The effects of temperature and light integral on early vegetative growth and chlorophyll fluorescence of four contrasting genotypes of cacao (*Theobroma cacao*)

By A J DAYMOND* and P HADLEY

School of Plant Sciences, The University of Reading, Whiteknights, Reading, RG6 6AS, UK

Summary

The effect of temperature on early vegetative growth, leaf chlorophyll fluorescence and chlorophyll content was examined on four genotypes of cacao (Amelonado, AMAZ 15-15, SCA 6 and SPEC 54/1). A controlled environment glasshouse was used to simulate the temperature conditions of three cacao-growing regions (Bahia, Brazil; Tafo, Ghana and Lower Perak, Malaysia) over the course of a year. Base temperatures calculated from increments in main stem growth varied from 18.6°C for AMAZ 15/15 to 20.8°C for SPEC 54/1. Temporal variation in Fv/Fm observed for two of the clones (SCA 6 and SPEC 54/1) in two of the compartments were correlated with temperature differences over time. Significant differences were also recorded between genotypes in leaf chlorophyll content. It was shown that variation over time in leaf chlorophyll content could be quantified accurately as a function of temperature and light integral. The results imply that genetic variability exists in cacao in response to temperature stress.

Introduction

The tropical tree crop cacao (*Theobroma cacao*) is endemic to the Amazon basin, although its area of cultivation is spread throughout the tropical and sub-tropical regions of Central and South America, West Africa and South-East Asia. Consequently, the temperature range experienced by cacao crops is often much greater than that of its natural habitat. For example, the main cacao-growing region of Brazil (located in the state of Bahia) experiences a cooler period between June and September due to its sub-tropical latitude (14° S), whilst in Ghana lower maximum temperatures are experienced between June and September due to the rainy season. In contrast, South-East Asia experiences continuously high temperatures all year round (Wood, 1985). Cacao has been shown to be sensitive to temperature in terms of its physiological and developmental processes (Hadley *et al*., 1994; Raja Harun & Hardwick, 1988). Consequently, temperature is the principal factor limiting the regions in which cacao may be grown and temperature stress is a factor affecting the seasonal variation in bean yield.

The technique of chlorophyll fluorescence has been shown to be a simple and reliable measure of physiological temperature stress by monitoring the function of photosystem II (PS II) in the thylakoid membrane (Maxwell & Johnson, 2000; Smillie & Hetherington, 1983), and has been used to assess tolerances to both low and high temperature conditions for a range of crops (e.g. Brennan & Jefferies, 1990; Hakam *et al*., 2000; Moffatt *et al*.,

1990; Yamada *et al*., 1996). Whilst chlorophyll fluorescence is indicative of more short-term responses to environmental stresses, the concentration of chlorophyll in leaves can represent developmental adaptation to the environment in which the leaf has developed (Hale & Orcutt, 1987).

Previous studies have shown that cacao exhibits considerable genetic variation in morphological and physiological characteristics (Daymond *et al*., 2002*a,b*; Yapp & Hadley, 1994). However, few studies have been carried out that consider the interaction between cacao genotype and environment; e.g. Galyuon *et al*. (1996) examined the effects of shade on several cacao clones. Little information is available specifically on the interaction with temperature. The present study therefore addresses the question of whether particular cacao genotypes are more susceptible to temperature stress than others by using chlorophyll fluorescence and leaf chlorophyll content as a probe and by evaluating early vegetative growth.

Here, a range of cacao genotypes was grown in semi-controlled environment greenhouses simulating the contrasting temperature regimes of three cacao-growing areas. Such an approach allows the effect of temperature to be studied whilst at the same time maintaining a similar solar radiation and nutrient regime between treatments throughout the time-course of the experiment.

Materials and Methods

Controlled environment greenhouse
The experiment was located within a triple-span

*Corresponding Author E-mail: a.j.daymond@reading.ac.uk

greenhouse (each compartment approx. 19 m × 6.5 m) located at the School of Plant Sciences, University of Reading. Heating in each compartment was provided by gas-powered hot-air heaters (150 000 btu) and cooling by motorised vents in the roof. Temperature control in the three bays was achieved automatically through a combined computer and microprocessor-based system (ADAM-5000, distributed data acquisition and control system, Semaphore Systems Ltd, London, UK) along with dedicated software (GENIDAQ, Semaphore Systems Ltd) operating through an IBM compatible computer.

