

# The impact of elevated CO<sub>2</sub> and water deficit stress on growth and photosynthesis of juvenile cacao (*Theobroma cacao* L.).

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## Abstract

Atmospheric CO<sub>2</sub> concentration continues to rise and is predicted to reach approximately 700 ppm by 2100. Some predictions suggest that the dry season in West Africa could be extended with climate change. This study examined the effects of elevated CO<sub>2</sub> concentration and water deficit on growth and photosynthesis of juvenile cacao. Light-saturated photosynthesis ( $P_{\max}$ ), quantum efficiency, and intrinsic water-use efficiency increased significantly in response to elevated CO<sub>2</sub>, as did a range of growth and development responses (*e.g.* leaf area and leaf number), but the magnitude of the increase was dependent on the water treatment. Stomatal index was significantly greater in the elevated CO<sub>2</sub> treatment; an atypical response which may be a reflection of the environment in which cacao evolved. This study shows a positive effect of elevated CO<sub>2</sub> on juvenile cacao which may help to alleviate some of the negative impacts of water deficit stress.

*Additional key words:* abiotic stress; climate change; gas exchange; growth; photosynthesis.

## Introduction

Cacao (*Theobroma cacao*) is one of the most important perennial cash crops grown in the world. Cacao is a tropical tree species which originates from the Upper Amazon (Motamayor *et al.* 2002). It is now grown in tropical regions in West Africa, Central and South America, and South-East Asia (Wood 1985a). The beans produced from the tree are used in the production of chocolate and confectionary. The majority of production comes from West Africa, which supplies about 74% of world cocoa output (ICCO 2017).

By the end of the century, atmospheric CO<sub>2</sub> concentration is predicted to reach about 700 ppm (IPCC 2013). Carbon dioxide is the primary substrate for photosynthesis and plant growth and, at current concentrations, limits photosynthetic rates in C<sub>3</sub> plants. Therefore, increases in the amount of CO<sub>2</sub> available in the atmosphere for uptake by plants will have considerable effects on photosynthesis and plant growth. Increases in atmospheric CO<sub>2</sub> generally impact on C<sub>3</sub> plant photosynthesis by increasing  $P_{\max}$ , reducing stomatal conductance ( $g_s$ ) and transpiration rate ( $E$ ) and often, improving water-use efficiency (WUE) as a consequence. An increase in CO<sub>2</sub> concentration from 380 ppm to about 550–700 ppm has the potential to increase photosynthetic rates in C<sub>3</sub> plants by an average of

about 38% (Long *et al.* 2004). Trees are predicted to have the greatest response, with an average 47% increase in  $P_{\max}$  (Ainsworth and Long 2005).

Little research has been carried out on tropical perennial plant responses to increasing atmospheric CO<sub>2</sub> concentrations. It has been suggested that warm tropical temperatures increase the potential for stimulation of net photosynthesis by elevated CO<sub>2</sub> through suppression of photorespiration compared to predictions for plants growing in cooler temperate environments (Cernusak *et al.* 2013). Short-term experiments have been carried out to examine responses of cacao to elevated CO<sub>2</sub>. In a study by Baligar *et al.* (2005) cacao seedlings were grown at CO<sub>2</sub> concentrations of 380 and 700 ppm for 57 d. Plants grown at 700 ppm had increased root, shoot and leaf dry masses, stem height, leaf area, shoot/root ratio and relative growth rate compared to plants grown at ambient CO<sub>2</sub>. Baligar *et al.* (2008) examined the instantaneous photosynthetic responses of 1.5 year old cacao seedlings to increasing CO<sub>2</sub> concentration. Photosynthesis was measured at CO<sub>2</sub> concentrations of 850, 680, 240, 160, and 85 ppm. Increasing CO<sub>2</sub> between 370–680 ppm resulted in higher  $P_{\max}$  (+33%), reduced  $g_s$  (–65%) along with reduced  $E$ . Increasing CO<sub>2</sub> beyond 680 ppm resulted in minimal

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*Abbreviations:* DAE – day of experiment;  $E$  – transpiration rate;  $g_s$  – stomatal conductance; WUE – instantaneous water-use efficiency; LCP – light-compensation point; LSP – light-saturation point;  $P_{\max}$  – light-saturated photosynthetic rate; SD – stomatal density; SI – stomatal index; SLA – specific leaf area; WUE<sub>i</sub> – intrinsic water-use efficiency;  $\Phi$  – quantum efficiency;  $\Psi$  – water potential.

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increase in photosynthesis (Baligar *et al.* 2008). Three different cacao genotypes were assessed in this study (CCN 51, LCT EEN 37/A, and VB-1117) but no differences in response to CO<sub>2</sub> were observed between these genotypes (Baligar *et al.* 2008).

Rainfall has been described as the most important environmental factor influencing cacao yields (Wood 1985b, Zuidema *et al.* 2005). An average rainfall of 1,400–2,000 mm per year is sufficient to support growth of cacao trees. Less than 1,200 mm per year has been reported to result in soil water deficits and reduced growth and yield (Alvim 1977). However, the distribution of rainfall throughout the year is also important. Cacao trees can tolerate dry periods (where evapotranspiration is greater than rainfall) of about 3 months. A long period of dry weather can have substantial negative impacts on cacao tree growth, with younger trees being more sensitive to soil moisture deficit than older, more established trees (Moser *et al.* 2010).

Reduced photosynthetic rates in cacao are associated with lower leaf water potential ( $\Psi$ ) values (Balasimha *et al.* 1991). At leaf  $\Psi$  below  $-1.5$  MPa,  $g_s$  and photosynthesis decline significantly in cacao (Deng *et al.* 1990, Mohd Razi *et al.* 1992). Stomatal closure reduces transpirational water loss to a greater extent than it does CO<sub>2</sub> uptake and WUE in cacao is often reported to increase under water stress conditions (Rada *et al.* 2005). Leaf area expansion is also reduced in water-stressed cacao plants (Joly and Hahn 1989, Deng *et al.* 1989), which occurs before a reduction in photosynthetic rate (Deng *et al.* 1989). A dry season is experienced in many cacao growing regions of

West Africa as well as some cacao growing regions in South America and Southeast Asia. Changes in precipitation due to climate change are predicted to vary greatly across the globe, with dry regions getting drier while wet regions are predicted to see increased rainfall (IPCC 2013). In West Africa the frequency and intensity of drought appears to have increased since 1950 (IPCC 2013).

