Evaluating the combined effects of climate change parameters on growth and physiology of *Theobroma cacao* L.

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Declaration

I confirm that this is my own work and the use of all material from other sources has been properly and fully acknowledged.

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Acknowledgements

Abstract

Climate change scenarios predict increases in the atmospheric carbon dioxide concentration [CO_2] leading to global warming and changes in rainfall patterns in tropical regions. This will potentially impact the sustainability of cacao production and the livelihoods of millions of smallholder cacao farmers. Through a series of experiments, carried out under controlled environment facilities (growth cabinets and greenhouses), this research aimed to examine the interactive effects of elevated [CO_2], temperature and water deficit on the growth and physiology of juvenile and mature plants of different cacao genotypes. Elevated [CO_2] improved photosynthesis and growth parameters in cacao plants. However, the enhancement of growth was more evident in seedlings than matures trees. In seedlings, elevated [CO_2] shifted the optimal temperature of photosynthesis by 2.5°C under warming conditions, suggesting potentially increased resilience of cacao to increased temperatures under higher [CO_2] when air humidity and soil water is not limited. However, above 36/27°C (day/night) the compensatory effect of elevated [CO_2] diminished. The negative effect of increased temperatures on growth and leaf area in juvenile and mature cacao plants, as well as on aspects of reproductive development (pollen viability and fertilisation success), pod growth and pod and bean biomass, were alleviated by elevated [CO_2]. However, the apparent susceptibility of some genotypes to increases in temperature seemed to regulate the extent to which elevated [CO_2] alleviated the negative impacts of temperature. Under the water-limited treatment, the compensatory effect of elevated [CO_2] on photosynthesis and growth of seedlings was still observed but at a lower magnitude. However, temperature increases above 36/27°C exacerbated the adverse effect of water deficit. Genotypic variation in response to the different climate parameters demonstrated the potential for breeding cacao to cope with future scenarios. The results have shown that the impact of climate parameters on cacao are dynamic and interactive in nature, and the effect of a single climate variable may be modulated by others when they occur in combination.

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1 General introduction

1.1 *Theobroma cacao* L.

Cacao (*Theobroma cacao* L.) is a small tropical tree whose natural habitat is the lower storey of the evergreen rain-forest (Carr and Lockwood, 2011). Its origin and main centre of diversity is the north-western Amazon rainforest in South America. Although it has been reported that cacao was domesticated about 3,000 years ago in Mesoamerica (Argout *et al.*, 2011; Thomas *et al.*, 2012), there is also archeological evidence of cacao cultivation in South America approximatelly 5,300 years ago (Zarrillo *et al.*, 2018). Today, cacao is one of the most economically important tree crops, proving a livelihood to between 5 and 6 million smallholder farmers worldwide. Cacao seeds are used to produce cocoa powder and butter which are the key ingredients of chocolate and are also used for pharmaceutical and cosmetic purposes (Li *et al.*, 1998). According to recent statistics, the estimated production of dried cacao beans between 2020/2021 was approximately 5.1 million tonnes (ICCO (International Cocoa Organization), 2021) with the primary growing regions in West Africa, Southeast Asia and Central and South America. Between 80 and 90% of cacao comes from small family-run farms. The average yield globally is 500 kg of dried cacao seeds per hectare, but this varies considerably by region, country, management and type of cacao planted (Daymond *et al.*, 2022).

The *Theobroma* genus has been placed into the subfamily Byttnerioideae Burnett, one of nine subfamilies within the family Malvaceae (Richardson *et al.*, 2015). This genus comprises 22 species, of which cacao has the greatest global economic importance (Zarrillo *et al.*, 2018). Cacao has traditionally been classified into three groups, which can be distinguished by fruit and seed descriptors: Forastero (from Upper and Lower Amazon), cacao Criollo and the hybrid Trinitario (Bartley, 2005; Yang *et al.*, 2013). The Forastero group comprises different populations spread across the Amazon region from Colombia to Guyana (Martínez, 2007) whilst the Criollo group was first domesticated and cultivated by the Mayas in Central America from Mexico to Costa Rica (Motamayor *et al.*, 2002). The Trinitario group includes natural hybrids between Criollo and Forastero, which were introduced to Trinidad to replace Criollo plantations partially destroyed by a disease disaster in 1727 (Martínez, 2007). Motamayor *et al.* (2008) proposed 10 major clusters or groups (Marañon, Curaray, Criollo, Iquitos, Nanay, Contamana, Amelonado, Purús, Nacional and Guiana) for *Theobroma cacao* based on genotyping accessions sampled from a large geographical area in Central and South America.

