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Genotypic variation in photosynthesis in cacao is correlated with stomatal conductance and leaf nitrogen

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Abstract

Variation in photosynthetic parameters was observed between eight contrasting cacao (*Theobroma cacao*) genotypes. Net photosynthetic rate (P_N) ranged from 3.4 to 5.7 μmol(CO_2) m^{-2} s^{-1} for the genotypes IMC 47 and SCA 6, respectively. Furthermore, genotypic differences were detected in quantum efficiency ranging from 0.020 to 0.043 μmol(CO_2) μmol^{-1}(photon) for UF 676 and AMAZ 15/15, respectively. Differences in P_N were correlated with both stomatal conductance (g_s) and leaf nitrogen per unit area. Some variation in water use efficiency was observed between genotypes, both intrinsic (P_N/g_s) and instantaneous (P_N/transpiration rate). Both measures of water use efficiency were a negative function of specific leaf area. Evidence was found for a trade-off mechanism between cacao genotypes in photosynthesis and leaf structure. High photosynthetic rate, expressed on a mass basis was associated with smaller leaves. Furthermore, thinner leaves were compensated for by a higher nitrogen content per unit mass.

Additional key words: light response curves, quantum efficiency, specific leaf area

Introduction

Cacao is native to the Amazon rainforest, where it grows in the understorey of larger trees (Toxopeus 1985). As such, the photosynthetic characteristics of cacao are those of a shade-adapted species with a low light compensation point and leaf photosynthesis that is usually light saturated at low irradiance, typically around 20 % of full sunlight (Hutcheon 1977, Raja Harun and Hardwick 1988a, Mielke *et al*. 2005). Light saturated photosynthetic rates in cacao have been recorded in the region of 2 - 8 μmol(CO_2) m^{-2} s^{-1} (*e.g*. Hutcheon 1977, Balasimha *et al*. 1991, Costa *et al*. 2001), compared with values in the region of 10 μmol (CO_2) m^2 s^{-1} for soybean (Huang *et al*. 2006) and 20 - 30 μmol(CO_2) m^{-2} s^{-1} for wheat (*e.g*. Yang *et al*. 2007).

Studies of genotypic variation in photosynthetic rate (P_N) in cacao are limited. Galyuon *et al*. (1996a) found no significant differences between five Trinidad cacao cultivars. Similarly Baligar *et al*. (2008) observed no significant differences in gas exchange parameters between three populations of seedlings. In contrast, Hadley and Yapp (1993) demonstrated differences amongst cacao genotypes both under greenhouse and in the field.

Variation in single P_N is often not directly correlated with yield, since P_N at the leaf level may be eclipsed by variation in canopy characteristics and biomass partitioning, both factors that are known to vary in cacao (Daymond *et al*. 2002a,b). However, an assessment of variability in photosynthetic characteristics determines the adaptation of the crop to particular growing conditions. Additionally, an assessment of the factors limiting photosynthesis (stomatal and non-stomatal) is relevant to the understanding of genotypic variation in responses to environmental stresses. Such stresses may arise in cacao since the variation in environmental conditions that cacao crops are subjected to in their principle areas of production (West Africa, South-East Asia and South America) is often greater than that experienced in the wild (Wood 1985). In particular, irradiance may be higher due to sparse overhead shade, and, in a number of cacao-growing regions, a defined dry season is experienced.

Here, we explore genotypic variation in responses of P_N to irradiance and in water use efficiency and examine factors underlying genotypic variation in P_N.

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Abbreviations: E - transpiration rate; g_s - stomatal conductance; IWUE - intrinsic water use efficiency; P_N - net photosynthetic rate; WUE - water use efficiency.

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Materials and methods

Eight cacao (*Theobroma cacao* L.) clones were selected for the experiment and propagated by means of patch budding onto seedlings of "GU" clones (originating from French Guiana; Lachenaud *et al*. 2007). All propagation and subsequent experimentation took place in temperature-controlled polythene clad greenhouses at the International Cacao Quarantine Centre at The University of Reading between September 2006 and March 2007. Plant culture was similar to that described in Daymond and Hadley (2004). The potting medium used was a mixture of sand, gravel and *Vermiculite* (1:2:2) and the plants were fed seven times per day with a modified Long Ashton solution (End 1990). Average minimum night temperature from propagation to the end of the experiment was 21.1 °C and average maximum day temperature was 30.7 °C.