The system controlled and monitored the temperature in each compartment to specific values by switching heaters on and opening vents as required. The computer program was designed in order to simulate the temperature regime of three different cacao-growing regions; these being Brazil (Itabuna, Bahia), Malaysia (Lower Perak, Sabah) and Ghana (Tafo). Diurnal temperature was simulated continuously in the form of a sine-wave such that the maximum temperature was reached at 14:00 h and the minimum at 06:00 h. Seasonal variation was simulated by changing the set point maximum and minimum temperatures at the beginning of each month. The glasshouse simulated temperature six months out of synchrony since in Brazil and Ghana the period from June to September is slightly cooler than the rest of the year. The cacao-growing region of Brazil shows seasonal variation in both maximum and minimum temperatures, that of Ghana shows seasonality in maximum temperatures, although little difference in minimum temperatures. In contrast, the temperature regime of the cacao-growing region of Malaysia is almost constant throughout the year (Table 1).

Twelve 400 W high pressure sodium vapour lamps provided supplementary lighting during the darkest 6 months of the year (21 October to 21 March) in order to maintain a 12-h day-length.

Environmental monitoring

The *ADAM-5000* control system also served as a datalogger. Temperature and humidity were monitored by PT100 sensors (S W Burrage, Kent, UK) located in an aspirated screen in the centre of each greenhouse compartment and logged at one minute intervals. Solar radiation was recorded by means of tube-solarimeters (in-house manufacture; Szeicz *et al.*, 1964), one in each bay positioned above the crop.

Plant culture and experimental design

Buds from three clones were patch-budded on to seedlings of the 'Amelonado' cocoa variety during May 2001 and raised in a common environment. The three clones used were SCA 6, AMAZ 15/15 and SPEC 54/1. In addition to the clonally propagated material, seedlings of the Amelonado variety were also raised during the same period for inclusion in the experiment. The plants were initially grown in 1 litre pots in a mixture of sand, gravel and vermiculite (1:2:2 v/v) and were fed hydroponically with a

modified Long-Ashton solution developed at Reading specifically for cocoa (End, 1990). The concentration of the nutrient system was controlled to an electrical conductivity of 2 mS cm^{-1} and maintained at a pH of 5.8. The automated system irrigated seven times per day such that on each irrigation excess solution drained from the pot to prevent a build up of nutrients in the pot.

Five replicates of each genotype were transferred to the three simulated environments in December 2001 and the three temperature regimes were imposed at this point. These were randomised within five blocks along the length of the glasshouse. The plants were re-potted into 10 litre pots in January 2002 and re-potted and re-spaced into 37 litre pots during August 2002.

Growth analyses

Measurements of main stem diameter of all plants were taken repeatedly at 4-wk intervals over the course of a year. Base temperatures (i.e. the theoretical temperature below which growth is zero) were then calculated by means of a linear regression between the main-stem cross-sectional area growth rate (cm^2 day^{-1}) and the corresponding temperature for that time interval.

Chlorophyll and chlorophyll fluorescence

Chlorophyll fluorescence measurements were taken at approximately monthly intervals during the course of a year from February 2002. For this, the youngest fully expanded leaf on an exposed branch was measured on each replicate plant. Different randomly chosen leaves were used on each occasion and data were taken between 09:00 h and midday in order to minimise temporal artefacts. Leaves were dark-adapted by attaching light-exclusion clips to the leaf surface for a period of 30 min. Chlorophyll fluorescence was then measured using a portable fluorescence spectrometer (Hansatech Instruments Ltd, Kings Lynn, UK). At the same time, chlorophyll content was estimated from four measurements per leaf using a chlorophyll content meter (Hansatech Instruments Ltd).