In its natural habit, wild cacao trees can reach 20 to 25 m in height, while under cultivation its height is typically maintained between 3 to 5 m (De Almeida and Valle, 2007). Usually cacao is cultivated under the shade of larger trees which impacts final yield (Cunningham and Burridge, 1960) due to competition

between the shade species and cacao for water, nutrients and light (Beer, 1987). Usually, temporary shade, such as plantain, is planted during field establishment, which is later removed. The amount of shade maintained over mature cacao trees is highly variable and can comprise forest remnants or intercropped tree crop species. Although it has been shown that under non- limiting water and nutrients, cacao trees can produce greater yields under full sunlight than under shade conditions (De Almeida and Valle, 2007), the number of years of cropping may be reduced in sunlight.

Cacao can be propagated from seed, rooted cuttings or grafts and is transplanted in the field after 4 to 6 months growth under nursery conditions. Cacao seedlings initially have an orthotropic growth habit and a dimorphism is initiated after around 1-2 years at which time the apical meristem stops growing and typically 3-5 plagiotropic branches are initiated from the axillary positions in the shoot tip forming a "jorquette" (Greathouse and Laetsch, 1969). As with some other tropical species, the growth pattern in cacao takes place in cycles of leaf flushing (Vogel, 1975). Although it has been noted that leaf emergence is under endogenous control (Vogel, 1975), leaf development may be also affected by environmental factors such as soil moisture (Alvim *et al.*, 1974). The shoot-growth rhythms in cacao pass through alternate periods of growth and apparent dormancy. During the growth period, expansion of leaves and shoot elongation occurs (flushing) whereas during dormancy (inter-flushing), the shoot is constant in length, and no new leaves expand (Greathouse *et al.*, 1971).

In the post jorquette phase, cacao produces caulescent flowers which form on the trunk and main branches of the tree (Toxopeus, 1985) and the floral cushion or meristem produces flowers throughout the life of the tree (Aneja *et al.*, 1999). Flowers are particularly small (between 1 and 2 cm) and contain an ovary that is surmounted by a thin style which is divided terminally into five stigmatic lobes. The stigma and style rarely reach 3 mm, and the whole structure is receptive to the pollen grains. Cacao flowers have 5 sepals, 5 petals, and 10 stamens (Glendinning, 1972). The flowers begin dehiscing in late afternoon and are fully open early morning of the following day releasing pollen to the receptive stigma. Pollination is most effective during the first 12 hours, after which pollen becomes less viable for fertilization (Aneja and Gianfagna, 1992). Flowers that are not pollinated may abscise 24-36 h after the anthesis (Bertolde *et al.*, 2012). Despite the large number of flowers produced by the cacao tree, less than 5% develop into cacao pods (Aneja *et al.*, 1999). If fertilization occurs, the ovary increases in size, and cacao fruits ("pods") mature over a period of 5 - 6 months and may contain about 30 - 40 seeds (or "cacao beans") (Toxopeus, 1985). During the period of cacao pod development, a proportion of pods can be lost through a process called "cherelle wilt", a physiological process to reduce the pod load on the tree, whereby pods shrivel and turn black whilst still attached to the tree (Nichols, 1964). It has been hypothesized that the intensity of cherelle wilt is related to assimilates or nutrient status and

competition for carbohydrates within the tree (Nichols and Walmsley, 1965; Alvim, 1977; Valle *et al.*, 1990).

