leguminous (Ormosia macrocalyx) and one nonleguminous (Swietenia macrophylla) tree species were grown under drought or well-watered conditions, with or without added fertilizer, at both ambient and elevated [CO\(_2\)]. In the second experiment, four leguminous (Albizia adinocephala, Dalbergia retusa, Inga punctata, and Schizolobium parahyba) and four nonleguminous (Chrysophyllum cainito, Coccoloba uvifera, Hieronyma alchorneoides, and Pachira quinata) tree species were grown at ambient and elevated [CO\(_2\)] in unfertilized soil under well-watered conditions.

RESULTS

Growth, Transpiration, and Water-Use Efficiency

Results for plants grown in unfertilized soil at high water supply are summarized in Figure 1 for final plant dry mass (\( M_p \)), cumulative transpiration over the course of the experiments (\( E_T \)), and \( W_p \). The \( M_p \) increased at elevated (70 Pa) compared with ambient (40 Pa) CO\(_2\) partial pressure (Fig. 1A; Table I), with mean values of 30.8 and 36.8 g, respectively. On the other hand, the \( E_T \) decreased in response to elevated [CO\(_2\)] (Fig. 1B; Table I). Mean \( E_T \) at ambient and elevated [CO\(_2\)] was 12.5 and 9.7 kg, respectively. As a result, the \( W_p \) showed a marked increase in response to elevated [CO\(_2\)] (Fig. 1C). Mean \( W_p \) at ambient and elevated [CO\(_2\)] was 1.6 and 2.4 mmol carbon mol\(^{-1}\) water, respectively. Thus, \( W_p \) increased by 54% in response to elevated [CO\(_2\)]. The \( M_p \), \( E_T \), and \( W_p \) all varied among species (Fig. 1; Table I). In addition, \( W_p \) was higher in legumes as a group than in nonlegumes. Mean \( W_p \) under ambient [CO\(_2\)] was 1.7 mmol carbon mol\(^{-1}\) water for legumes and 1.4 mmol carbon mol\(^{-1}\) water for nonlegumes. The \( W_p \) also increased more under elevated [CO\(_2\)] in legumes than in nonlegumes, resulting in a significant [CO\(_2\)] \times legume interaction in the ANOVA (Table I). Mean \( W_p \) under elevated [CO\(_2\)] was 2.7 mmol carbon mol\(^{-1}\) water for legumes and 2.1 mmol carbon mol\(^{-1}\) water for nonlegumes.

Results for \( M_p \), \( E_T \), and \( W_p \) of S. macrophylla grown in fertilized and unfertilized soil, and at high and low water supply, are summarized in Figure 2. The \( M_p \) of S. macrophylla increased in response to elevated [CO\(_2\)] and in response to high water supply and fertilizer addition (Fig. 2A; Table II); there was also a significant water \( \times \) nutrient interaction (Table II). The \( E_T \) for S. macrophylla did not vary significantly in response to elevated [CO\(_2\)]. The \( E_T \) was higher in S. macrophylla at high compared with low water supply and in fertilized compared with unfertilized plants, and there was a significant water \( \times \) nutrient interaction (Fig. 2C; Table II). The \( W_p \) of S. macrophylla increased in response to elevated [CO\(_2\)] for all combinations of water and nutrient treatments (Fig. 2E). Across all water and nutrient treatments, mean \( W_p \) of S. macrophylla was 1.7 mmol carbon mol\(^{-1}\) water under ambient [CO\(_2\)] and 2.6 mmol carbon mol\(^{-1}\) water under elevated [CO\(_2\)]. Thus, \( W_p \) showed a mean increase of 54% in response to elevated [CO\(_2\)] for this species.

The \( M_p \), \( E_T \), and \( W_p \) of O. macrocalyx in fertilized and unfertilized soil, and at high and low water supply, are also summarized in Figure 2. The \( M_p \) of O. macrocalyx increased strongly in response to elevated [CO\(_2\)] (Fig. 2B; Table III). The \( E_T \) of O. macrocalyx showed modest increases in response to elevated [CO\(_2\)] and in response to high water supply (Fig. 2D; Table III). Marked increases in \( W_p \) were recorded in response to elevated [CO\(_2\)] across all water and nutrient treatments (Fig. 2F; Table III). At ambient [CO\(_2\)], mean \( W_p \) of O. macrocalyx was 1.7 mmol carbon mol\(^{-1}\) water, whereas at elevated [CO\(_2\)], mean \( W_p \) was 3.3 mmol carbon mol\(^{-1}\) water, for a mean increase of 91% in response to elevated [CO\(_2\)]. Overall, the dependence of \( M_p \) and \( W_p \) on fertilizer addition was much more pronounced in the nonleguminous S. macrophylla compared with the nodule-forming legume O. macrocalyx (Fig. 2; Tables II and III).

Nodulation Patterns among Legumes

Four of the legume species included in the study formed nodules on their roots. These species were O. macrocalyx (Fig. 3A), I. punctata (Fig. 3B), A. adinocephala (Fig. 3C), and D. retusa (Fig. 3D). The only legume species that did not form nodules was S. parahyba (Fig. 3E). Nodule mass ratio did not vary in response to elevated [CO\(_2\)] for the nodulated species (\( P = 0.32, n = 35 \)). The nodule mass ratio of O. macrocalyx decreased sharply in fertilized compared with unfertilized plants (\( P < 0.001, n = 40 \)). Mean nodule mass ratio for unfertilized O. macrocalyx plants was 48.9 mg g\(^{-1}\), whereas that for fertilized plants was 2.8 mg g\(^{-1}\).
Nitrogen Uptake, Nitrogen Concentration, and $\delta^{15}N$

For unfertilized plants at high water supply, final plant nitrogen ($N_F$) did not vary in response to elevated $[\text{CO}_2]$, on average (Fig. 4A; Table I). However, $N_F$ varied among species and was higher in legumes as a group than in nonlegumes. On the other hand, whole-plant $[N]$ decreased significantly in response to elevated $[\text{CO}_2]$ across all species (Fig. 4B; Table I). The $[N]$ was also higher in legumes than in nonlegumes and varied among species within the two groups. Whole-plant $\delta^{15}N$ decreased in response to elevated $[\text{CO}_2]$. Average $\delta^{15}N$ for plants grown at ambient $[\text{CO}_2]$ was 4.6‰ and that for plants grown at elevated $[\text{CO}_2]$ was 4.1‰. The $\delta^{15}N$ was lower in legumes than in nonlegumes and varied among species within the two groups (Fig. 4C; Table I).