All gas exchange measurements were made using an LC_{pro} portable gas exchange system fitted with a light attachment (*ADC BioScientific*, Great Amwell, Herts., UK). An initial screen of light saturated photosynthesis was made on 9 August 2007. For this, two leaves were tagged on each plant (the youngest or second youngest fully expanded, fully hardened leaf in each case). During the gas exchange measurements the irradiance was 1044 µmol m^{-2} s^{-1} (photosynthetically active radiation), leaf chamber temperatures 29.5 - 30.1 °C and reference CO_2 concentrations 371 - 394 µmol mol^{-1}.

Responses of P_N to irradiance were measured on the same tagged leaves between 13 - 18 August 2007. For these, irradiances of 696, 435, 261, 174, 87, 44, 26 and 0 µmol m^{-2} s^{-1} were used. Chamber temperatures were maintained within a range of 29.1 - 30.5 °C and CO_2 between 362 and 398 µmol mol^{-1}. Chlorophyll content was measured on the same leaves using a *Hansatech CL-01* chlorophyll content meter (*Hansatech Instruments*, Kings Lynn, UK). The chlorophyll content meter was calibrated against values obtained using an independent set of leaves using the method of Arnon (1949). Chlorophyll content (c) was described by the following linear regression: c = (1.945 × chlorophyll meter reading) + 11.392 (r^2 = 0.988, d.f. = 7).

Leaf impressions were made on one mature leaf per plant using clear nail polish on 22 August 2007. Digital images were then obtained using an *Axioscope 2* microscope with an *Axiocam* camera attached (*Carl Zeiss*, Jena, Germany), (three images per impression) using *Axio Vision 3.1* (*Image Associates*, Oxfordshire, UK) software and the number of stomata per unit area counted. Guard cell length was measured using *ImageJ* software (*ImageJ 1.37v*, National Institute of Health, USA) for 20 guard cell pairs per clone. At the same time, specific leaf area was determined by punching leaf discs of known area in-between mid-ribs and drying these to a constant mass at 70 °C in a ventilated oven. Specific leaf area was then calculated as the ratio of area to dry mass. Nitrogen content of dried leaf samples was determined by a Kjeldhal method. An independent set of leaves was detached (six randomly selected leaves per clone; the youngest fully hardened and expanded leaf in each case) and their area measured using a leaf area meter (*Delta T Devices*, Cambridge, UK).

Chlorophyll fluorescence was measured on an independent set of leaves (two per plant; the youngest or second youngest from two flushes) on 3 September 2007 using a *Plant Efficiency Analyser* (*PEA*, *Hansatech Instruments*). Measurements were made between 09:00 and 10:00 and the leaves were dark adapted using leaf clips for 15 min before taking the measurements.

The dried leaves of two contrasting cacao clones ICS 1 and IMC 47 (previously used for gas exchange measurements) were ground into a fine homogenous powder. Weighed samples (~1 mg) were analysed for $\delta^{13}C$ using a *Europa 20-20* isotope ratio mass-spectrometer (*Europa Scientific*, Crew, UK) coupled to a *Sercon* elemental analyser.

Gas exchange parameters were analysed by analysis of variance (*ANOVA*) using *GENSTAT 8.1*. Photosynthetic light response curves were fitted by means of a non-rectangular hyperbola in the form: $P_N = [\theta Q + P_{Nmax} - SQRT\{(\theta Q + P_{N\text{-}max})^2 - 4\theta Q k P_{N\text{-}max}\}/2k] - R$, where θ is apparent quantum efficiency, Q is irradiance, P_{Nmax} is light saturated photosynthetic rate, k is convexity and R is respiration rate. The non-rectangular hyperbola was fitted to photosynthetic data using *Photosyn Assistant* software (Dundee Scientific, Dundee, UK).

Factors underlying genotypic differences in light-saturated photosynthesis and stomatal conductance were analysed by regression analysis using *Microsoft Excel* and *GENSTAT 8.1*.

Results

Light saturated photosynthetic rate (P_{Nmax}) varied significantly between cacao clones ($P < 0.01$); average values over the two sampling periods ranged from 3.4 for IMC 47 to 5.7 µmol(CO_2) m^{-2} s^{-1} for SCA 6 (Fig. 1). There were no differences between sampling times for the following light-saturated gas exchange parameters: photosynthetic rate, transpiration rate, water use efficiency. Stomatal conductance (g_s) was significantly greater on the first sampling date compared with the second ($P < 0.001$; data not shown). However there was no significant interaction between sampling time and clone.