The most obvious effect of elevated CO<sub>2</sub> with regard to water relations is an increase in whole plant WUE due to greater growth rates at elevated CO<sub>2</sub> (Chaves and Pereira 1992). Partial stomatal closure brought about under both water deficit and elevated CO<sub>2</sub> reduces plant water loss and the higher atmospheric CO<sub>2</sub> concentration increases C assimilation/biomass production. To date no research has been reported on how elevated CO<sub>2</sub> and soil moisture deficit interact to influence photosynthesis and growth in cacao.

This study aimed to examine the growth and photosynthetic responses of juvenile cacao to growth in an elevated CO<sub>2</sub> environment alone (experiment A) and in combination with soil water deficit (experiment B). The hypotheses tested in this study were: (1) growth of juvenile cacao plants in an elevated CO<sub>2</sub> environment would enhance  $P_{max}$ ; (2) growth parameters of these plants would be enhanced as a result of increased assimilation; (3) WUE<sub>i</sub> of cacao would be improved in an elevated CO<sub>2</sub> environment due to increased CO<sub>2</sub> uptake and/or reduced rates of  $g_s$ ; (4) the negative impact of soil water deficit on growth and photosynthesis in cacao plants would be ameliorated when grown at elevated CO<sub>2</sub> due to enhanced CO<sub>2</sub> uptake and an improvement in WUE<sub>i</sub> resulting from increased  $P_{max}$  and reduced water loss through  $g_s$ .

## Materials and methods

**Greenhouse conditions:** The experiments were conducted within four compartments in a 2 × 2 square arrangement of a greenhouse suite specifically designed to study the effects of climate change on cacao. Two compartments were maintained at elevated CO<sub>2</sub> (700 ppm) and two at ambient CO<sub>2</sub>, each treatment being represented on either side of the square. The CO<sub>2</sub> enrichment system was controlled by a centralised computer system (T200, Tomtech, Spalding, Lincs, UK). In all compartments a wall-mounted infrared gas analyser (MYCO<sub>2</sub> Gascard II, Edinburgh Sensors, Livingstone, UK) was installed which continually measured the CO<sub>2</sub> concentration of the air within each compartment. The target CO<sub>2</sub> concentration in the elevated CO<sub>2</sub> compartments was set to 700 ppm. The flue gas from the natural gas boiler within each compartment was used for CO<sub>2</sub> enrichment. CO<sub>2</sub> enrichment was achieved by blowing flue gases from the heater *via* a fan into the compartment through plastic lay-flat ducts which ran along either side of the compartment about 30 cm from the floor. The flue gas produced initially after heater ignition was directed out of the compartment; only once clean combustion gas was produced, the CO<sub>2</sub> unit was switched on to direct CO<sub>2</sub> into the compartment. Air

circulatory fans were suspended from the ceiling in each compartment to improve air mixing. Temperature conditions within each compartment were set to mimic typical conditions in Ghana in January through a combination of heating and venting. The target temperature was set to cycle between a minimum of 19°C and a maximum of 32°C. The minimum temperature was reached at 06:00 h and the maximum temperature at 14:00 h.

## Plant culture

**Experiment A:** Seeds of *T. cacao* (var. Amelonado) were sown into a 1:2:2 (v/v) mixture of sand, gravel, and vermiculite on 25 July 2011 at the International Cocoa Quarantine Centre at the School of Agriculture, Policy and Development Field Unit, Shinfield, UK. These plants were repotted into 5-L pots on 14 November 2011 and transferred to a greenhouse facility at the University of Reading where temperature conditions were maintained as described above under ambient CO<sub>2</sub> concentration. They were watered six times daily with a modified Long Ashton solution (End 1990) throughout the experimental period.

The four-month-old plants were grown under two different CO<sub>2</sub> concentrations for 154 d (between

Table 1. Environmental conditions within each greenhouse compartment during the experimental period (Experiment A).

		CO <sub>2</sub> [ppm]	Temperature [°C]	Relative humidity [%]
Ambient CO <sub>2</sub> compartment 1	Average	410	26.8	51
	Min.	345	19.4	34
	Max.	464	34.1	70
Ambient CO <sub>2</sub> compartment 2	Average	380	26.8	51
	Min.	344	19.6	34
	Max.	431	33.7	73
Elevated CO <sub>2</sub> compartment 1	Average	724	27.2	50
	Min.	594	19.4	34
	Max.	865	35.5	68

30 November 2011 and 2 May 2012). Eighteen plants were placed in each of the four greenhouse compartments. Due to a breakdown in the CO<sub>2</sub> system in one compartment early on in the experiment, 36 plants were grown in a single compartment with elevated CO<sub>2</sub> facilities.

**Experiment B:** Seeds of *T. cacao* (var. Amelonado) were sown in a sand:gravel:vermiculite (1:2:2) mixture on 5 January 2012 and transferred into 5 L pots containing a 2:1

mixture of John Innes No.2 and vermiculite on 7 August 2012. This potting mix allowed for better control of soil moisture content than that used in the experiment A. Prior to the beginning of the experiment, the plants were transferred into 10-L pots on 23 October 2012 containing the same growing medium. The pots were watered daily and supplemented with a modified Long Ashton solution (End 1990) as necessary. The experiment began on 6 November 2012 and ran for 13 weeks until 7 February 2013.

Table 2. Environmental conditions within each greenhouse compartment during the experimental period (Experiment B).