The nonlegume $S. \ macrophylla$ and the legume $O. \ macrocalyx$ differed in responses of $N_F$ to elevated $[\text{CO}_2]$ (Fig. 5, A and B; Tables II and III). The $N_F$ of $S. \ macrophylla$ did not increase significantly in response to elevated $[\text{CO}_2]$, whereas that of $O. \ macrocalyx$ showed a pronounced increase in response to elevated $[\text{CO}_2]$. The $N_F$ of both species increased in response to fertilizer addition and at high compared with low water supply. However, interactions between fertilizer addition and water supply differed between species (Tables II and III). The $[N]$ of both $S. \ macrophylla$ and $O. \ macrocalyx$ decreased in response to elevated $[\text{CO}_2]$ (Fig. 5, C and D; Tables II and III). The $[N]$ was also higher in fertilized compared with unfertilized plants for both species. Responses of whole-plant $\delta^{15}N$ to elevated $[\text{CO}_2]$ in $S. \ macrophylla$ and $O. \ macrocalyx$ were variable, depending on the nutrient and water treatments (Fig. 5, E and F; Tables II and III). The $\delta^{15}N$ of $O. \ macrocalyx$ was near 0‰, except in fertilized plants at high water supply.

Among legume species, variation in nodule mass ratio explained much of the variation in responses of both $N$ uptake and growth to elevated $[\text{CO}_2]$. The ratio of $N_F$ at elevated to ambient $[\text{CO}_2]$ ($N_F/N_A$) is plotted against nodule mass ratio in Figure 6A. $N_F/N_A$ was less than unity in the non-nodule-forming species $S. \ parahyba$. It then increased exponentially as nodule mass ratio increased across the nodule-forming species. A very similar relationship existed between the ratio of final dry mass at elevated to ambient $[\text{CO}_2]$ ($M_F/M_A$) and nodule mass ratio (Fig. 6B). The $M_F/M_A$ was 0.92 in $S. \ parahyba$ and increased exponentially as a function of nodule mass ratio, reaching a maximum of 2.35 in $O. \ macrocalyx$. In contrast, nonlegumes showed a much narrower range of $M_F/M_A$, with values ranging from 1.21 for $S. \ macrophylla$ to 1.29 for $C. \ uvifera$.

Leaf Gas Exchange and Whole-Plant Carbon-Isotope Discrimination

The drawdown in $\text{CO}_2$ partial pressure from air outside the leaf to the leaf intercellular air spaces ($p_a - p_i$), as assessed by instantaneous gas-exchange measurements, was a good predictor of variation in $W_P$, determined over the full course of the experiment ($r^2 = 0.60, P < 0.001, n = 171$). All data from both experiments were included in the analysis. The $W_P$ normalized for variation in growth $\text{CO}_2$ partial pressure ($W_P/p_s$) showed a strong correlation with whole-plant carbon-isotope discrimination ($\Delta^{13}C; r^2 = 0.54, P < 0.001, n = 171$). Finally, the $\Delta^{13}C$ was closely correlated with the instantaneous $p_i/p_s$ ($r^2 = 0.62, P < 0.001, n = 171$). Together, these analyses demonstrate consistency between instantaneous gas-exchange measurements and $\Delta^{13}C$ as useful predictors of variation in the experiment-long $W_P$.
Patterns of variation in leaf-level gas exchange supported observations at the whole-plant level for plants grown in unfertilized soil at high water supply. Stomatal conductance \( \left( g_s \right) \) decreased and net photosynthesis \( (A) \) increased in response to elevated \([CO_2]\) (Fig. 7, A and B; Table I). The mean \( g_s \) decreased from 0.382 to 0.241 \( \text{mol m}^{-2} \text{s}^{-1} \) in response to elevated \([CO_2]\), and the mean \( A \) increased from 12.2 to 15.2 \( \mu\text{mol m}^{-2} \text{s}^{-1} \). The \( p_i/p_a \) did not vary significantly in response to elevated \([CO_2]\) (Fig. 7C; Table I), whereas the mean \( p_a \) increased from 8.0 to 15.6 Pa in response to elevated \([CO_2]\). Whole-plant \( \Delta^{13}\text{C} \) also did not vary significantly between ambient and elevated \([CO_2]\) (Fig. 7D; Table I), consistent with instantaneous assessments of \( p_i/p_a \). Overall, legumes displayed lower \( g_s \) compared with nonlegumes, whereas \( A \) was similar between the two groups. As a result, \( p_i/p_a \) and \( \Delta^{13}\text{C} \) were both lower in legumes than in nonlegumes (Fig. 7; Table I).

The \( g_s \) decreased in \( S. \ macrophylla \) and in \( O. \ macrocalyx \) in response to elevated \([CO_2]\) in plants under high water supply but not in plants under low water supply (Fig. 8, A and B). Thus, there was a significant \([CO_2] \times \) water interaction for \( g_s \) in both species (Tables II and III). On the other hand, the \( A \) increased in response to elevated \([CO_2]\) across all water and nutrient treatments for both species (Fig. 8, C and D). For \( S. \ macrophylla \), the \( A \) was also higher at high compared with low water supply and in fertilized compared with unfertilized plants (Table II). For \( O. \ macrocalyx \), the \( A \) increased at high compared with low water supply but did not vary in response to fertilizer addition (Table III). The \( p_i/p_a \) did not vary in response to elevated \([CO_2]\) in \( S. \ macrophylla \) (Fig. 8E; Table II) and decreased in response to elevated \([CO_2]\) in \( O. \ macrocalyx \) (Fig. 8F; Table III). This indicated large increases in \( W_i \) in both species in response to elevated \([CO_2]\) caused by large increases in \( p_i/p_a \). In \( S. \ macrophylla \), the \( p_i/p_a \) also decreased at low compared with high water supply and decreased in fertilized compared with unfertilized soil (Fig. 8E; Table II). In \( O. \ macrocalyx \), the \( p_i/p_a \) decreased at low compared with high water supply but did not vary in response to fertilizer addition (Fig. 8F; Table III). Patterns of \( \Delta^{13}\text{C} \) were generally consistent with patterns of \( p_i/p_a \) for the two species. The \( \Delta^{13}\text{C} \) decreased in response to elevated \([CO_2]\), decreased at low compared with high water supply, and decreased in response to fertilizer addition (Fig. 8, G and H; Tables II and III).