1.2   Climate change

The concentration of CO_2 in the atmosphere (hereafter referred to as $[CO_2]$) has increased rapidly since pre-industrial-era (for more than 10,000 years), from 280 parts per million (ppm) (Lüthi *et al.*, 2008) to values that exceeded 400 ppm for the first time recorded in May 2013 (NOAA, 2014). The increase has varied from year to year with an average $[CO_2]$ growth rate of 1.7 ppm year^{-1} for the last century. However, rates of 2 ppm year^{-1} were reported between 2001 and 2011 (Hartmann *et al.*, 2013). Depending on the emissions scenario based on the Representative Concentration Pathways (RCPs), the atmospheric $[CO_2]$ is projected to range from 550 ppm in 2050 (RCP 2.6) to 970 ppm in 2100 (RCP 8.5) (Collins *et al.*, 2013). Elevation of atmospheric $[CO_2]$ and other greenhouse gases are impacting the global climate. Temperatures are expected to increase by approximately 1.5 °C to 5.8 °C by the end of the 21st century (RCP 2.5 and RCP 8.5, respectively), whilst there will be a higher frequency of heat waves and fewer cold temperatures episodes in many areas. There will also be shifts in precipitation patterns, such that some areas will have prolonged drought episodes with more heavy rainfall episodes in others (IPCC, 2021). Impacts of climate changes on agriculture are already being seen and gaps in our knowledge of how agricultural systems will be affected in the short and long-term, and its implications for rural livelihoods have emerged from the last decade (Wassmann *et al.*, 2009). Crops can respond nonlinearly to changes in their climatic conditions, being subjected to interactions of stress factors that impact the growth and final yield (Porter and Semenov, 2005).

1.2.1   Temperature effects on plants

Plants have optimal temperature requirements for their general growth and performance (Hatfield *et al.*, 2008). The literature demonstrates a wide range of responses to temperature in terms of growth and development for different tropical species. Maximum/minimum temperatures of 33°/28° compared with 18°/13°, 23°/18° and 28°/23°C accelerated stem extension and node production as well as floral formation in nine *Coffea Arabica* L. cultivars (Drinnan and Menzel, 1995). Seasonal growth patterns of stem circumference growth was detected in semi deciduous trees (Blagitz *et al.*, 2016) and tropical rainforest trees (Shimamoto *et al.*, 2015) in Southern Brazil. Furthermore, the interaction of temperature with other climatic factors may impact on phenology and development. For instance, temperature and rainfall patterns have been associated with growth cycles in tropical forests (Lechowicz, 1995) and flowering and leaf flushing were correlated with day length and water availability in woody trees in China, rain forest trees in Brazil and Moriche Palm (*Mauritia flexuosa*) in the Colombian Amazon (Morellato *et al.*, 2000; Urrego *et al.*, 2016; Mohandass *et al.*, 2018).

The net photosynthetic rate of plants often increases in the short-term with warming up to an optimum temperature, above which it drops off as temperature rises above that point (Sage and Kubien, 2007). However, plants can show considerable capacity to adjust their photosynthetic characteristics to their thermal growth conditions, allowing more efficient photosynthesis at their new temperature in a process called acclimation (Yamori *et al.*, 2013). Slot and Winter (2017) reported photosynthetic acclimation to warming in three tropical trees through measuring photosynthetic temperature-response curves. The authors also stated that although photosynthesis can acclimate to moderate warming, carbon gain decreases with more severe warming.

Elevated temperatures can also lead to an increase in the vapour pressure deficit (VPD) which also can affect photosynthesis. In reviewing the impacts of rising temperatures on tropical trees, Lloyd and Farquhar (2008) concluded that reductions in photosynthesis may occur at warmer temperatures due to increases in VPD which induce stomatal closure. However, the authors also pointed out the direct effect of temperature on photosynthetic metabolism resulting from changes in the activity of ribulose-1,5 - carboxylase/oxygenase (Rubisco) as well as processes associated with the regeneration of Rubisco's substrate, ribulose-1,5-bisphosphate (RuBP) through the Calvin cycle. These processes are reversible at moderately high temperatures but becomes increasingly irreversible with length and intensity of high temperature exposure (Berry and Bjorkman, 1980).

Temperature changes can be associated with a temperature-induced metabolic response that leads to shifts in carbohydrate allocation within trees (Dietze *et al.*, 2014). In "normal" or "optimal" growth conditions, there is a balance between carbon sources and sinks and temperature stresses may affect this balance (Ericsson *et al.*, 1996). Corrections in the metabolic process and export of assimilates from carbon sources occurs causing a new carbon balance between source and sink (Geiger and Servaites, 1991). Sperling *et al.* (2017) studied whether carbohydrate allocation within trees is assisted by temperature variations in pistachio (*Pistacia integerrima*). The authors reported that warm branches had less sugar in their sap than cold branches and spring conditions promoted allocation of carbohydrates from cold roots to the warm canopy. These seasonal responses were also highlighted by Liu *et al.* (2018) who reported that starch and non-structural carbohydrate (NSC) concentrations were strongly affected by dry (cold temperatures) and wet periods (warm temperatures) amongst twenty dominant species in monsoon evergreen forest in China.