Statistical analyses

The effects of genotype and growing environment (glasshouse compartment) on growth parameters were tested for by means of ANOVA using *GENSTAT*. Similarly, the effects of genotype, growing environment (glasshouse compartment) and time of

Table 1. *Variation in the seasonal temperature in three cacao-growing regions (Wood, 1985)*

Region	July temperature (°C)		January temperature (°C)	
	Maximum	Minimum	Maximum	Minimum
Itabuna, Brazil	26.5	17	30.5	20.5
Tafo, Ghana	28	21.5	31	20
Lower Perak, Malaysia	32.5	22.5	31.5	22.5

year on chlorophyll fluorescence and chlorophyll meter reading were tested by ANOVA. The effects of temporal variation in temperature and light integral on chlorophyll fluorescence and chlorophyll meter reading were tested for by means of simple and multiple regressions within *EXCEL* spreadsheets.

Results

Environmental control

The mean monthly temperatures in each of the three simulated environments over the experimental period are shown in Fig. 1. On average, temperatures in the 'Brazil' compartment deviated from the set point by + 0.6°C, in the 'Ghana' compartment by + 0.2°C and in the 'Malaysia' compartment by 0.0°C. Relative humidity showed little difference between compartments (mean of 60% in the 'Brazil' compartment and 61% in the 'Ghana' and 'Malaysia' compartments). Solar radiation was slightly higher in the 'Ghana' compartment (average PAR of 16.6 M m^{-2} day^{-1}) compared with the 'Brazil' and 'Malaysia' compartments (PAR of 14.4 and 14.5 M m^{-2} day^{-1} respectively).

Growth analyses

The increase in main stem cross-sectional area over a year differed significantly ($P < 0.001$) between genotypes varying from 2.6 cm^2 for SCA 6 to 8.3 cm^2 for Amelonado (Fig. 2). There were also clear differences in the response to the growing environment. For example, AMAZ 15/15 and SCA 6 exhibited a higher growth rate in the simulated 'Malaysia' conditions although little difference was observed between the 'Brazil' and 'Ghana' conditions whereas SPEC 54/1 showed different growth rates in each of the three simulated environments (Fig. 2). Amelonado showed higher growth rates than the clonally propagated cocoa in the cooler 'Brazil' conditions, whilst in the warmer 'Malaysia' conditions was not significantly different from SPEC 54/1.

Calculated base temperatures, based on regression analysis of growth increments against temperature, indicated differences in base temperature between genotypes, the most temperature-sensitive being SPEC 54/1 (Table 2).

Chlorophyll fluorescence

Differences were observed in the ratio of variable to maximal fluorescence between simulated environments, which were more evident during the early part of the year corresponding to cooler periods in the 'Brazil' and 'Ghana' compartments. These differences were particularly pronounced for the genotypes SCA 6 and SPEC 54/1 where the Fv/Fm ratios were depressed in the 'Brazil' and Ghana' compartments during the measurement periods between February and April (Fig. 3). Hence, the interaction between genotype and time was significant ($P < 0.001$).

The variation in growing temperature over time in each compartment was compared with Fv/Fm for each

genotype by regression analysis. Variation in Fv/Fm for the clone SPEC 54/1 in the 'Brazil' compartment was shown to be a curvilinear function of temperature rising to approximately 24.5°C and declining under hotter conditions ($P < 0.01$, r^2 = 0.73; Fig. 4a). Across the three compartments, variation in Fv/Fm for SPEC 54/1 was also a curvilinear function of temperature although with a lower percentage of the variation accounted for by the regression (Fig. 4b, $P < 0.01$, r^2 = 0.30). Variation in Fv/Fm for the clone SCA 6 in the 'Ghana' compartment was a similar curvilinear function of temperature ($P < 0.05$, r^2 = 0.61; Fig. 4c). No effects of light integral were noted for any of the genotypes.