Stomatal Density, Stomatal Index, and \( \delta^{18}\text{O} \)

Stomatal density (SD) did not show a significant response to elevated \([CO_2]\), but stomatal index (SI) decreased significantly at elevated compared with ambient \([CO_2]\) (Table I). Mean values for SD were

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**Table I. Results of ANOVAs for 10 tropical tree species grown at either ambient (40 Pa) or elevated (70 Pa) CO\(_2\) partial pressure**

Plants analyzed here were grown in unfertilized soil at high water supply. Species were nested into legumes and nonlegumes to test for differences between these two groups. Five to six individuals of each species were grown at each \([CO_2]\) partial pressure. Statistically significant results are shown in boldface.

| Parameter                  | [CO\(_2\)] | Legume        | Species | [CO\(_2\)] × Legume | [CO\(_2\)] × Species |
|----------------------------|------------|---------------|---------|----------------------|----------------------|
| \( \log_{10} M_p \) \( (\text{g dry mass}) \) | \( F = 7.6 \) | \( F = 3.7 \) | \( F = 56.1 \) | \( F = 0.1 \) | \( F = 1.3 \) |
| \( \log_{10} F_{\text{p}} \) \( (\text{kg water}) \) | \( P < 0.01 \) | \( P < 0.06 \) | \( P < 0.001 \) | \( P = 0.82 \) | \( P = 0.24 \) |
| \( W_{\text{f}} \) \( (\text{mmol carbon mol}^{-1} \text{ water}) \) | \( F = 11.7 \) | \( F = 0.0 \) | \( F = 46.8 \) | \( F = 0.5 \) | \( F = 1.4 \) |
| \( \log_{10} N_p \) \( (\text{mg N}) \) | \( P < 0.001 \) | \( P < 0.01 \) | \( P < 0.001 \) | \( P < 0.001 \) | \( P < 0.05 \) |
| \( [\text{N}] \) \( (\text{mg N g}^{-1} \text{ dry mass}) \) | \( F = 0.0 \) | \( F = 116.8 \) | \( F = 26.5 \) | \( F = 0.6 \) | \( F = 1.0 \) |
| \( \delta^{13}\text{N} \) \( (\%) \) | \( P < 0.001 \) | \( P < 0.001 \) | \( P < 0.001 \) | \( P = 0.16 \) | \( P < 0.05 \) |
| \( g_s \) \( (\text{mol water m}^{-2} \text{s}^{-1}) \) | \( F = 21.3 \) | \( F = 2.8 \) | \( F = 6.4 \) | \( F = 0.0 \) | \( F = 0.5 \) |
| \( A \) \( (\mu\text{mol CO}_2 \text{ m}^{-2} \text{s}^{-1}) \) | \( P < 0.001 \) | \( P < 0.001 \) | \( P < 0.001 \) | \( P = 0.96 \) | \( P = 0.86 \) |
| \( p_i/p_a \) \( (\text{Pa Pa}^{-1}) \) | \( F = 6.5 \) | \( F = 266.9 \) | \( F = 51.5 \) | \( F = 1.1 \) | \( F = 0.3 \) |
| \( \Delta^{13}\text{C} \) \( (\%) \) | \( P < 0.001 \) | \( P < 0.001 \) | \( P < 0.001 \) | \( P = 0.28 \) | \( P = 0.97 \) |
| SD \( (\text{mm}^{-2}) \) | \( F = 2.8 \) | \( F = 134.9 \) | \( F = 18.2 \) | \( F = 12.6 \) | \( F = 3.0 \) |
| SI \( (\%) \) | \( P < 0.10 \) | \( F = 4.5 \) | \( F = 6.9 \) | \( F = 70.2 \) | \( F = 0.6 \) | \( F = 1.8 \) |
| \( \delta^{18}\text{O} \) \( (\%) \) | \( F = 0.6 \) | \( F = 69.8 \) | \( F = 6.4 \) | \( F = 0.0 \) | \( F = 0.5 \) |

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54.0 and 51.0 mm\(^{-2}\) at ambient and elevated [CO\(_2\)], respectively, and those for SI were 19.9% and 19.0%. Both SD and SI varied between legumes and nonlegumes and among species within these groups (Table I). The \(\delta^{18}O\) of stem dry matter showed a small but significant increase in response to elevated [CO\(_2\)] (Table I). Mean values were 22.2‰ and 22.7‰ at ambient and elevated [CO\(_2\)], respectively. Stem \(\delta^{18}O\) also varied between legumes and nonlegumes and among species within these groups (Table I). Across the full data set, SD explained 29% of variation in stem dry matter \(\delta^{18}O\) (Fig. 9), whereas \(g_s\) and \(\delta^{13}O\) were not correlated in these plants (\(P = 0.08, n = 80\)).

**DISCUSSION**

Legume-Versus-Nonlegume Responses to Elevated [CO\(_2\)]

As a group, leguminous tropical tree seedlings did not show a stronger growth response to elevated [CO\(_2\)] than nonleguminous seedlings for plants grown in unfertilized soil at high water supply (Fig. 1A; Table I). However, within legumes, there was a large range of variation among species in growth response to elevated [CO\(_2\)]. This was closely related to their capacity for nodule formation, as quantified by nodule mass ratio (Fig. 6B). These results suggest that predictions of legume responses to elevated [CO\(_2\)] in tropical forests could benefit by taking into account interspecific patterns in nodulation potential. Such patterns have been reasonably well documented (de Faria et al., 1989; Moreira et al., 1992; Sprent, 2009). Our results for tropical tree legumes are consistent with observations in herbaceous legumes, where wide-ranging capacities for N\(_2\) fixation among species and among genotypes within species influenced growth responses to elevated [CO\(_2\)] (Ainsworth et al., 2004; Niklaus and Körner, 2004; Reich et al., 2006; Rogers et al., 2009). A similar pattern was also observed for several temperate Acacia species (Schortemeyer et al., 2002).