1.2.2 Carbon dioxide (CO_2) effects on plants

Several reviews have summarized the responses to elevated [CO_2] in crops (Kimball *et al.*, 2002; Ainsworth and Rogers, 2007; Xu *et al.*, 2015; van der Kooi *et al.*, 2016). Plants respond differently to increases in [CO_2] according to their mechanism of carbon fixation. Generally, C_4 plants respond less

than C_3 plants (Poorter and Navas, 2003). The short-term increase in net photosynthesis rate in C_3 plants is due to the higher carboxylation rate of ribulose- 1.5 bisphosphate carboxylase/oxygenase (Rubisco), which originates from the simultaneous increases in substrate availability and competitive inhibition to Oxygen (O_2) (Drake *et al.*, 1997; Kirschbaum, 2011). Norby *et al.* (1999) reported that in open-top chamber experiments, photosynthesis of trees was stimulated by between 40 - 80% in most of the studies reviewed, although in some cases the enhancement was considerably greater. In a meta-analysis, of photosynthetic responses of different functional groups using free-air CO_2 enrichment (FACE) experiments, Ainsworth and Long (2005) concluded that trees were more reactive than other functional species to elevated [CO_2] with increases of 47% in photosynthesis, which was higher than the formerly reported 31% increase for FACE experiments carried out by Curtis and Wang (1998).

Whilst large short-term responses to increased [CO_2] have been observed, long-term photosynthetic responses to elevated [CO_2] can be affected by biochemical processes and physiological reactions that balance carbon assimilation with growth (i.e. sink) demand (Stitt, 1991; Sage, 1994; Körner, 2003). This phenomenon, called acclimation of photosynthesis, or downregulation is accompanied by higher non-structural carbohydrate (NSC) concentrations, lower concentrations of soluble proteins and Rubisco, and inhibition of photosynthetic capacity (Drake *et al.*, 1997). Various specific reasons have been cited for the occurrence of acclimation. Firstly, the plant may be unable to use all the additional carbohydrates from the photosynthetic activity (Drake *et al.*, 1997) and this exceeds the sink capacity to utilize the photosynthates for growth (Makino and Mae, 1999) and secondly, acclimation has been attributed to decreases in Rubisco and leaf Nitrogen (N) concentrations (Stitt, 1991; Sage, 1994; Drake *et al.*, 1997; Makino and Mae, 1999). Nevertheless, Saxe *et al.* (1998), stated that downregulation of photosynthetic capacity was mainly associated with stressed plants, at least for trees species. This was corroborated in FACE experiments on sweet gum tree and coffee plants which showed increases in net photosynthesis rate without downregulation after 3 and 4 years of exposure to elevated [CO_2] respectively (Sholtis *et al.*, 2004; Rakocevic *et al.*, 2018).

It has been suggested that stomata respond to several environmental factors including [CO_2] enrichment. Reduced stomatal conductance (g_s) has been reported as a short-term responses to increased [CO_2] whilst long-term responses have also been reported such as morphological changes in size and stomatal density (SD) (Woodward and Kelly, 1995; Kimball *et al.*, 2002; Ainsworth and Rogers, 2007; Ramalho *et al.*, 2013). For example, using 41 observations from 28 different species, Drake *et al.* (1997) found that the average reduction of g_s at elevated [CO_2] (ranging from 542 ppm to 986 ppm) was 20% across the species analysed. This was similar to the analysis presented by Medlyn *et al.* (2001) who reported a reduction of 21% (estimated at 700 ppm CO_2) in g_s in trees, and the average reduction of 22% (estimated at 567 ppm CO_2) among a wide range of species reported by Ainsworth and Rogers (2007). In

addition, an improvement of water use efficiency (WUE) has been shown to be associated with reduced g_s at elevated $[CO_2]$ through a reduction in transpirational water loss. Improved WUE would benefit plant performance within a climate change scenario where the water availability is sometimes expected to be reduced (Xu *et al.*, 2016). It should be noted that the magnitude of the change in stomatal density in response to elevated $[CO_2]$ might vary according to the duration of the experiment, species or even genotype, and interactions with other environmental factors (Xu *et al.*, 2016).