		CO <sub>2</sub> [ppm]	Temperature [°C]	Relative humidity [%]
Ambient CO <sub>2</sub> compartment 1	Average	453	26.2	53.3
	Min.	408	19.3	41.4
	Max.	493	32.2	65.7
Ambient CO <sub>2</sub> compartment 2	Average	480	26.8	52.7
	Min.	439	20	40.7
	Max.	548	32.7	66.9
Elevated CO <sub>2</sub> compartment 1	Average	738	26.6	52.2
	Min.	615	19.9	40.4
	Max.	916	32.5	62.6
Elevated CO <sub>2</sub> compartment 2	Average	731	26.4	52.8
	Min.	620	20	42
	Max.	910	32.3	64.2

Ten plants were placed in one of four greenhouse compartments. Five plants in each compartment were designated for the well-watered treatment and five were designated for the water deficit treatment. All pots were watered to saturation on the afternoon of 5 November 2012 and allowed to drain overnight. A plastic cover was placed on top of the soil in order to prevent evaporative water loss from the soil surface. On the morning of 6 November 2012 each pot was weighed to determine the field capacity mass. Throughout the experiment each pot was weighed every second day to determine water use, at the same time the soil moisture within the pot was also measured using a soil moisture sensor (*SM300*, *Delta T Devices*, Cambridge, UK). Field capacity of the soil on day 1 of the experiment (DAE) was 40%. The well-watered plants received the same amount of water as was used in order to return the

soil back to field capacity. The plants under the water deficit treatment received 25% of the water used until the soil moisture within the pot fell to between 15–20% (as determined by moisture probe measurements). When this point was reached the pots were then watered back to the mass corresponding to soil moisture of approximately 20% at each watering event. The average soil moisture (measured before watering) throughout the whole experiment was 34.4 and 16.4%, for well-watered and water deficit pots, respectively. To maintain an adequate nutrient supply the irrigation water was supplemented with 100 ml of modified Long Ashton nutrient solution once a week.

**Photosynthetic light-response curves:** Photosynthetic light-response curves were carried out on the youngest fully matured and hardened leaf on each plant, which had

fully developed under experimental conditions, using an *LCpro+* portable infrared gas analyser fitted with a light attachment and an internal CO<sub>2</sub> source (*ADC BioScientific*, Great Amwell, Herts, UK). Measurements were made between 07:00 and 14:00 h. Photosynthetic rate was measured at eight irradiance intensities [696, 435, 261, 174, 87, 44, 26, 0 μmol(photon) m<sup>-2</sup> s<sup>-1</sup>] and at growth CO<sub>2</sub> concentration. The photosynthetic rate was allowed to stabilise at each irradiance level before a record was taken.

Photosynthetic light-response curves were fitted by means of a nonrectangular hyperbola in the form:  $P = \{\Phi Q + P_{\max} - \sqrt{[(\Phi Q + P_{\max})^2 - 4 \Phi Q k P_{\max}]/2k}\} - R$ , where  $\Phi$  is apparent quantum efficiency,  $Q$  is irradiance,  $P_{\max}$  is light-saturated photosynthetic rate,  $k$  is convexity, and  $R$  is respiration rate. *Photosyn Assistant* software (*Dundee Scientific*, Dundee, UK) was used to fit a nonrectangular hyperbola to the photosynthetic data. Photosynthetic parameters  $P_{\max}$ ,  $\Phi$ , respiration rate, light-compensation point (LCP), and light-saturation point (LSP) were estimated from the data.

**Experiment A:** Photosynthesis measurements were carried out between 12 April 2012 and 1 May 2012. Twelve plants in each ambient greenhouse compartment and 24 plants in the one elevated CO<sub>2</sub> greenhouse compartment were measured. The average CO<sub>2</sub> concentration within the chamber was 409 ppm and 711 ppm for the ambient and elevated CO<sub>2</sub> grown plants, respectively. Average temperature within the IRGA chamber was 31°C and average relative humidity was 47%. Stomatal conductance ( $g_s$ ) and  $E$  were recorded at the highest irradiance level [696 μmol(photon) m<sup>-2</sup> s<sup>-1</sup>] when photosynthesis was light-saturated.

**Experiment B:** Photosynthesis measurements were carried out between 26 January 2013 and 6 February 2013. The average CO<sub>2</sub> concentration in the IRGA chamber for the ambient and elevated CO<sub>2</sub> grown plants was 459 ppm and 712 ppm, respectively. The temperature in the IRGA chamber ranged from 29–31°C during measurements. Average relative humidity was 55% during measurements.

**Growth measurements:** Similar growth measurements were recorded in both experiments A and B. Interflush period was measured as the number of days between the unrolling of the last leaf of flush 1 and the first leaf of flush 2. The plants were destructively harvested following 154 and 90 DAE under experimental conditions in experiment A and B, respectively. Plants were cut from the base of the stem and leaf number, stem length, leaf and stem fresh and dry mass recorded for each plant. Total leaf area was measured for each plant using a *WD3 WinDIAS* leaf area meter (*Delta T Devices Ltd.*, Cambridge, UK). The fresh material was oven dried at 70°C to a constant

mass to determine leaf and stem dry mass. Specific leaf area (SLA) was calculated as total leaf area divided by total leaf dry mass. In experiment B, stem diameter increase was calculated by measuring the diameter (using callipers, at 10 cm from the base of the plant) at the beginning of the experiment and again prior to harvest. In experiment B, the leaves used for photosynthetic measurements were oven dried and ground to a fine powder for carbon and nitrogen analysis. The carbon and nitrogen content of the powdered samples were analysed using a *Leco SC-144DR* carbon analyser and a *Leco FP-328* nitrogen analyser (*LECO Corporation*, Michigan, USA).

**Stomatal measurements:** Epidermal impressions were made on the abaxial surface of one mature leaf per plant which had developed in the most recent flush and under experimental conditions. A thin layer of clear nail polish was applied, allowed to dry and removed with sellotape and mounted onto a glass slide. Three digital images of each peel were obtained using an *Axioscope 2* microscope with an *Axiocam* camera attached (*Carl Zeiss*, Jena, Germany) using *Axio Vision 3.1* software (*Image Associates*, Oxon, UK). In experiment A, the number of stomata per unit area was counted and guard cell length was measured using *ImageJ* software. Guard cell length of ten stomata per image was measured. In experiment B, both stomatal and epidermal cells were counted in each image. Stomatal index (SI) was calculated according to the formula: [stomata number/(epidermal cell number + stomata number)] × 100 (Salisbury 1927).