We assumed that the nodule mass ratio provided an index in our study of the capacity of the leguminous tree species to fix atmospheric N\(_2\). This assumption was supported by measurements of the \(\delta^{15}N\) of plant biomass. Of the legume species, \(O.\) macrocalyx had the highest nodule mass ratio and also had whole-plant \(\delta^{15}N\) nearest to 0‰ (Fig. 4C), which is the \(\delta^{15}N\) of atmospheric N\(_2\). In addition, the nonnodulating \(S.\) parahyba had whole-plant \(\delta^{15}N\) furthest from 0‰ of the legume species. The other three legume species were intermediate between these two for both nodule mass ratio and whole-plant \(\delta^{15}N\). Thus, \(\delta^{15}N\) patterns among legume species provided evidence for a positive relationship between N\(_2\) fixation and nodule mass ratio. Taking the non-N\(_2\)-fixing legume \(S.\) parahyba as a reference plant (Pons et al., 2007) and assuming a discrimination of 1‰ during N\(_2\) fixation (Yoneyama et al., 1986), we estimated the proportion of plant N acquired by N\(_2\) fixation to be 84% for \(O.\) macrocalyx, 73% for \(A.\) adinocephala, 52% for \(D.\) retusa, 46% for \(I.\) punctata, and 0% for \(S.\) parahyba. However, these values should be considered as indicative only, because the non-N\(_2\)-fixing species included in the study showed a relatively large range of plant \(\delta^{15}N\), similar to previous observations for non-N\(_2\)-fixing tropical tree species (Guehl et al., 1998; Cernusak et al., 2007a, 2009c; Pons et al., 2007). This introduces uncertainty into the estimate of \(\delta^{15}N\) for soil-derived plant N (Shearer and Kohl, 1986).

The interspecific nodulation pattern observed in our experiment was entirely consistent with observations of field-grown trees. Thus, \(S.\) parahyba did not nodulate in our experiment (Fig. 3E) and does not nodulate in the field (Allen and Allen, 1981; Moreira et al., 1992; Aguilar et al., 1994); \(A.\) adinocephala nodulated in our
Table II. Results of ANOVAs for S. macrophylla grown at ambient (40 Pa) or elevated (70 Pa) CO₂ partial pressure, in fertilized or unfertilized soil, and at high or low water supply

For each treatment combination, n = 5. Significance tests for the three-way interaction terms are not shown. Statistically significant results are shown in boldface. Units are as in Table I.

| Parameter | [CO₂] | Water | Nutrient | [CO₂] × Water | [CO₂] × Nutrient | Water × Nutrient |
|-----------|-------|-------|----------|---------------|-----------------|-----------------|
| Log₁₀ Mₚ | F = 15.4 | F = 37.1 | F = 249.6 | F = 0.3 | F = 1.6 | F = 19.5 |
| P < 0.001 | P < 0.001 | P < 0.001 | P = 0.61 | P = 0.22 | P < 0.001 |
| Log₁₀ Eᵣ | F = 88.3 | F = 16.0 | F = 126.9 | F = 4.0 | F = 9.1 | F = 2.8 |
| P = 0.83 | P < 0.001 | P < 0.001 | P = 0.76 | P = 0.36 | P < 0.001 |
| Wᵣ | F = 2.8 | F = 46.8 | F = 304.2 | F = 0.3 | F = 1.2 | F = 13.2 |
| P = 0.11 | P < 0.001 | P < 0.001 | P = 0.59 | P = 0.27 | P < 0.01 |
| [N] | F = 38.1 | F = 4.6 | F = 26.3 | F = 0.4 | F = 1.3 | F = 2.1 |
| P < 0.001 | P < 0.001 | P < 0.001 | P = 0.55 | P = 0.26 | P = 0.16 |
| δ¹³C | F = 3.7 | F = 105.3 | F = 18.9 | F = 4.7 | F = 2.7 | F = 0.7 |
| P = 0.06 | P < 0.001 | P < 0.001 | P < 0.001 | P = 0.11 | P = 0.41 |

Table III. Results of ANOVAs for O. macrocalyx grown at ambient (40 Pa) or elevated (70 Pa) CO₂ partial pressure, in fertilized or unfertilized soil, and at high or low water supply

For each treatment combination, n = 5. Significance tests for the three-way interaction terms are not shown. Statistically significant results are shown in boldface. Units are as in Table I.

| Parameter | [CO₂] | Water | Nutrient | [CO₂] × Water | [CO₂] × Nutrient | Water × Nutrient |
|-----------|-------|-------|----------|---------------|-----------------|-----------------|
| Log₁₀ Mₚ | F = 62.7 | F = 28.7 | F = 5.0 | F = 1.5 | F = 0.1 | F = 0.9 |
| P < 0.001 | P < 0.001 | P < 0.001 | P = 0.23 | P = 0.78 | P = 0.36 |
| Log₁₀ Eᵣ | F = 10.0 | F = 32.5 | F = 3.4 | F = 0.7 | F = 0.0 | F = 0.7 |
| P < 0.001 | P < 0.001 | P < 0.001 | P = 0.42 | P = 0.96 | P = 0.42 |
| Wᵣ | F = 285.0 | F = 0.1 | F = 8.8 | F = 4.2 | F = 3.3 | F = 0.0 |
| P < 0.001 | P = 0.71 | P < 0.01 | P < 0.05 | P = 0.08 | P = 0.83 |
| Log₁₀ Nᵣ | F = 50.3 | F = 39.2 | F = 14.7 | F = 6.3 | F = 0.0 | F = 1.0 |
| P < 0.001 | P < 0.001 | P < 0.001 | P < 0.05 | P = 1.0 | P = 0.32 |
| [N] | F = 29.7 | F = 0.7 | F = 14.5 | F = 8.1 | F = 1.1 | F = 0.1 |
| P < 0.001 | P = 0.41 | P < 0.001 | P < 0.01 | P = 0.31 | P = 0.75 |
| δ¹³C | F = 0.3 | F = 39.0 | F = 41.8 | F = 1.9 | F = 0.0 | F = 47.2 |
| P = 0.56 | P < 0.001 | P < 0.001 | P = 0.18 | P = 0.83 | P < 0.001 |

Ormosia survey revealed that in natural forest stands in Guyana, Ormosia

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had the highest estimate for the proportion of leaf N derived from symbiotic N$_2$ fixation (Pons et al., 2007). These comparisons demonstrate that our experiment effectively replicated field-based patterns of nodulation and N$_2$ fixation among the legume species included in the study. This result was achieved without artificially inoculating the seedlings with rhizobia, indicating that compatible N$_2$-fixing bacteria were sufficiently abundant in the forest topsoil used for the experiment.

Nodulation of leguminous tree species in a lowland tropical forest in Panama was observed to be most pronounced at disturbed sites and in forest gaps (Barron et al., 2011). Nodule formation was promoted when N availability in the soil was comparatively low (Barron et al., 2011) and when plant demand for N was high due to the stimulation of plant growth by sunlight. Thus, nodule formation can be suppressed by increasing the availability of N in the soil, as demonstrated for fertilized O. macrocalyx in this study, and by growth under low irradiance (McHargue, 1999). The unfertilized treatments in our experiments replicated a low-N gap environment, insofar as there was high irradiance (approximately 80% of full sunlight) and low soil N availability, caused by the addition of rice husks to the soil mixture (Cernusak et al., 2007b). Therefore, these experimental conditions should have led to maximal expression of nodule mass ratio and N$_2$ fixation, when compared with the range of conditions likely to be encountered by these species in their native environments.