Elevated $[CO_2]$ increases photosynthesis and this may result in increased aboveground biomass and final yield (Baker and Allen, 1994; Makino and Mae, 1999). Singh and Jasrai (2012) stated that elevated $[CO_2]$ has generally been shown to enhance crop growth although with large intra/inter-specific variation. Saxe *et al.* (1998) indicated that elevated $[CO_2]$ significantly increased tree biomass with increased exposure time. After 338 days, an increase in biomass of 130% was observed with elevated $[CO_2]$ for conifers, whereas deciduous trees exposed over 329 days showed an increase of only 49%. Ainsworth and Long (2005) reported that, using large-scale FACE experiments, growth and above-ground biomass generally increased at elevated $[CO_2]$ but the magnitude varied according to the species, growing season and experimental conditions. In addition, plants that are exposed to elevated $[CO_2]$ are capable of absorbing more nutrients because more carbon is allocated below-ground, leading to higher number of fine roots (Ceulemans *et al.*, 1999).

Finally, elevated $[CO_2]$ may also impacts on the timing of phenological stages such bud burst (Jach and Ceulemans, 1999), flowering (Springer and Ward, 2007), and fruit development (Schaffer *et al.*, 1999) due to changes in physiology and plant biochemistry. Authors have stated that changes in starch or hormonal levels could alter dormancy and growth responses by shifting the timing and duration of different vegetative phases (Saxe *et al.*, 1998; Norby *et al.*, 1999).

1.2.3 CO_2 and temperature effects on plants

Increasing $[CO_2]$ in the atmosphere is associated with an increase in the mean global temperature. Therefore it is important to consider interaction between $[CO_2]$ and temperature on physiological responses. Models of the C_3 photosynthetic pathway predict that increased $[CO_2]$ enhances photosynthesis and this should increase further at elevated temperatures (Long, 1991; Kirschbaum, 1994). Based on simulations, Long (1991) showed that increases in leaf photosynthesis with increases in $[CO_2]$ from 350 to 650 ppm varied at temperature level. They noted increases in photosynthesis by 20% at 10°C and by 105 % at 35°C and also that the temperature optimum increased for photosynthesis by 3°C at 500 ppm to 5°C at 650 ppm. Furthermore, they suggested that at the canopy level, the magnitude of the responses can vary between species. Ainsworth and Long (2005), analysing data from FACE

experiments on more than 40 species showed that photosynthesis under elevated $[CO_2]$ was enhanced by 19% for experiments carried out below 25°C and by 30% for those carried out above 25°C.

Idso and Kimball (1992), working with orange trees (*Citrus aurantium* L.), reported that a rise of 300 ppm of CO_2 at mean leaf temperatures of 31°C, 35°C and 42°C, increased the photosynthesis rate of leaves exposed to full sun by 75, 100 and 200% respectively and demonstrated an increase in the upper temperature limit for growth by 7°C under elevated $[CO_2]$. Furthermore, a study of the physiological performance of two well-watered and fertilized eucalyptus species (faster growing *Eucalyptus saligna* and slower growing *Eucalyptus sideroxylon*) under three $[CO_2]$ (290, 400 and 650 ppm) and two day/night temperatures (26/18 °C and 30/22°C), showed that, at the high temperature, the thermal optimum of photosynthesis increased by 2-7°C across the $[CO_2]$ treatments, suggesting that eucalyptus seedlings will remain strongly responsive to an increase of atmospheric $[CO_2]$ in a future warmer climate (Ghannoum *et al.*, 2010). However, an interaction between temperature and $[CO_2]$ has not always been observed. For example Tjoelker *et al.* (1998) evaluating acclimation and ontogenetic drift of photosynthesis rate in seedlings of several boreal species grown at ambient and elevated $[CO_2]$ and a combination of 5 day/night temperatures (18/12°C, 21/15°C, 24/18°C, 27/21°C, and 30/24°C), showed that increases in photosynthesis rate varied from 13 to 36% among the species at elevated $[CO_2]$. Nonetheless, increasing growth temperature did not enhance the response of photosynthesis rate in 4 of the 5 species studied.