**Data analysis:** Data were analysed for differences and interactions between CO<sub>2</sub> and water treatments by analysis of variance using *Genstat 13<sup>th</sup> edition* (where unequal replicates were available unbalanced ANOVA was performed). Data transformation (log or square-root) was carried out in order to normalise the data where appropriate. Judgement of normality was based on the distribution of residuals in plotted residual histograms and fitted-value plots in *Genstat*. All analyses were performed, where necessary on the transformed data; untransformed data means and standard errors are reported. *Kruskal-Wallis* one-way ANOVA was carried out on non-normal data which could not be normalised through transformation. It was assumed that there was no variation between the four greenhouse compartments other than the different environmental treatments imposed. To test for positional effects in experiment B, ANOVA analysis was carried out twice on all data, applying both vertical and horizontal blocking. The block effect was not significant in any of the analyses providing confidence that observed effects were due to the treatments imposed rather than positional effects of the greenhouse.

## Results

### Experiment A

**Photosynthesis:** Overall, growth in the elevated CO<sub>2</sub> environment enhanced  $P_{\max}$  by an average of 105%, while  $\Phi$  was increased by 78% in plants grown at elevated CO<sub>2</sub> compared with plants grown under ambient CO<sub>2</sub> conditions; this was also accompanied by a decline in LCP [from 11.7 ( $\pm$  1.1) to 6 ( $\pm$  0.09)  $\mu\text{mol m}^{-2} \text{s}^{-1}$ ] (Fig. 1). Water-use efficiency, both intrinsic ( $P_{\max}/g_s$ ) and instantaneous ( $P_{\max}/E$ ), increased significantly by 242.6 and 65.8%, respectively, compared to plants grown under ambient CO<sub>2</sub> conditions, due to the enhancement of photosynthesis at elevated CO<sub>2</sub>, as neither  $g_s$  or  $E$  were significantly affected.

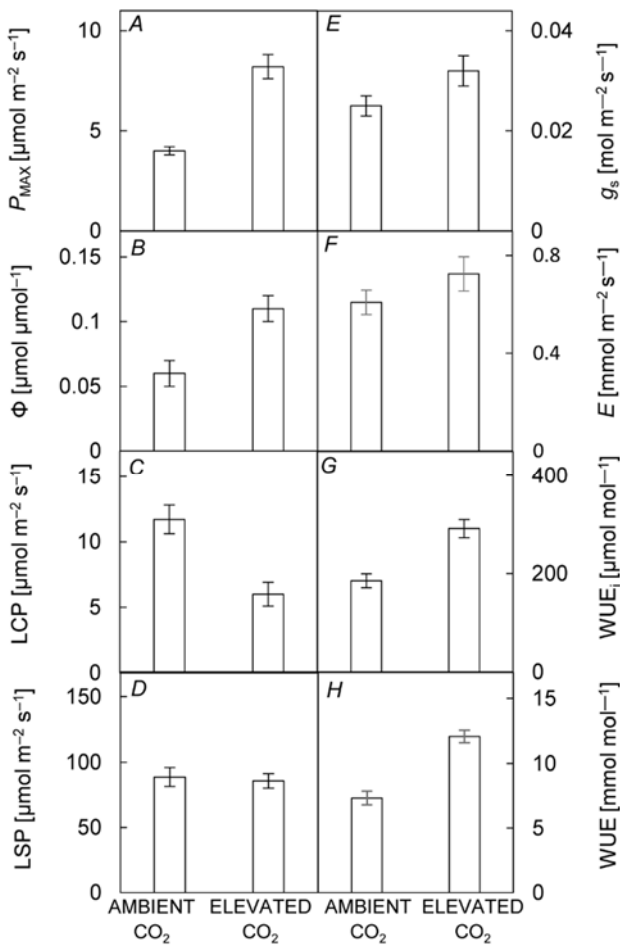


Fig. 1. Photosynthetic responses of cacao plants (var. Amelonado) grown at elevated and ambient CO<sub>2</sub>. (A) light-saturated photosynthetic rate ( $P_{\max}$ ); (B) quantum efficiency ( $\Phi$ ); (C) light-compensation point (LCP); (D) light-saturation point (LSP); (E) stomatal conductance ( $g_s$ ); (F) transpiration rate ( $E$ ); (G) intrinsic water-use efficiency ( $WUE_i$ ); (H) instantaneous water-use efficiency ( $WUE$ ).  $n = 24$ . Error bars represent SE (Experiment A).

**Growth:** Leaf production and stem elongation was greater in plants grown at elevated CO<sub>2</sub> compared to those grown at ambient (Fig. 2). In flush 1, an average of eight and nine leaves were produced in ambient and elevated CO<sub>2</sub>, respectively. This increased to an average of 12 and 15 leaves per flush in flush 2 in the ambient and elevated CO<sub>2</sub> environments, respectively. In flush 1, average stem elongation was 17.5 ( $\pm$  0.91) cm and 24.4 ( $\pm$  1.37) cm in ambient and elevated CO<sub>2</sub>, respectively. In flush 2, average stem elongation was 32.4 ( $\pm$  1.31) cm and 39.2 ( $\pm$  1.65) cm in ambient and elevated CO<sub>2</sub>, respectively. Total leaf fresh and dry mass were both significantly increased in the elevated CO<sub>2</sub> treatment (26.9 and 28.5%, respectively) compared with plants grown under ambient conditions. Total leaf area increased significantly by 18.7% in the elevated CO<sub>2</sub> grown plants (Fig. 2).