Differences between clones in g_s were on the borderline of significance (P = 0.087; Fig. 1). Transpiration rate (E) ranged from 1.06 for IMC 47 to 1.56 mmol(H_2O) m^{-2} s^{-1} for SCA 6 ($P < 0.05$; Fig. 1),

whilst instantaneous water use efficiency (WUE = P_N/E) varied between 3.1 for IMC 47 to 4.2 mmol(CO_2) mol^{-1}(H_2O) for ICS 1 ($P < 0.001$; Fig. 1). The clonal differences in the intrinsic water use efficiency (IWUE = P_N/g_s) were also significant ($P < 0.01$); the average of the two sampling times ranged from 49.4 for MAN 15/2 to 63.2 µmol(CO_2) mol^{-1}(H_2O) for ICS 1 (Fig. 1).

The responses of P_N to irradiance were described well

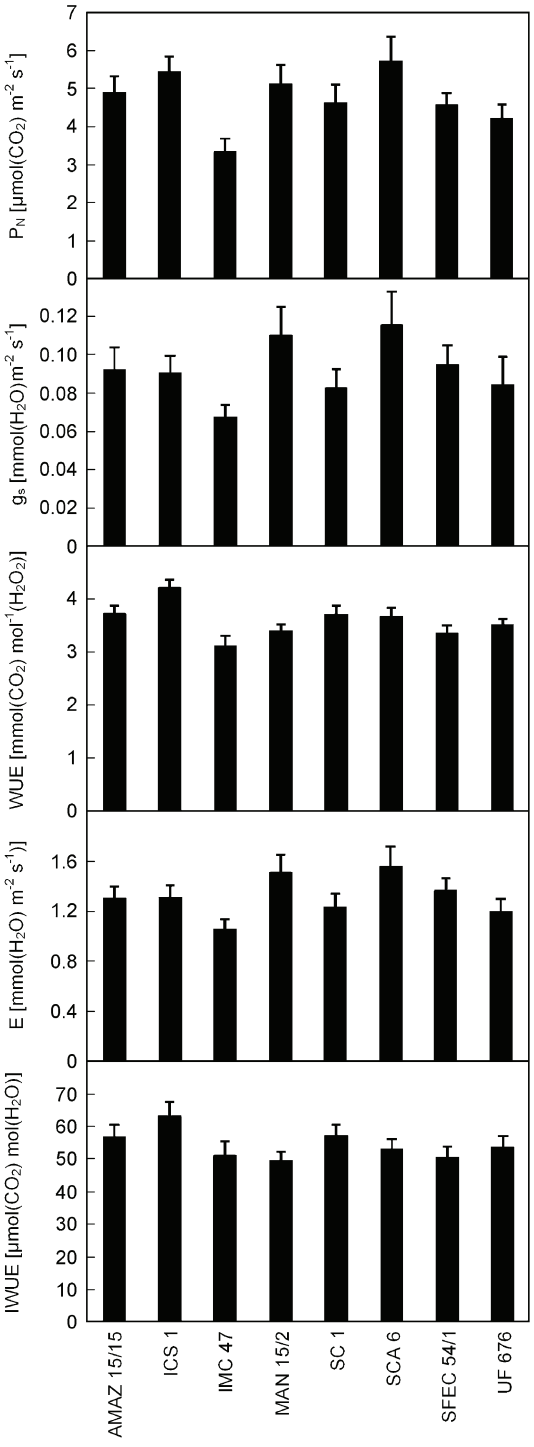

Fig. 1. Genetic variation in net photosynthetic rate (P_N), stomatal conductance (g_S), transpiration rate (E), water use efficiency (WUE) and intrinsic water use efficiency (IWUE) of eight cacao clones measured under saturating irradiance. Means ± SE of 8 replicates, measured on two occasions.