Chlorophyll concentration

There were significant differences in chlorophyll content between genotypes ($P < 0.001$); average chlorophyll content over the three simulated environments and over the time course ranged from 17.0 for Amelonado to 23.7 meter units for SCA 6 (Fig. 5). Chlorophyll concentrations were generally higher ($P < 0.001$) in the warmer 'Malaysia'
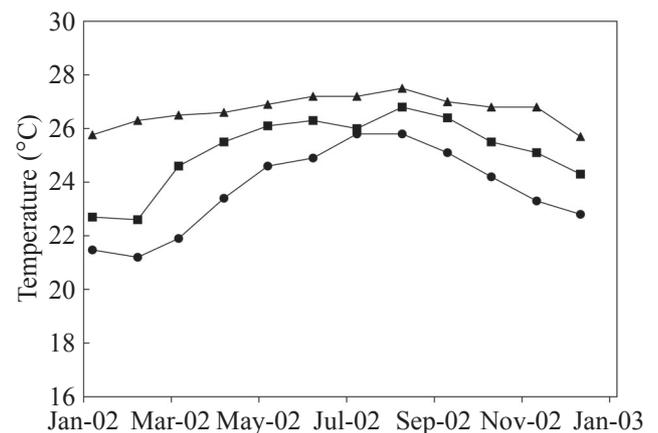

Fig. 1. Mean monthly temperatures of the three glasshouse compartments in which the temperature regimes of the cacao-growing regions of Brazil (●), Ghana (■) and Malaysia (▲) were simulated.
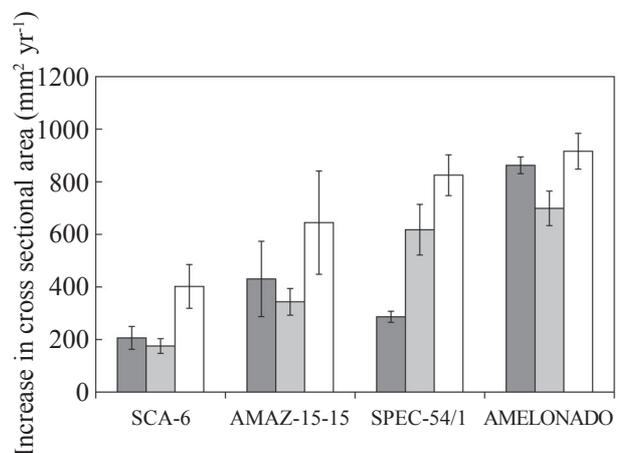

Fig. 2. Increase in the main stem cross-sectional area over the course of a year from the time of introduction into the thermal environments. Each point represents the mean of five observations. Brazil = dark grey bars; Ghana = light grey bars; Malaysia = unshaded bars.

compartment. There was a significant trend of chlorophyll concentrations increasing over the experimental time period ($P < 0.001$).

Multiple regression analyses revealed that, in the majority of cases, temporal variation in chlorophyll content within each compartment could be explained by both temperature and light integral. The forms of the equations were such that chlorophyll was a positive linear function of temperature and a negative linear or curvilinear function of light integral. For each of the four genotypes, variability in chlorophyll content could be explained by a common equation across the three compartments (Table 3).

Discussion

Main stem cross-sectional area is commonly used as an index of vegetative growth rates of trees (e.g. Larsen & Fitts, 1987). The observed differences in growth rate, as measured by the increase in main stem cross-sectional area, are indicative of the wide range of vigour that exists amongst cacao germplasm (Daymond *et al.*, 2002*b*), although the higher rate observed for Amelonado is probably due more to the fact that this was seed raised. The values calculated for base temperatures were considerably higher than that obtained by Alvim (1977), who calculated a base temperature of 9°C for the 'Catongo' variety based on pod growth. The high base temperatures calculated here are probably due to the fact that the present study was conducted under lower light conditions than that of Alvim (1977) which was carried out in the field in the tropics. The genetic differences observed here in base temperature indicate varying temperature sensitivies, as reflected by the different growth responses to the thermal environments.

When considering chlorophyll fluorescence, the two genotypes that had the greatest growth response to temperature (SPEC 54/1 and SCA 6) also showed a considerable suppression of Fv/Fm during the early part of the year in the 'Brazil' and 'Ghana' compartments. It is reasonable to assume that this suppression was primarily due to the cooler temperatures experienced at this time of the year in these two compartments. The only other environmental variable that fluctuated throughout the course of the experiment was light intensity.