Stomatal density (SD) increased significantly from an average of 754 ( $\pm$  28) stomata  $\text{mm}^{-2}$  at ambient CO<sub>2</sub> to an average of 830 ( $\pm$  20) stomata  $\text{mm}^{-2}$  in leaves which developed under elevated CO<sub>2</sub>. Average stomatal length was 12.7  $\mu\text{m}$  and did not change in response to the CO<sub>2</sub> treatment.

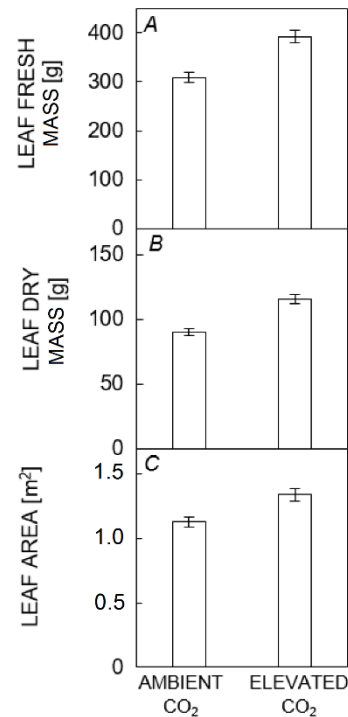


Fig. 2. Growth responses of cacao plants (var. Amelonado) grown at ambient and elevated CO<sub>2</sub>. (A) leaf fresh mass; (B) leaf dry mass; (C) leaf area.  $n = 36$  (ambient CO<sub>2</sub>),  $n = 34$  (elevated CO<sub>2</sub>), Error bars represent SE (Experiment A).

**Experiment B**

**Photosynthesis:** Cacao plants grown at elevated CO<sub>2</sub> had a significantly higher  $P_{\max}$  than those grown at ambient CO<sub>2</sub>. Average  $P_{\max}$  in ambient CO<sub>2</sub>-grown plants was  $2.78 (\pm 0.23) \mu\text{mol m}^{-2} \text{s}^{-1}$ ; this increased by 56% to  $4.33 (\pm 0.34) \mu\text{mol m}^{-2} \text{s}^{-1}$  in plants grown at elevated CO<sub>2</sub>. In well-watered plants the average  $P_{\max}$  was  $4.44 (\pm 0.32) \mu\text{mol m}^{-2} \text{s}^{-1}$ .  $P_{\max}$  decreased significantly in the water deficit treatment to an average of  $2.67 (\pm 0.21) \mu\text{mol m}^{-2} \text{s}^{-1}$ . The percentage increase in  $P_{\max}$  in response to elevated CO<sub>2</sub> was similar in both water treatments hence there was no interaction between the treatments (Fig. 3). There was no effect of CO<sub>2</sub> concentration or water treatment on the respiration rate in the plants (Fig. 3). The  $\Phi$  of plants grown at elevated CO<sub>2</sub> ( $0.064 \pm 0.006 \mu\text{mol} \mu\text{mol}^{-1}$ ) was significantly greater than that of plants grown at ambient CO<sub>2</sub> ( $0.049 \pm 0.005 \mu\text{mol} \mu\text{mol}^{-1}$ ). The effect of the water deficit treatment on  $\Phi$  was on the borderline of significance ( $P=0.057$ ). On average there was a ~22% decline in  $\Phi$  in response to the water deficit treatment. However, the decrease in response to water deficit appeared to be greater in plants grown at elevated CO<sub>2</sub> which experienced a 28% decrease compared to an 11% decrease in ambient CO<sub>2</sub> grown plants, but the interaction term was not significant (Fig. 3). LCP decreased by 30% from an average of  $10.2 \pm 1.1 \mu\text{mol m}^{-2} \text{s}^{-1}$  at ambient CO<sub>2</sub>

to an average of  $6.8 \pm 0.6 \mu\text{mol m}^{-2} \text{s}^{-1}$  in plants grown at elevated CO<sub>2</sub>. The water deficit treatment caused a significant increase in LCP compared to the well-watered plants, from  $7.1 \pm 0.7 \mu\text{mol m}^{-2} \text{s}^{-1}$  to  $9.9 \pm 1.0 \mu\text{mol m}^{-2} \text{s}^{-1}$ . Although the interaction was not significant, the decrease in LCP at elevated CO<sub>2</sub> appeared to be greater in the well-watered than that of the water-deficit plants (–40 and –27%, respectively) (Fig. 3). There was no effect of CO<sub>2</sub> treatment on LSP. There was a slight reduction (–17%) in LSP in the plants grown under water deficit which was approaching significance ( $P=0.054$ ) (Fig. 3). CO<sub>2</sub> concentration did not have an effect on  $g_s$ . The water deficit treatment resulted in a significant reduction in  $g_s$ , from an average of  $0.031 \pm 0.003 \text{mmol m}^{-2} \text{s}^{-1}$  in well-watered plants to  $0.015 \pm 0.001 \text{mmol m}^{-2} \text{s}^{-1}$  in the water deficit-treated plants (Fig. 3).

Intrinsic water-use efficiency ( $WUE_i$ ) was significantly greater in plants grown at elevated CO<sub>2</sub>. Plants grown at ambient CO<sub>2</sub> had an average  $WUE_i$  of  $115.7 \pm 7.4 \mu\text{mol mol}^{-1}$ , this increased by 44% to  $166.2 \pm 12.5 \mu\text{mol mol}^{-1}$  in those grown at elevated CO<sub>2</sub>. The increase is mainly due to an increase in  $P_{\max}$  as there was no difference measured in  $g_s$  between the ambient and elevated CO<sub>2</sub> treatment. The water deficit treatment did not have a significant effect on  $WUE_i$  (Fig. 3).