The increasing productivity response to elevated [CO$_2$] with increasing nodule mass ratio among the

Figure 3. Examples of root systems for plants growing in unfertilized soil showing different nodulation patterns among the legume species O. macrocalyx (A), I. punctata (B), A. adinocephala (C), D. retusa (D), and S. parahyba (E). Species are ordered from left to right from highest to lowest nodule mass ratio. S. parahyba on the far right did not form nodules.

Figure 4. Final plant N mass (A), final plant [N] (B), and final plant $\delta^{15}$N (C) of seedlings of 10 tropical tree species grown at either ambient (40 Pa) or elevated (70 Pa) CO$_2$ partial pressure. Plants were grown in unfertilized soil at high water supply. The top five species are legumes and the bottom five species are nonlegumes. Each bar represents the average of five or six plants. Error bars represent se.
legume species resulted from two components. The first component was a stimulation of N acquisition by elevated \([\text{CO}_2]\) at high nodule mass ratio, as can be clearly seen in Figure 6A. Moreover, the \(N_E/N_A\) correlated negatively with plant \(\delta^{15}\text{N}\) \((r^2 = 0.81, P = 0.04, n = 5)\), indicating that species with high \(N_E/N_A\) acquired a larger proportion of their N from N\(_2\) fixation than species with low \(N_E/N_A\). Therefore, legume species with large nodule mass ratio had a greater capacity to up-regulate N\(_2\) fixation under elevated \([\text{CO}_2]\) than legume species with little or no nodule mass ratio. The second component was a greater stimulation of whole-plant N-use efficiency by elevated \([\text{CO}_2]\) \((r^2 = 0.98, P < 0.001, n = 5)\), which can also be seen by comparing the y axes in Figure 6. Thus, biomass production per unit of N taken up was stimulated by elevated \([\text{CO}_2]\), and the extent of stimulation increased with increasing nodule mass ratio. This was likely caused by reduced sink limitation to photosynthesis under elevated \([\text{CO}_2]\) in plants with large nodule mass ratio compared with those with little or no nodule mass ratio \((\text{Ainsworth et al., 2004})\). This second component of the productivity response is further evidenced by a negative correlation among legume species between the ratio of plant carbon to N at elevated to ambient \([\text{CO}_2]\) and plant \(\delta^{15}\text{N}\) \((r^2 = 0.77, P < 0.05, n = 5)\). This indicates that

Figure 5. Final plant N mass (A and B), final plant \([\text{N}]\) (C and D), and final plant \(\delta^{15}\text{N}\) (E and F) of seedlings of two tropical tree species grown at either ambient (40 Pa) or elevated (70 Pa) \([\text{CO}_2]\) partial pressure and in four soil treatments. Soil treatments were as follows: high water supply, fertilizer addition (+W+N); high water supply, no fertilizer addition (+W−N); low water supply, fertilizer addition (−W+N); and low water supply, no fertilizer addition (−W−N). \(O.\text{ macrocalyx}\) is a legume and \(S.\text{ macrophylla}\) is a nonlegume. Each bar represents an average of five plants. Error bars represent se.

Figure 6. The ratio of final plant N mass at elevated (70 Pa) to that at ambient (40 Pa) \([\text{CO}_2]\) partial pressure (A), and the ratio of final plant dry mass at elevated to that at ambient CO\(_2\) partial pressure (B) plotted against the nodule mass ratio at elevated \([\text{CO}_2]\) for the five leguminous tree species included in the study. Plants were grown in unfertilized soil at high water supply. The line in A represents a nonlinear regression equation: 
\[N_E/N_A = 0.80 + 0.038\text{exp}(0.060NMR),\]
where \(NMR\) is nodule mass ratio. The line in B represents a similar equation: 
\[M_E/M_A = 0.88 + 0.047\text{exp}(0.062NMR),\]
greater increases in plant carbon-to-N ratio under elevated \([\text{CO}_2]\) were achieved in plants with high \(\text{N}_2\) fixation rates compared with those with little or no \(\text{N}_2\) fixation.

The growth response of \(S.\) *macrophylla* to elevated \([\text{CO}_2]\) was larger in fertilized soil than in unfertilized soil at high water supply (Fig. 2A). For this species, the \(M_e/M_A\) was 1.76 in fertilized soil and 1.21 in unfertilized soil. Other nonleguminous tropical tree seedlings also showed little growth stimulation under elevated \([\text{CO}_2]\) in unfertilized soil but large responses in fertilized soil (Lovelock et al., 1998; Winter et al., 2000, 2001a, 2001b; Körner, 2009). In contrast, the legume \(O.\) *macrocalyx* showed a similar proportional increase in biomass under elevated \([\text{CO}_2]\) in both fertilized and unfertilized soil at high water supply. Here, the \(M_e/M_A\) was 2.35 in fertilized soil and 2.33 in unfertilized soil. At low water supply, on the other hand, \(S.\) *macrophylla* enjoyed a similar proportional increase in biomass under elevated \([\text{CO}_2]\) in both fertilized and unfertilized soil, with \(M_e/M_A\) of 1.55 and 1.53, respectively, whereas \(O.\) *macrocalyx* had \(M_e/M_A\) of 3.33 and 2.87 in fertilized and unfertilized soil at low water supply. These data suggest that tropical tree seedlings may experience growth stimulation in response to elevated \([\text{CO}_2]\) during dry spells even when nutrient availability is relatively low. This could have important consequences for distribution patterns of tropical tree species, because drought sensitivity of seedlings has been shown to play an important role in structuring tropical tree communities (Engelbrecht et al., 2007).

We observed that legumes as a group had higher \(W_P\) than nonlegumes when grown in unfertilized soil at high water supply (Fig. 1C; Table I). Consistent with this, legumes also had lower \(p_i/p_a\) and lower \(\Delta^{13}\text{C}\) (Fig. 7, C and D; Table I). This agrees with previous observations for a smaller number of species (Cernusak et al., 2007a, 2008, 2009b). When plants were grown in fertilized soil, the legume species appeared to lose their advantage in \(W_P\), as shown for the comparison between \(O.\) *macrocalyx* and \(S.\) *macrophylla* in this study (Fig. 2, E and F) and for comparisons between the legume species \(Platymiscium\) *pinnatum* and the nonlegume species \(Tectona\) *grandis* and \(S.\) *macrophylla* in previous studies (Cernusak et al., 2009a, 2009b). Interestingly, the higher \(W_P\) of legumes than nonlegumes in unfertilized soil in this study did not appear to be associated exclusively with \(\text{N}_2\) fixation, because the nonnodulating legume \(S.\) *parahyba* had the highest \(W_P\) of any of the studied species (Fig. 1C). These results suggest that high water-use efficiency may be a general trait of tropical legume trees, consistent with generally high foliar N concentrations and high photosynthetic capacity (McKey, 1994).