Table 2. *Calculated base temperatures of four cocoa genotypes based on a linear regression of stem cross-section growth increment (I) against corresponding temperature (T)*

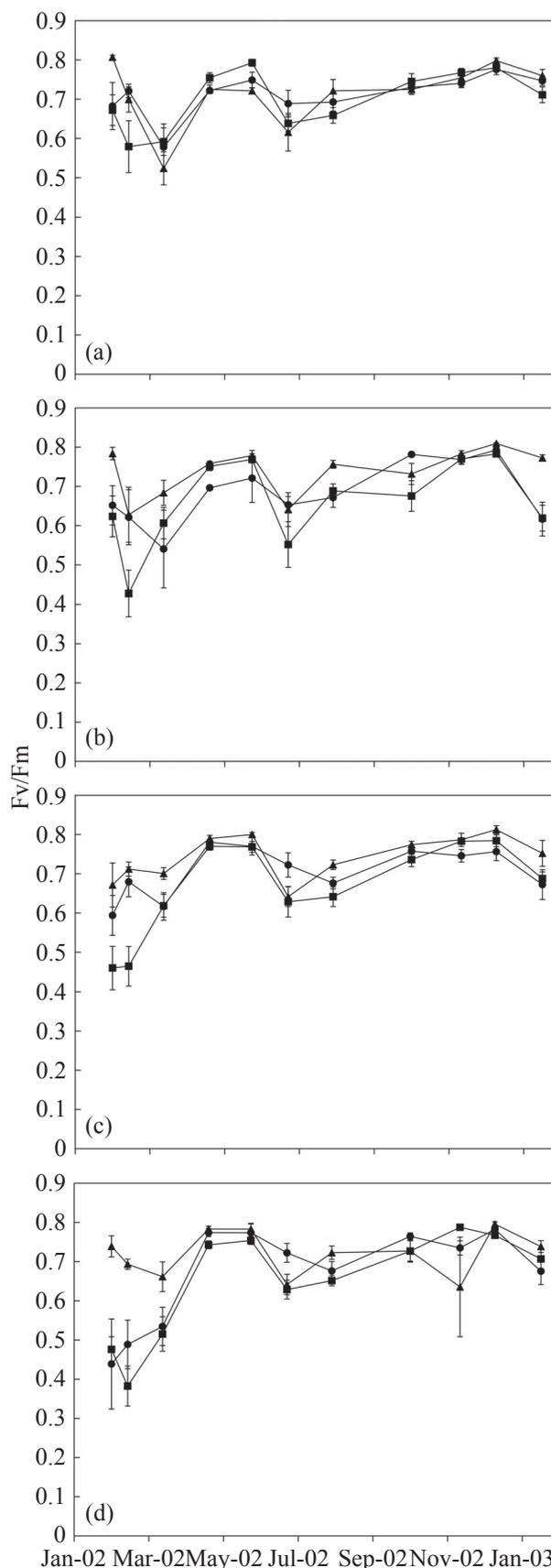| Genotype	Equation	Base temperature (°C)
Amelonado	I = 0.40T-7.85	19.7
AMAZ 15/15	I = 0.21T-3.89	18.6
SCA 6	I = 0.14T-2.82	19.9
SPEC 54/1	I = 0.37T-7.65	20.8