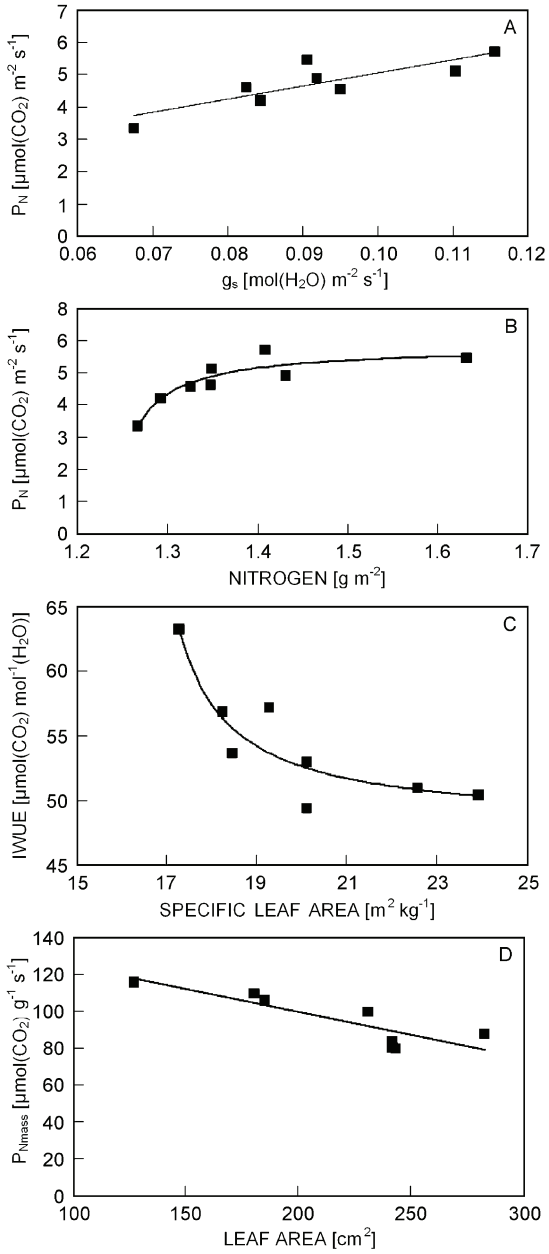

Fig. 2. Factors underlying genotypic difference in maximum photosynthetic rates. *A* - Relationship between P_N and g_S (the fitted function is $P_N = ag_S + b$, where a = 40.9 and b = 0.968, $r^2 = 0.66$, $P < 0.01$). *B* - The relationship between P_N and leaf nitrogen per area ($P_N = a + b[1+(dNitrogen_{area})]$, where a = 5.81, b = 0.10 and d = -0.82, $r^2 = 0.81$, $P < 0.01$). *C* - The relationship between intrinsic water use efficiency (IWUE) and specific leaf area (SLA) (IWUE = a + b/[1+(dSLA)], where a = 48.1, b = 1.10 and d = -0.0621. $r^2 = 0.75$, $P < 0.05$). *D* - Relationship between photosynthetic rate on a mass basis (P_{Nmass}) and leaf area (LA) (P_{Nmass} = aLA + b, where a = -0.248 and b = 149.0. $r^2 = 0.70$, $P < 0.01$).

Table 1. Parameters derived from the fitting of photosynthetic light response curves in eight cacao clones. Means ± SE, $n = 8$.

Clone	Respiration rate [μmol(CO_2) m^{-2} s^{-1}]	Quantum efficiency [μmol(CO_2) μmol^{-1}(photon)]	Convexity	Comp. irradiance [μmol m^{-2} s^{-1}]	Sat. irradiance [μmol m^{-2} s^{-1}]
AMAZ 15/15	-0.79 ± 0.07	0.043 ± 0.006	0.56 ± 0.14	20.5 ± 2.8	189.3 ± 23.6
ICS 1	-0.66 ± 0.07	0.035 ± 0.004	0.75 ± 0.11	22.3 ± 4.7	213.8 ± 21.0
IMC 47	-0.45 ± 0.11	0.026 ± 0.008	0.67 ± 0.15	24.1 ± 6.9	275.6 ± 52.0
MAN 15/2	-0.81 ± 0.26	0.032 ± 0.004	0.61 ± 0.12	23.6 ± 6.0	237.6 ± 29.6
SC 1	-0.50 ± 0.10	0.032 ± 0.008	0.55 ± 0.15	20.3 ± 5.7	276.1 ± 46.3
SCA 6	-1.39 ± 0.10	0.041 ± 0.002	0.67 ± 0.09	11.7 ± 2.6	174.0 ± 15.4
SPEC 54/1	-0.59 ± 0.06	0.042 ± 0.003	0.55 ± 0.14	14.4 ± 1.4	163.8 ± 14.8
UF 676	-0.46 ± 0.06	0.020 ± 0.003	0.70 ± 0.15	24.7 ± 3.9	304.0 ± 44.4
P value	n.s	0.05	n.s	n.s	0.05

Table 2. Leaf traits of eight cacao clones. Means ± SE, $n = 8$ (SLA and Chl) and 4 (% N, N_{area} and stomatal density).