**Water-Use Efficiency Responses to Elevated \([\text{CO}_2]\)**

We observed that \(p_i/p_a\) either remained constant or tended to decrease in tropical tree seedlings grown at elevated compared with ambient \(\text{CO}_2\) partial pressure (Figs. 7 and 8). Constant or decreasing \(p_i/p_a\) should lead to a marked increase in \(W_P\) under increasing \(p_a\). This is consistent with our observations (Fig. 1C). If other determinants of \(W_P\), in addition to \(p_i/p_a\), also remained constant at elevated compared with ambient \([\text{CO}_2]\), an increase in \(W_P\) of 75% would be predicted for an increase in \(p_a\) from 40 to 70 Pa. For the 10 species in our study grown in unfertilized soil at high water supply, we observed an average increase in \(W_P\) of 54% for growth at \(p_a\) of 70 compared with 40 Pa. This discrepancy between prediction and observation is most likely explained by an increase...
We observed approximately constant $p_i/p_a$ in response to an increase in $p_a$ from 40 to 70 Pa. The $p_i/p_a$ of tropical trees also appears to have remained approximately constant in response to variation in $p_a$ from preindustrial times to the present, as inferred from tree-ring $\Delta ^{13}C$ (Hietz et al., 2005; Nock et al., 2010; Brienen et al., 2011). A similar pattern was inferred for subtropical vegetation from preindustrial times to the present, based on changes in SD and morphology (de Boer et al., 2011; Lammertsma et al., 2011). Together, these observations suggest that $W_p$ of tropical trees has increased in response to increasing $p_a$ over the past 200 years and will continue to increase in the future as $p_a$ continues to increase. If increases in tree growth in response to increasing $p_a$ are smaller than increases in $W_p$, as was the case in our study (Fig. 1), canopy water use will decrease. This will likely have important consequences for the hydrological cycle and the biogeochemistry of tropical forests, although the implications of the manifold feedbacks between water, carbon, and nutrient cycles are complex and difficult to predict (Galbraith et al., 2010). Nevertheless, the apparently conservative nature of $p_i/p_a$ in tropical trees in response to variable $p_a$ could provide an effective set point for modeling responses of tropical tree growth and water use to variations in atmospheric $\left[ \text{CO}_2 \right]$ (Buckley, 2008).

Figure 8. Stomatal conductance (A and B), net photosynthesis (C and D), $p_i/p_a$ (E and F), and $\Delta ^{13}C$ (G and H) of seedlings of two tropical tree species grown at either ambient (40 Pa) or elevated (70 Pa) CO$_2$ partial pressure and in four soil treatments. Soil treatments were as follows: high water supply, fertilizer addition (+W+N); high water supply, no fertilizer addition (+W−N); low water supply, fertilizer addition (−W+N); and low water supply, no fertilizer addition (−W−N). *O. macrocalyx* is a legume and *S. macrophylla* is a nonlegume. Each bar represents an average of five plants. Error bars represent SE.

Figure 9. Stem dry matter $\delta ^{18}O$ plotted against SD for seedlings of seven tropical tree species. The figure includes plants grown at both ambient (40 Pa) and elevated (70 Pa) CO$_2$ partial pressure. SD was measured on abaxial leaf surfaces. The line is a least-squares linear regression: $\delta ^{18}O = -0.03SD + 23$ ($r^2 = 0.29$, $P < 0.001$, $n = 80$).
We observed a 37% mean reduction in $g_s$ in response to elevated [CO$_2$] for plants grown in unfertilized soil at high water supply (Fig. 7A). For the most part, this reduction in $g_s$ was not caused by reduced SD in response to elevated [CO$_2$]. SD showed a mean reduction of only 6% in response to elevated [CO$_2$], and SI showed a similar mean reduction of only 5%. These results are similar to observations in temperate species, where growth at [CO$_2$] above ambient did not lead to significant reductions in SD or SI (Eistiarte et al., 1994; Reid et al., 2003; Marchi et al., 2004; Tricker et al., 2005; Ainsworth and Rogers, 2007).

The $\delta^{18}O$ of leaf water, and consequently organic material, is expected to increase with decreasing $g_s$, all else being equal (Farquhar and Lloyd, 1993; Barbour and Farquhar, 2000; Cernusak et al., 2004; Farquhar et al., 2007). We observed a mean increase in stem dry matter $\delta^{18}O$ of 0.5% in response to elevated [CO$_2$], qualitatively consistent with this prediction. However, we also observed that some of the variation among species in stem dry matter $\delta^{18}O$ was explained by SD (Fig. 9), whereas the $\delta^{18}O$ did not correlate with $g_s$ across the full data set. This suggests that leaf anatomy may play an important role in determining variations among species in $\delta^{18}O$ enrichment. The relationship shown here between stem dry matter $\delta^{18}O$ and SD may provide a promising avenue for future research into variation in $\delta^{18}O$ enrichment among terrestrial plant species.

**CONCLUSION**

The legume species with the highest nodulation capacity, *O. macrocarpa*, was by far the strongest responder to elevated [CO$_2$] in this study (Figs. 1 and 6). We conclude that legume species with capacity to achieve large nodule mass ratios will respond more strongly to elevated [CO$_2$] than other species; they will likely be among the winners in tropical forests as atmospheric [CO$_2$] rises. Across all species, $p_{L}/p_{x}$ was approximately constant in response to elevated [CO$_2$], and this was supported by measurements of $\Delta^{13}C$ (Figs. 7 and 8). As a result, $W_p$ increased markedly in response to elevated [CO$_2$] (Fig. 1). Similar increases were recorded in fertilized and unfertilized soil and at both high and low water supply (Fig. 2). We conclude that tropical forest trees will likely experience large increases in $W_p$ as atmospheric [CO$_2$] continues to rise over the coming century.