Increasing air temperatures and atmospheric $[CO_2]$ has been reported to induce changes in g_s over short and long periods of time (Way *et al.*, 2015). In the short-term increases in air temperature typically lead to a reduction in g_s (Way *et al.*, 2015; Slot and Winter, 2017) but under high temperature stress g_s may actually increase (Urban *et al.*, 2017; Drake *et al.*, 2018). On the other hand, it has generally been observed that instantaneous responses to elevated $[CO_2]$ result in a decrease of g_s (Fauset *et al.*, 2019). In a review of climate change impacts on vegetation, Baker and Allen (1994) stated that stomatal closure at elevated $[CO_2]$ results in increased leaf temperature, and consequently increases in the vapour pressure gradient between air and leaf, thereby impacting the plant water status at warm temperatures. In this way, elevated $[CO_2]$ under high temperatures may exaggerate heat damage partly due to decreased g_s (Warren *et al.*, 2011).

A meta-analysis carried out by Wang *et al.* (2012) examined the interaction between elevated $[CO_2]$ and temperatures on the physiology and growth of different species grouped according to their photosynthetic pathways (C_3, C_4), functional types (legumes, non-legumes) and growth forms (herbaceous, woody). They categorized $[CO_2]$ as ambient (<400 ppm) or elevated (>560 ppm) and temperature levels as ambient, elevated (ambient +1.4-6°C) and heat stress (ambient + >8°C). The

enhancement of photosynthesis by elevated [CO_2] was found to be greater for woody species than for herbaceous species at elevated temperature and heat stress. Additionally, the total dry weight of above and belowground biomass was increased by elevated [CO_2] for most species groups at all temperatures, especially for C_3 species. Specifically, working on perennial Chinese yam, Thinh *et al.* (2017) grew plants at two [CO_2] (ambient, ambient+200 ppm) and under two temperature regimes (day/night: 29.1/24.1°C and 24.9/20.2°C). The authors reported that leaf area, leaf dry weight and total dry weight were significantly higher at elevated [CO_2] compared with ambient [CO_2] for both temperature regimes.

Vegetation models have incorporated the interaction between high atmospheric [CO_2] and temperature (Long, 1991) on leaf photosynthesis and predict a positive response on net primary productivity in warm tropical forests with a doubling of the [CO_2] (from 350 to 700 ppm) (McMurtrie and Wang, 1993; Hickler *et al.*, 2008). However, Baig *et al.* (2015) challenged these assumptions after carrying out a meta-analysis to test vegetation models to predict net primary productivity (NPP). Firstly, the growth responses in factorial combinations of elevated [CO_2] x temperature showed a positive but not significant interaction between temperature and elevated [CO_2]. Additionally, examining field-based experiments, the authors reported a similar but non-significant correlation with mean annual temperature.

1.2.4 Drought effects on plants

The term drought can be classified in several ways, but for agronomic purposes the conceptual definition involves physical processes such as a lack of precipitation over a region for a period of time (meteorological drought) or shortages of soil moisture (agricultural drought) (Mukherjee *et al.*, 2018). Drought exerts changes in physiological, morphological, biochemical and molecular traits in plants, which have varied responses depending on plant species (Salehi-Lisar and Bakhshayeshan-Agdam, 2016). For short-term crops under drought, there is an inhibition of floral induction, reproductive development and final yield in rice and maize and decreases in photosynthesis rate and leaf water potential (Ψ) in soybean (Saini, 1997; Saini and Westgate, 1999; Liu *et al.*, 2004). This reduction in photosynthesis has been attributed to stomatal closure to prevent the water loss from the leaves, which also limits CO_2 uptake (Chaves *et al.*, 2002). Working on trees, Larcheveque *et al.* (2011) found that three different poplar clones showed varying degrees of regulation of leaf water potential, differences in shedding leaves, root growth and stem growth, and differences in water use efficiency in response to drought. A study on Aspen (poplar) seedlings addressed the issue of whether drought causes carbon starvation by examining root carbohydrates (Galvez *et al.*, 2011). The authors found that drought quickly lowered stomatal conductance, photosynthesis and tree height, but that root carbohydrate reserves increased in the drought treatment compared to the controls.

For tropical species such as Guava and Coffee, the pattern of wet and dry periods contributes to the seasonal responses of growth, flowering and fruiting (Alvim, 1960; Opler *et al.*, 1976; Mercado-Silva *et al.*, 1998). Alvim (1960) suggested that there was a need for a dry period in cacao which is similar to the chilling requirement seen in temperate species for flowering stimulation. In tropical rainforest species, strong seasonal variation in tree growth has been observed: decreasing during the drought periods and significantly increasing in the rainy season (Tian *et al.*, 1998; Baker *et al.*, 2003; Nepstad *et al.*, 2004).