Clone	Leaf area [cm^2]	SLA [m^2 kg^{-1}]	N [%]	N_{area} [g m^{-2}]	Chlorophyll [μg cm^{-2}]	Stomatal density [mm^{-2}]	Guard cell length [μm]
AMAZ 15/15	241.7 ± 48.3	18.2 ± 0.4	2.61 ± 0.13	1.43 ± 0.09	47.7± 1.9	1009 ± 39	13.4 ± 0.2
ICS 1	230.9 ± 15.2	17.3 ± 0.2	2.82 ± 0.06	1.63 ± 0.05	56.8 ± 2.8	1081 ± 62	13.4 ± 0.4
IMC 47	241.8 ± 30.6	22.6 ± 0.7	2.86 ± 0.12	1.27 ± 0.09	39.3 ± 3.7	983 ± 43	14.0 ± 0.3
MAN 15/2	180.3 ± 10.9	20.1 ± 0.9	2.71 ± 0.14	1.35 ± 0.13	43.2 ± 3.3	1040 ± 81	12.6 ± 0.3
SC 1	282.9 ± 20.5	19.3 ± 0.6	2.60 ± 0.11	1.35 ± 0.08	60.7 ± 3.9	908 ± 55	14.0 ± 0.3
SCA 6	126.7 ± 7.3	20.1 ± 0.7	2.83 ± 0.09	1.41 ± 0.04	56.6 ± 2.2	1056 ± 92	12.6 ± 0.3
SPEC 54/1	184.9 ± 16.6	23.9 ± 1.0	3.17 ± 0.10	1.33 ± 0.07	51.1 ± 4.6	788 ± 56	12.9 ± 0.3
UF 676	243.2 ± 11.6	18.5 ± 0.4	2.39 ± 0.08	1.29 ± 0.05	52.9 ± 2.9	816 ± 17	15.1 ± 0.3
P value	<0.001	<0.001	<0.01	0.06	<0.001	<0.05	<0.001

by the non-rectangular hyperbola (r^2 varied from 0.928 to 0.999 for the responses of individual leaves). The parameters estimated from the light-response curves are summarised in Table 1. Significant differences were observed between clones in quantum efficiency ($P < 0.05$) varying between 0.020 for UF 676 to 0.043 μmol(CO_2) μmol^{-1}(photon) for AMAZ 15/15. Additionally, there was a significant clonal effect on estimated saturation irradiance ($P < 0.05$) (ranging from 164 for SPEC 54/1 to 304 μmol m^{-2} s^{-1} for UF 676).

Small but significant differences were observed in the efficiency of photosystem 2 (variable to maximum fluorescence ratio, F_v/F_m; $P < 0.001$), ranging from 0.79 for SCA 6 to 0.83 for SC 1. However, differences in electron transport rate between clones (ET_o/CS) were not significant (data not shown).

Most of the leaf traits measured varied between clones. Specific leaf area ranged from 17.3 for ICS 1 to 23.9 m^{-2} kg^{-1} for SPEC 54/1 ($P < 0.001$; Table 2). Leaf nitrogen, when expressed as a percentage of dry matter differed significantly between clones, ranging from 2.39 for UF 676 to 3.17 % for SPEC 54/1 ($P < 0.001$; Table 2). When expressed as leaf nitrogen per area, the differences were on the borderline of significance (range 1.27 for IMC 47 to 1.63 g m^{-2} for ICS 1; $P = 0.06$). Absolute leaf area ranged from 126.7 for SCA 6 to 282.9 cm^2 for SC 1 ($P < 0.001$; Table 2). Leaf nitrogen (expressed as a percentage of dry matter) increased linearly with specific leaf area ($r^2 = 0.46$, $P < 0.05$).

Leaf chlorophyll content varied considerably between clones ($P < 0.001$), ranging from 39.3 for IMC 47 to 60.7 μg cm^{-2} for SC 1. Stomatal density also varied between clones, ranging from 788 for SPEC 54/1 to 1081 stomata mm^{-1} for ICS 1 ($P < 0.05$). Furthermore, stomatal guard cell length ranged from 12.6 to 15.1 μm ($P < 0.001$; Table 2).