Fig. 3. Variation in the ratio of variable to maximum fluorescence (Fv/Fm) of four cacao genotypes grown under the three temperature regimes. a. Amelonado, b. AMAZ 15-15, c. SCA 6, d. SPEC 54/1. Each point represents the mean of five observations. ● = Brazil; ■ = Ghana; ▲ = Malaysia.
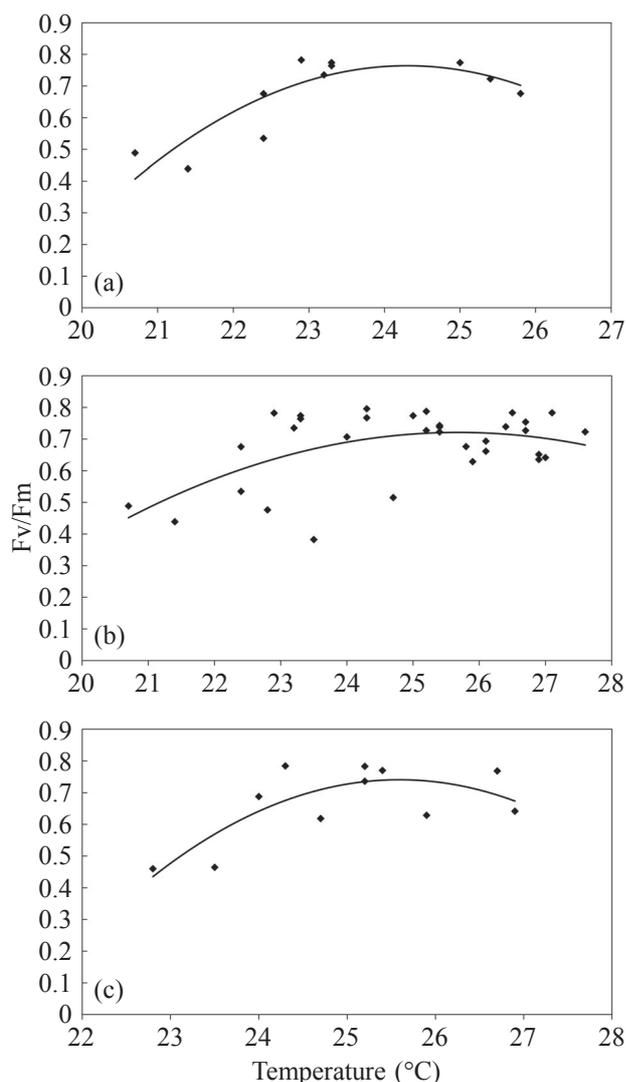
Fig. 4. Relationship between the temporal variation in the ratio of variable to maximum fluorescence (Fv/Fm) and temperature (T). a. SPEC 54/1 in the 'Brazil' compartment; Fv/Fm = $-0.0276T^2 + 1.3398T - 15.514$ ($r^2 = 0.73$). b. SPEC 54/1 across the three compartments; Fv/Fm = $-0.01087\ T^2 + 0.5572T - 6.4345$ ($r^2 = 0.30$). c. SCA 6 in the 'Ghana' compartment; Fv/Fm = $-0.0393T^2 + 2.0095T - 24.972$ ($r^2 = 0.61$). The temperature values (T) represent the mean value over 30 days up to the day of measurement.

Table 3. *Relation between temperature (T) and light integral (L) with chlorophyll content meter reading (Chl) across the greenhouses. Temperatures are in °C and are mean values between d-30 and d where 'd' is the day on which the chlorophyll measurements were taken. Light integrals are for PAR in mol m^{-2} day^{-1} and are mean values between d-30 and d*