**MATERIALS AND METHODS**

**Plant Material and Experimental Treatments**

Experiments took place at the Santa Cruz Experimental Field Facility, Smithsonian Tropical Research Institute, Gamboa, Panama (9°07'N, 79°42'W). The study site is located at an altitude of approximately 28 m above sea level. Plants were grown in two glasshouses, one of which had CO$_2$ partial pressure similar to ambient (40 Pa) and the other of which had an elevated CO$_2$ partial pressure (70 Pa), maintained by releasing CO$_2$ gas from a high-pressure cylinder when the CO$_2$ partial pressure in the glasshouse declined below 69 Pa. The glasshouses were air conditioned, with the air conditioners programmed to turn on when the air temperature exceeded 30°C. Air temperature and relative humidity were recorded in the two glasshouses every 15 min during experiments with a data logger (CR10X; Campbell Scientific). Average daytime air temperature was 30.4°C in the ambient [CO$_2$] glasshouse and 30.3°C in the elevated [CO$_2$] glasshouse, with average nighttime air temperatures of 27.1°C and 27.2°C in the two glasshouses, respectively. Average daytime relative humidity was 71% in the ambient [CO$_2$] glasshouse and 72% in the elevated [CO$_2$] glasshouse, with average nighttime relative humidity of 86% and 90% in the two glasshouses, respectively. The average CO$_2$ partial pressure recorded in the elevated [CO$_2$] glasshouse was 69 ± 1 Pa (mean ± so); that recorded in the ambient [CO$_2$] glasshouse was 39 ± 3 Pa.

Two experiments were conducted. In the first experiment, 20 seedlings each of *Swietenia macrophylla* (Meliaceae) and *Ormosia macrocarpa* (Fabaceae--Papilionoideae) were grown in each of the two glasshouses. Seeds were collected from trees growing in the Panama Canal watershed. Seedlings were grown individually in 18-L pots. Each pot contained 13.5 kg of dry homogenized soil mixture. The soil mixture comprised 60% by volume dark, air-dried forest topsoil and 40% by volume air-dried rice husks. The rice husks were added to improve soil structure and drainage. The pots were saturated with water and drained overnight to establish the pot water content at field capacity, which was determined to be 5.0 kg. The soil surface of each pot was then covered with 1.5 kg of gravel to reduce evaporation. Ten control pots with no plants were placed in each glasshouse along with the 40 pots containing plants to estimate pot water loss due to evaporation from the soil surface.

Within each glasshouse, two fertilizer treatments and two water supply treatments were deployed. At the beginning of the experiment, 10 of the 20 pots for each species were randomly chosen to receive approximately 12 g of Osmocote-Plus controlled-release fertilizer (Scotts-Sierra). The fertilizer contained by weight 15% N, 9% P, and 12% potassium and had an estimated release time of 5 to 6 months. Five fertilized and five unfertilized pots from each species were then randomly allocated to receive reduced water supply. All pots started the experiment watered to field capacity. Those receiving high water supply were weighed each week and rewatered to near field capacity. Later in the experiment, pots were weighed and rewatered at shorter intervals, depending on water loss rates. Pots receiving low water supply were allowed to dry down to pot water contents of less than 1.5 kg, or approximately 30% of field capacity, over several weeks. Thereafter, they were weighed and rewatered to this pot water content each week, or at shorter intervals, as necessary. Pots were weighed to the nearest 5 g with a 64-kg-capacity balance (Sartorius Q564B; Thomas). Drain holes at the bases of the pots were sealed for the duration of the experiment.

Pots were placed in the ambient and elevated [CO$_2$] glasshouses on January 8, 2007, approximately 1 month after seedlings had germinated. Within each glasshouse, the pots were placed on plastic tables, such that they were elevated approximately 1 m above the glasshouse floor. Initial plant dry mass was estimated by harvesting five representative individuals of each species. Mean values were 0.3 g and 0.2 g for *O. macrocarpa* and *S. macrophylla*, respectively. Plants were harvested on April 26, 2007, after more than 3 months of growth at either ambient or elevated [CO$_2$].

The second experiment was similar to the first, except that six individuals of each of eight tree species were grown in each of the two glasshouses. The eight species were *Albizia adianthifolia* (Fabaceae--Mimosoideae), *Chrysophyllum cainito* (Sapotaceae), *Coccoloba uvifera* (Polygonaceae), *Dalbergia retusa* (Fabaceae--Papilionoideae), *Hieronyma alchorneoides* (Phyllanthaceae), *Inga punctata* (Fabaceae--Mimosoideae), *Pachira quinata* (Malvaceae), and *Schizolobium parahyba* (Fabaceae--Caesalpinioideae). This experiment involved only one soil treatment for each species, corresponding to the high-water-supply, unfertilized treatment for each species. Preparation of the pots followed the same method as in the first experiment. Seedlings of the eight species were obtained from the nursery of the Native Species Reforestation Project, based at the Smithsonian Tropical Research Institute. They were approximately 2 months old when the experiment began. The seedlings were transplanted to the experimental pots on June 20, 2007, with one seedling in each pot. They were allowed 2 weeks to recover from transplanting before being placed in the ambient and elevated [CO$_2$] glasshouses on July 3, 2007. Five control pots with no plants were placed in each glasshouse to estimate evaporation from the soil surface. Initial dry mass at the beginning of the experiment for each species was 0.2, 0.6, 0.6, 0.3, 0.5, 0.2, 0.2, and 1.0 g, given in the same order as the species names are given above. Plants were harvested on October 1, 2007, after 3 months of growth under ambient or elevated [CO$_2$].
The forest topsoil used in the experiments had a relatively high P availability. Soil obtained from the same forest area and used in a recent pot experiment had a mean extractable P concentration of 15 μg P g⁻¹ dry soil, determined by extraction in Bray solution (30 mM NH₄F and 25 mM HCl). This extractable P concentration is in the high range of values typically observed for neotropical rainforest soils (Clinebell et al., 1995; Turner and Engelbrecht, 2011). We previously observed that the addition of rice husks to the soil mixture differentially reduced N availability relative to P availability, presumably due to microbial immobilization of available N (Cernusak et al., 2007b). Based on these considerations, the unfertilized soil treatments in our experiments likely had relatively low N availability compared with P availability. The mean leaf N:P ratio of all plants grown in unfertilized soil at high water supply in the two experiments was 10.8 g g⁻¹, consistent with this assessment.

We did not add an artificial source of rhizobia to the experimental soil mixture but rather relied on bacteria already present in the forest topsoil to induce nodulation in the leguminous seedlings.