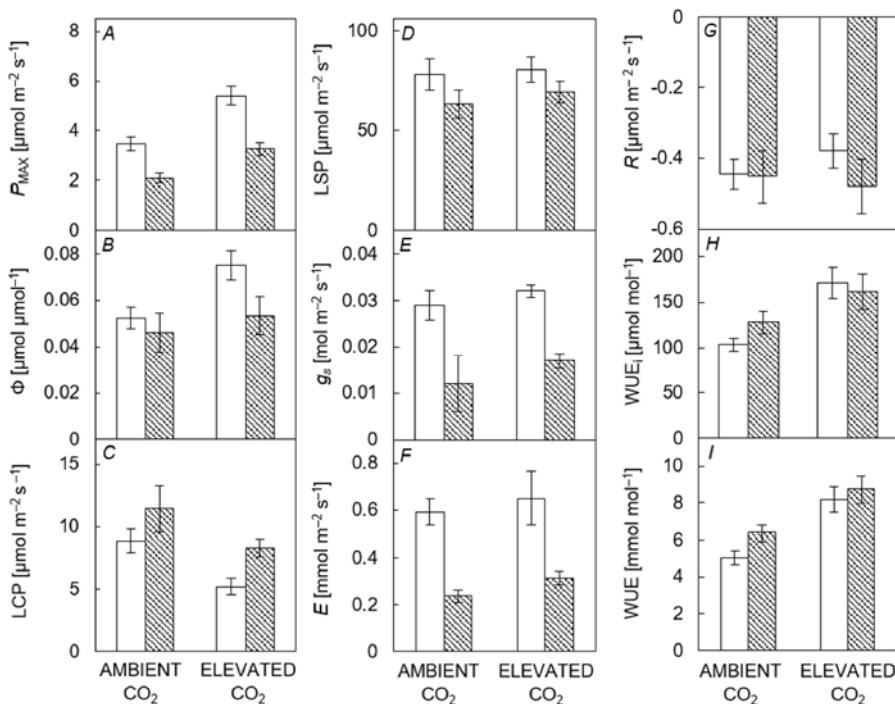


Fig. 3. Photosynthetic responses of well-watered and water deficit-treated cacao plants (var. Amelonado) grown at ambient and elevated CO<sub>2</sub>. (A) light-saturated photosynthesis ( $P_{\max}$ ); (B) quantum efficiency ( $\Phi$ ); (C) light-compensation point (LCP); (D) light-saturation point (LSP); (E) stomatal conductance ( $g_s$ ); (F) transpiration rate ( $E$ ); (G) respiration rate ( $R$ ); (H) intrinsic water-use efficiency ( $WUE_i$ ); (I) instantaneous water-use efficiency ( $WUE$ ). White bars: well-watered treatment; hatched bars: water-deficit treatment.  $n = 10$ ; error bars represent SE (Experiment B).

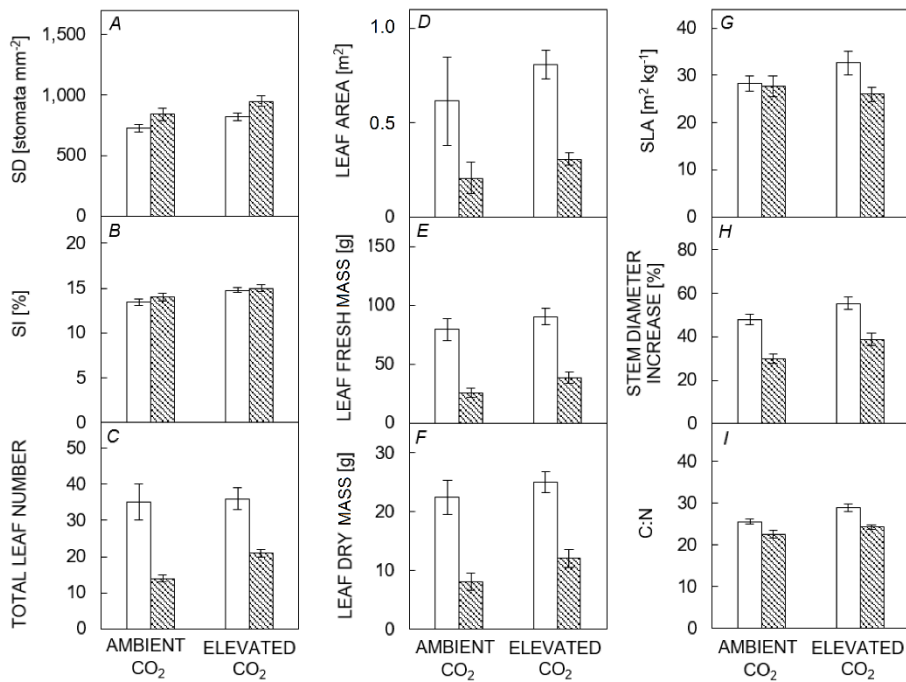


Fig. 4. Growth responses of well-watered and water deficit-treated cacao plants (var. Amelonado) grown at ambient and elevated CO<sub>2</sub>. (A) stomatal density (SD); (B) stomatal index (SI); (C) total leaf number; (D) leaf area; (E) leaf fresh mass; (F) leaf dry mass; (G) specific leaf area (SLA); (H) stem diameter increase; (I) carbon:nitrogen ratio (C:N). White bars: well-watered treatment; hatched bars: water-deficit treatment.  $n = 10$ ; error bars represent SE (Experiment B).

**Stomatal responses:** Average SD of ambient CO<sub>2</sub> grown leaves was  $786 \pm 31$  stomata  $\text{mm}^{-2}$ , this increased by 13% to an average of  $885 \pm 29$  stomata  $\text{mm}^{-2}$  in leaves grown at elevated CO<sub>2</sub>. The water deficit treatment also significantly increased SD from  $776 \pm 24$  stomata  $\text{mm}^{-2}$  in well watered plants to an average of  $896 \pm 34$  in water stressed plants (Fig. 4). SI significantly increased in leaves which developed under elevated CO<sub>2</sub>. The average SI in plants grown at ambient CO<sub>2</sub> was  $13.7 \pm 0.3\%$ , in plants grown at elevated CO<sub>2</sub> the SI increased by 8% to an average of  $14.9 \pm 0.2\%$ . While epidermal cell number remained similar between the ambient and the elevated CO<sub>2</sub> grown leaves, a greater number of stomatal cells were formed in leaves grown under elevated CO<sub>2</sub>. When SD was converted to SI, the effect of the water treatment was no longer significant; however, there was a trend towards higher SI in the water deficit plants (Fig. 4).