No significant differences were observed between the two clones studied in $\delta^{13}C$ (-28.4 ± 0.7 for ICS 1, -29.0 ± 0.3 for IMC 47).

A large proportion of the observed variation in P_N between cacao clones could be explained by variation in g_s ($r^2 = 0.66$, $P < 0.01$; Fig. 2*A*) and leaf nitrogen per unit area (Fig, 2B; $r^2 = 0.81$, $P < 0.01$). In contrast, no significant relationship was found between fluorescence parameters and photosynthesis.

Intrinsic water use efficiency was negatively correlated with specific leaf area, the relationship being described by rectangular hyperbola ($r^2 = 0.75$; $P < 0.05$; Fig. 2*C*). Specific leaf area was also negatively correlated with P_N/E ($r^2 = 0.55$; $P < 0.05$; data not shown). To examine how cacao leaf structure and photosynthetic rate per mass unit (P_{Nmass}) are inter-related, the relationship

between leaf area (youngest fully-expanded leaf) and P_{Nmass} of the different cacao clones was examined. P_{Nmass} was a negative linear function of leaf area (r^2= 0.70; $P < 0.01$; Fig. 2*D*).

Differences between cacao clones in g_s were not correlated with stomatal density but were a negative function of guard cell length, the relation being described by a rectangular hyperbola (r^2 = 0.69; $P < 0.05$; data not shown).

Discussion

Genotypic variation in photosynthetic rates has been demonstrated for a number of species, for example, cotton (Pettigrew and Turley 1998), grapevine (Bota *et al*. 2001), wheat (Morgan and LeCain 1991), grain amaranth (Harley and Ehleringer 1987) and common bean (Santos *et al*. 2009). Here, it was demonstrated that such variation exists for cacao under uniform growth and measurement conditions.

When considering factors underlying photosynthesis, the results indicate that genotypic variation in leaf photosynthesis in cacao is brought about by both differences in g_s and CO_2 fixation. In our cacao leaves, P_N was correlated with N content below a particular N threshold, a pattern seen in a number of other species (Kanemura *et al*. 2007).

Genetic variation in cacao leaf traits, such as specific leaf area and stomatal frequency has previously been reported (Balasimha *et al*. 1985, Galyuon *et al*. 1996b). The lack of correlation observed here between stomatal number and stomatal conductance is similar to that reported by Ohsumi *et al*. (2007) for rice and by Morgan and LeCain (1991) for winter wheat. Furthermore, the negative relationship between guard cell length and stomatal conductance implies that the degree of stomatal opening was a greater factor underlying variation in stomatal conductance amongst the genotypes studied. Stomatal conductance, as mediated by environmental factors such as relative humidity, has been shown to be correlated with photosynthetic rate within particular varieties of cacao (Sena Gomes *et al*. 1987, Raja Harun and Hardwick 1988b). Here it has been shown that there is also stomatal limitation to genotypic variation in photosynthesis in cacao.

The present study has shown that there is a trade-off between cacao genotypes in P_N and leaf structure in that, when P_N was expressed on a mass basis (to normalise the wide range of specific leaf areas observed). A higher P_{Nmass} was associated with smaller leaves. Thinner leaves were partially compensated for by a higher nitrogen concentration per unit mass. Such trade-off mechanisms have previously been observed at an inter-species level (Poorter and Bongers 2006).

The observed genotypic variation in water use efficiency (expressed both as the ratio of P_N/E and intrinsic water use efficiency, P_N/g_s) was not reflected by the $\delta^{13}C$ composition of the leaves of two clones that exhibited variation in WUE. This may be due to differential partitioning of $\delta^{13}C$ between different plant organs, as observed in grapevines by (De Souza *et al*. 2005). Genotypes with high water use efficiency (alongside other water-stress tolerance traits) could be utilised in more marginal growing areas where rainfall patterns are less consistent. Since specific leaf area was correlated with intrinsic water use efficiency, low SLA might therefore represent a selection criterion in breeding programmes.

To conclude, this study has shown that cacao exhibits significant genotypic variation in a number of photosynthetic traits, in particular light saturated photosynthesis, quantum efficiency and water use efficiency. Breeding for these traits can contribute to improved productivity and to optimisation of performance under particular growing conditions.

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