### Water Use and Biomass Measurements

Cumulative plant water use 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. Pot water loss was determined for each weekly or subweekly interval in the experiment from the gravimetric measurements described above. Half the control pots in the first experiment were allowed to dry down to a pot water content of 1.5 kg to estimate soil evaporation for the low-water-supply treatment. The other half were returned to a pot water content of 5.0 kg each week, as done for the high-water-supply treatment. Immediately following the harvest in each experiment, total leaf area of each plant was measured with a Li-3100 leaf area meter (Li-Cor). Harvested plants were dried to constant mass at 70°C, and dry masses of leaves, stems, and roots was determined separately for each plant to the nearest 0.02 g. For the nodulated legumes, nodules were removed from dried roots, and the nodule dry mass was determined. Nodule mass ratio was then calculated as nodule dry mass divided by total plant dry mass. For *D. retusa*, nodules were removed from roots of only one plant per [CO₂] treatment, because the small size and disaggregated distribution of nodules in this species made them very difficult to separate from roots.

The water-use efficiency of each plant was calculated as \( W_u = \frac{M_n - M_c}{M_o} \), where \( M_n \) and \( M_c \) are the plant carbon mass at the beginning and end of the experiment, \( M_o \) is the carbon mass of leaves absconded over the course of the experiment, and \( E_u \) is the sum of water transpired over the course of the experiment.

### Leaf Gas-Exchange Measurements

The \( A_{\text{soil}}, g_{\text{soil}}, \) and \( p_{\text{soil}}/p_{\text{air}} \) were measured on the youngest fully expanded leaf on each plant using a Li-6400 portable photosynthesis system (Li-Cor). Measurements were made at a photon flux of 1,500 μmol m⁻² s⁻¹, which was supplied by an artificial light source (Li-Cor). Mean \( \delta^13C \) during measurements was 2.0 ± 0.4 kPa (mean ± so), and mean leaf temperature was 34°C ± 1°C. The [CO₂] in the gas-exchange cuvette during measurements was similar to the growth [CO₂]; the mean value for measurements in the ambient [CO₂] glasshouse was 381 ± 11 μmol mol⁻¹; that for measurements in the elevated [CO₂] glasshouse was 701 ± 12 μmol mol⁻¹. Measurements in the first experiment took place on April 18 and 20, 2007, and the two sets of measurements were averaged for each plant. Measurements for the second experiment took place on September 22, 2007. Thus, in each experiment, leaf gas-exchange measurements took place following approximately 3 months of growth in the respective glasshouses, and the leaves that were measured had expanded under the conditions imposed by the different experimental treatments.

### Stable Isotope and Elemental Analyses

Leaves, stems, and roots of each plant were ground separately to a fine, homogeneous powder. Samples of approximately 2 mg were analyzed for \( \delta^13C \), \( \delta^15N \), [C], and [N] with an elemental analyzer (CE Instruments) coupled to an isotope ratio mass spectrometer (Delta V; Thermo Fisher Scientific) in the Stable Isotope Laboratory of the Smithsonian Tropical Research Institute. Approximately 1 mg of stem dry matter of each plant was analyzed for \( \delta^18O \) on an isotope ratio mass spectrometer (Delta XP; Finnigan MAT) following pyrolysis in a high-temperature furnace (Thermoquest TC/EA; Finnigan MAT) at the Stable Isotope Core Laboratory, Washington State University. The \( \delta^13C \), \( \delta^18O \), and \( \delta^15N \) values were expressed relative to standards of PeeDee Belemnite, Vienna Standard Mean Ocean Water, and air, respectively. The \( \Delta^13C \) of plant material was calculated as \( \Delta^13C = (\delta^13C - \delta^13C_{\text{soil}})/(1 + \delta^13C_{\text{soil}}) \), where \( \delta^13C \) is the δ13C of CO₂ in air in each glasshouse and \( \delta^13C_{\text{soil}} \) is the δ13C of plant carbon. The \( \delta^13C \) was estimated by growing two species of *C. retusa* plant in each glasshouse and outside in the open air. The *C. retusa* plant species were *Saccharum spontaneum* and *Portulaca oleracea*. The δ13C of source CO₂ for plants grown outside was assumed to be ~8‰. This value of \( \delta^13C \) was used to calculate the δ13C for each C₄ species. The δ13C for the C₃ species was assumed to be the same in the glasshouse and outside and was then used to estimate \( \delta^13C \) in each glasshouse. Using this method, we estimated that \( \delta^13C \) in the ambient [CO₂] glasshouse was ~9‰ and that \( \delta^13C \) in the elevated [CO₂] glasshouse was ~7‰. The supplier of the tank CO₂ used to fumigate the elevated [CO₂] glasshouse (Acré-Oxygen) indicated that the CO₂ originated from a CO₂ spring, and not from fossil fuel combustion, consistent with our estimation of \( \delta^13C \) in the elevated [CO₂] glasshouse.

### SD and SI

In the second experiment, leaves were sampled from plants grown in each glasshouse for measurements of SD and SI. Leaves were collected on September 25, 2007, approximately 1 week before the plants were harvested. The sampled leaves were the same as those that had been measured for leaf gas exchange (i.e. the youngest fully expanded leaf on each plant). 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 observed with a light microscope (Nikon Eclipse 200) at 40× magnification. Three fields were observed for each leaf. The total number of stomatal and epidermal cells was counted in each field. The three fields together covered an area appropriate for calculation of the SI. The SI was defined as SD (volume [%]) = [SD/(SD + ED)] × 100, where SD is the SD (mm⁻²) and ED is the epidermal cell density (mm⁻²). The species *C. cinifolium* was excluded from these analyses, because the pubescence on the abaxial surface of its leaves obscured the epidermal impressions.

### Statistical Analyses

Relationships between continuous variables were analyzed by least-squares linear regression. A nested ANOVA was used to test for effects of elevated [CO₂] on growth, leaf gas exchange, stable isotope composition, water-use efficiency, and other response variables under conditions of high water supply in unfertilized soil. The eight species from the second experiment and the two species from the first experiment with the corresponding soil treatment were analyzed together. Species were nested into legumes and nonlegumes to test whether the two groups differed in their responses to elevated [CO₂]. Separate ANOVAs were conducted to test for effects of the different soil treatments and elevated [CO₂] on response variables in *S. macrophylla* and *O. macrocalyx* in the first experiment. Where necessary, data were log transformed prior to analyses to meet assumptions of normality and homogeneity of variance. Results were considered statistically significant at \( P < 0.05 \). Statistical analyses were carried out in Systat 12 (Systat Software).

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