**Carbon and nitrogen content:** Carbon content of the leaf was not affected by CO<sub>2</sub> concentration. Carbon content increased significantly from 43.2% in well-watered plants to 44.5% in water-deficit plants. Leaf nitrogen content decreased significantly from an average of  $1.86 \pm 0.06\%$  in ambient CO<sub>2</sub> grown leaves to  $1.67 \pm 0.05\%$  in leaves of plants grown at elevated CO<sub>2</sub>. The water deficit treatment resulted in a significant increase in the nitrogen content of the leaf. The nitrogen content increased from an average of  $1.6 \pm 0.04\%$  in the well-watered plants to an average of  $1.9 \pm 0.05\%$  in the water deficit-treated plants. The C:N ratio

increased significantly in plants grown at elevated CO<sub>2</sub> and decreased in response to the water deficit treatment (Fig. 4).

**Growth:** Plant growth was significantly affected by both elevated CO<sub>2</sub> and water deficit (Fig. 4). A number of growth parameters were significantly greater in plants grown in the elevated CO<sub>2</sub> environment, including leaf area (+35.6%), leaf fresh and dry mass (+22.9 and +21.6%, respectively) compared to plants grown under ambient conditions. In both the ambient and elevated CO<sub>2</sub>-grown plants, the water deficit treatment reduced growth and caused a significant decrease in each of these parameters. Leaf area declined by 197.8 and 162.7% in response to water deficit in the ambient and elevated CO<sub>2</sub>-grown plants, respectively. Similarly, leaf fresh and dry mass declined by over 100% in response to water deficit in both the ambient and elevated CO<sub>2</sub> treatments. Slightly more leaves were produced in the elevated CO<sub>2</sub> treatment compared to ambient, however, the increase was mainly seen between the plants grown under water deficit. The interaction could not be investigated here due to the use of a non-parametric statistical test. The increase in stem diameter was significantly greater (+20.6%) in plants grown at elevated CO<sub>2</sub> and significantly reduced by the water deficit treatment (-33.6%). SLA was not affected by the CO<sub>2</sub> treatment. The water deficit treatment caused a slight reduction in SLA which was on the borderline of significance. The interval between leaf flushes was not significantly influenced by the CO<sub>2</sub> or water treatments.

## Discussion

While much research has focused on temperate crop responses to changing environmental conditions, little data are currently available on tropical crops such as cacao. The photosynthetic response of juvenile cacao plants to elevated CO<sub>2</sub> studied here was similar to that of other C<sub>3</sub> plant species, resulting in an increase in light-saturated photosynthetic rate, although the magnitude of the response differed between the two experiments (+105% and +56% in experiment A and B, respectively). Meta-analyses of tree responses to elevated CO<sub>2</sub> have found an average 47% increase in photosynthesis in FACE studies (Ainsworth and Long 2005) and 54% increase in greenhouse or open top chamber studies (Curtis and Wang 1998). Baligar *et al.* (2008) measured instantaneous photosynthetic responses in cacao seedlings and reported a 33% increase in  $P_{\max}$  between CO<sub>2</sub> concentrations of 370 and 680 ppm. Rubisco is not saturated at current CO<sub>2</sub> concentrations and elevated CO<sub>2</sub> concentration around the leaf commonly enhances photosynthetic rate through competitive inhibition of oxygenation in favour of carboxylation, thereby enhancing carboxylation efficiency of Rubisco and increasing photosynthetic rate (Farquhar and Sharkey 1982). The increase in  $P_{\max}$  in response to CO<sub>2</sub> reported here was also maintained under the water deficit conditions imposed. Although water deficit reduced  $P_{\max}$ , a positive response to elevated CO<sub>2</sub> was observed in both well-watered (+55.5%) and water deficit-treated (+56.7%) plants. This positive effect of CO<sub>2</sub> may ameliorate growth reductions caused by water limitation in the future. Indeed, in this study the reduction in a number of growth parameters (*e.g.* leaf area, leaf dry mass) due to water deficit was smaller in plants grown at elevated CO<sub>2</sub> compared to those grown at ambient CO<sub>2</sub>.

Along with higher  $P_{\max}$ ,  $\Phi$  increased and a concurrent decrease in LCP was seen in plants grown at elevated CO<sub>2</sub> relative to ambient grown plants. Quantum efficiency is an important photosynthetic parameter, especially in shaded conditions, as is the case to a greater or lesser extent on cacao farms. When cacao is grown under shade,  $\Phi$  provides an indication of how efficiently available light is utilised. It has been demonstrated in other studies that more shade-tolerant species tend to have a greater relative response to increasing CO<sub>2</sub> concentration in terms of  $\Phi$  and hence light utilisation than shade intolerant species. Sefcik *et al.* (2006) found that as light levels increased, the long-term enhancement in photosynthesis due to CO<sub>2</sub> elevation decreased. Ellsworth *et al.* (2012) also reported shade-tolerant species being more responsive to CO<sub>2</sub> elevation for photosynthetic rate and  $\Phi$ . A study of four tree species with varying shade tolerance found that the most shade-tolerant species opened their stomata at lower light levels which allowed for more efficient use of available light (Woods and Turner 1971). In the case of the cacao plants studied here the increase in  $\Phi$  in response to elevated CO<sub>2</sub> was greater (+44%) in the well-watered plants compared

to those grown with a water deficit (+15%). This may be due to stomatal limitations on CO<sub>2</sub> uptake in the water deficit-treated plants, but this does not provide a complete explanation as  $g_s$  was slightly higher in the water deficit-elevated CO<sub>2</sub>-grown plants compared to ambient grown plants when light was saturated. It is also likely that the prolonged period of growth with water limitation may have led to a down-regulation of the photosynthetic machinery in the water deficit plants making these plants unable to utilise the lower light levels as effectively for photosynthesis. The beneficial effects of elevated CO<sub>2</sub> on light utilisation may therefore be less evident in cacao during dry periods.