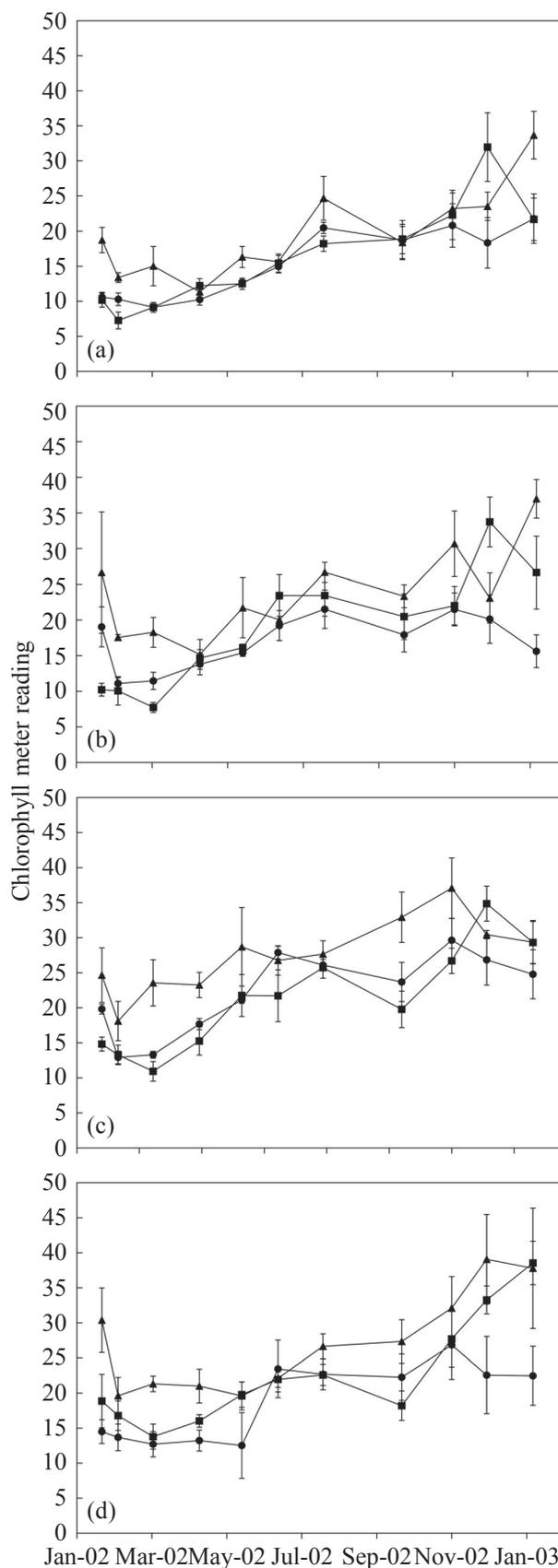| Genotype	Model	P	r^2
Amelonado	chl = 1.280T-2.126L+0.057L^2	< 0.001	0.50
AMAZ 15/15	chl = 1.021T-0.367L	< 0.01	0.32
SCA 6	chl = 1.412T-0.267L	< 0.05	0.24
SPEC 54/1	chl = 1.613T-2.460L-0.064L^2	< 0.001	0.55

Fig. 5. Variation over time of leaf chlorophyll content of four cacao genotypes grown under the three temperature regimes. a. Amelonado, b. AMAZ 15-15, c. SCA 6, d. SPEC 54/1. Each point represents the mean of five observations. ● = Brazil; ■ = Ghana; ▲ = Malaysia.

However, the fact that no suppression of Fv/Fm was seen in the 'Malaysia' compartment at this time of the year (where the temperature was fairly constant) suggests that light intensity did not effect Fv/Fm. Additionally, regression analysis showed no significant effect of light integral on Fv/Fm. The fact that better fits in the regression analysis were achieved using mean temperatures within compartments compared to between compartments may reflect the different maximum and minimum temperatures of the three simulated environments.

Cacao growth and development, in common with other tropical woody species, is highly temperature dependent and this manifests itself in terms of reduced photosynthetic rate at super- and sub-optimal temperatures (Raja Harun & Hardwick, 1988). The present study demonstrates that chlorophyll fluorescence may be used as a simple and highly sensitive technique of assessing temperature stress in cacao. The technique was able to elicit a proportionally large response in two of the clones to mean daily temperature differences of around 5°C.

The observation of genetic variation in chlorophyll content is consistent with a previous study of a different set of cacao clones (Daymond, 2000). Moreover, all genotypes showed a consistent decline in chlorophyll content with increasing light integral. Previous studies have demonstrated a higher concentration of chlorophyll in cacao grown under shaded conditions compared with those grown under full sunlight (Guers, 1974; Galyuon et al., 1996). Here, it has been demonstrated that the chlorophyll concentration of cacao leaves can vary quite considerably over time and that, in contrast to Fv/Fm, both seasonal variation in temperature and light integral contribute to these differences. The fact that better model fits were obtained when a time lag was used between environment factor and chlorophyll content as a variable is reflective of the extended period of chlorophyll production that is typical of cacao leaves (Baker & Hardwick, 1973). These models implied genotypic differences in the response to the photo-thermal environment.

In order to achieve optimal matching of cacao genotypes with their growing environment a number of factors clearly need to be taken into account which, in addition to temperature, may include tolerance to water stress and high light intensity. Nevertheless, the apparent genetic difference in the response of cacao to temperature demonstrated here implies that some potential exists for better matching of germplasm with growing conditions. Such a potential could be exploited in breeding and selection programmes using chlorophyll fluorescence as a selection tool. As a first step towards this, a screening of a wider range of cacao germplasm for high and low temperature tolerance is required.

Acknowledgements

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