A common response to elevated CO<sub>2</sub> is a reduction in  $g_s$  accompanied by increased photosynthesis resulting in improved leaf-level WUE (Ainsworth and Long 2005). Lower  $g_s$  is due, in the long term, to fewer stomata on a leaf (Woodward 1987) and in the short term, to a reduction in stomatal aperture (Bettarini *et al.* 1998, Haworth *et al.* 2013). Baligar *et al.* (2008) have previously reported an average reduction of 65% in  $g_s$  in response to increasing instantaneous CO<sub>2</sub> concentration in three different cacao varieties which in turn improved WUE<sub>i</sub>. Similarly, in oil palm and the tropical tree species *Luehea seemannii*,  $g_s$  declined with increasing CO<sub>2</sub> concentration (Jaafar and Ibrahim 2012, Lovelock *et al.* 1999). An increase in WUE in response to elevated CO<sub>2</sub> has been widely reported in a number of species due to an enhancement of  $P_{\max}$ , a reduction in  $g_s$  or a combination of both (Wullschleger *et al.* 2002). In this study the increase in WUE in response to elevated CO<sub>2</sub> was due only to the higher photosynthetic rate at elevated CO<sub>2</sub> as  $g_s$  actually increased slightly in response to CO<sub>2</sub>. This is similar to conclusions drawn by Hietz *et al.* (2005) following a study of intrinsic WUE in tropical trees in Brazil which found that WUE<sub>i</sub> had increased in the past few decades through increased assimilation rates rather than a reduction in  $g_s$ . This increase in  $g_s$  may have been caused by the higher SI in leaves grown at elevated CO<sub>2</sub>. In the majority of plant species that have been studied, an inverse relationship has been found between CO<sub>2</sub> concentration and SD (Royer *et al.* 2001). Increases in SI in response to increasing CO<sub>2</sub> have been reported previously in rice; however, despite this,  $g_s$  was still lower in the elevated CO<sub>2</sub> plants relative to the ambient CO<sub>2</sub>-grown plants (Upreti *et al.* 2002). Other species, which demonstrate an increase in SD at increased CO<sub>2</sub> concentrations, include *Sanguisorba minor* (Ferris and Taylor 1994), *Convolvulus arvensis* and *Conyza canadensis* (Bettarini *et al.* 1998). In the case of *C. canadensis*,  $g_s$  also increased (Bettarini *et al.* 1998). The results observed in the present study for cacao could be a result of its evolution in the humid understory of the tropical rainforest where water is rarely, if ever, limiting. In such an environment, selection for lower SI and  $g_s$  at elevated CO<sub>2</sub> concentrations in order to maximise



assimilation but reduce water loss may not prove advantageous, especially in a species which has such low rates of photosynthesis and  $g_s$ . In this study,  $g_s$  decreased more in response to water deficit in the ambient grown trees compared to the elevated CO<sub>2</sub>-grown trees. This response is not unique to cacao and has been reported previously in beech. The leaves of beech trees grown at elevated CO<sub>2</sub> and under drought stress had higher  $g_s$  than trees grown at ambient CO<sub>2</sub> (Heath and Kerstiens 1997). The authors suggested that whole plant water relations may have been improved in the elevated CO<sub>2</sub> environment through more effective water uptake by the roots or through increased xylem conductivity which would improve water supply to the leaves during dry conditions.

Leaf area increased in the cacao plants grown at elevated CO<sub>2</sub>. Greater leaf area, along with higher  $g_s$ , could have implications for water use in the future. It is important to note that an increase in WUE at elevated CO<sub>2</sub> does not necessarily equate to improved water stress tolerance, especially as water loss through the leaf is not reduced. It has previously been reported that cacao trees increased WUE with increasing water limitation through a greater reduction in  $E$  than  $P_{max}$  (Rada *et al.* 2005). In this study, water stress failed to improve WUE<sub>i</sub> in cacao plants due to strong reductions in both  $g_s$  and  $P_{max}$ . It is possible that the degree of water stress used here restricted  $g_s$  too much to maintain photosynthesis. Selection for improved WUE<sub>i</sub> would be beneficial in the future, especially where it can be achieved through both an increase in photosynthesis and a decline in  $g_s$ .

Leaves grown at elevated CO<sub>2</sub> had an increased C:N

relative to ambient CO<sub>2</sub>-grown leaves due to a reduction in N content as there was no change in leaf C content. Leaf photosynthetic capacity is often correlated with leaf nitrogen content due to the essential role of nitrogen in light harvesting and CO<sub>2</sub> fixation. In cacao, leaf nitrogen content is positively correlated with photosynthetic rate (Daymond *et al.* 2011). The fact that biomass production and photosynthesis increased despite a reduction in N indicates an improvement in N-use efficiency at elevated CO<sub>2</sub>. Improved N-use efficiency could be beneficial in the nutrient depleted soils in many cacao growing regions in West Africa. Several explanations have been suggested to explain the general decline in leaf N content at elevated CO<sub>2</sub> including, the accumulation of nonstructural carbohydrates which occurs due to greater photosynthesis may result in a dilution of N (Wong 1990), reduced N uptake due to reduced  $E$  at elevated CO<sub>2</sub> (McDonald *et al.* 2002) or altered root uptake capacity (Bernston and Bazzaz 1995).

From the results of this study elevated CO<sub>2</sub> appears to be beneficial in enhancing photosynthesis and growth in cacao, similar to observations in other C<sub>3</sub> plants. In combination with water deficit, this enhancement was still evident, thus CO<sub>2</sub> partially mitigated the effects of water stress. This study was carried out on young cacao plants which had not yet reached reproductive maturity and so, whether this will translate into increased pod and bean yield is not certain. If assimilation rate is increased at elevated CO<sub>2</sub> it would be logical to hypothesise that this could potentially improve pod yields by supporting a larger number of pods per tree.

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