Based on the different mechanisms that plants have to overcome water deficit or drought conditions, various drought-related traits have been identified (Fang and Xiong, 2015). For drought avoidance, plants have the capability to maintain physiological processes by adjusting morphological structures or growth in order to maintain adequate plant water potential such as stomata closure, leaf rolling (Tardieu, 2013), increasing wax accumulation on the leaf surface to reduce transpiration or a well-developed and deep root system to enhance water uptake (Fang and Xiong, 2015). On the other hand, drought tolerance involves physiological processes under severe stress conditions through the regulation of metabolic pathways to reduce or repair the resulting stress damage. In this way, plants also apply protoplasmic tolerance by increasing osmoregulatory molecules in the cells to maintain the cell turgor pressure (Luo, 2010).

1.2.5 CO_2, temperature and drought effects on plants

As discussed, climatic models predict that frequency, intensity and duration of dry periods will increase with elevated $[CO_2]$ and high temperatures (IPCC, 2021). Moreover, the interaction between these climatic parameters are difficult to predict due to the fact that increases in $[CO_2]$ and temperature have complex effects on plant growth, gas exchange, and plant biochemistry (Duan *et al.*, 2013). In general, elevated $[CO_2]$ may benefit plants under drought stress through decreases in water use and favourable leaf water relations (Atwell *et al.*, 2007) and lead to increased carbon assimilation (Wertin *et al.*, 2010), while warming has often been reported to exacerbate drought stress due to increases the evaporative demand (Allen *et al.*, 2010; Will *et al.*, 2013). In this way, combined effects of high $[CO_2]$ and warming temperatures under drought conditions may vary depending on the balance between $[CO_2]$ and temperature. Research using perennial species have shown contrasting responses. For example, the expected positive impact of elevated $[CO_2]$ on *Pinus halepensis* (Aleppo pine) was moderated and mostly disappeared under advancing high temperatures and water deficit (Birami *et al.*, 2020) while the combination of both elevated $[CO_2]$ and temperature did not affect the drought responses in *Pinus taeda* L. seedlings (Wertin *et al.*, 2012a), and temperature exacerbated drought stress in *Eucalyptus radiata* seedlings (Duan *et al.*, 2014).

Inter specific responses were explored by Duan *et al.* (2015), analysing two different gymnosperm species (*Pinus radiata* D. Don and *Callitris rhomboidea* R. Br) that differed in stomatal regulation strategies. They reported that elevated temperature (+4°C) had greater influence than elevated $[CO_2]$ (640 ppm) on the final drought response for both species. This was also noted previously by Lewis *et al.* (2013) who showed that elevated temperature under drought conditions reduced photosynthesis in *Eucalyptus saligna* Sm. (faster growing species) and *Eucalyptus. sideroxylon* (A.Cunn. ex Woolls) (slower growing species). In this work, the authors suggested that the beneficial effects of rising $[CO_2]$ and negative effects of high temperature on seedling responses to drought were generally balanced. However, another recent study using *Eucalyptus sideroxylon* A. Cunn. ex Woolls in a full-factorial combination of $[CO_2]$ and temperature, stated that elevated $[CO_2]$ significantly exacerbated drought stress when combined with elevated temperature (Duan *et al.*, 2018). Physiological processes and plant growth responses to future scenarios may not be predicted from a single experiment and multi-factorial experiments are important to make clear the integrated responses (Xu *et al.*, 2014).

1.3 Climate change in cacao regions

Global climate change is significantly affecting tropical ecosystems with extreme events such as drought, storms, cyclones and wildfires which fundamentally alter species distribution, composition, phenology and structure (Deb *et al.*, 2018). As *Theobroma cacao* is grown widely across the tropics, it is expected to be subjected to extreme climatic conditions. Indeed, studies based on surveys and interviews report that farmers are already experiencing the effects of climate change on cocoa development and yield due to changes in weather patterns, rainfall distribution, prolonged dry periods and increases of diseases in plantations (Läderach *et al.*, 2013; Anim-Kwapong and Frimpong, 2014; Hutchins *et al.*, 2015; Jacobi *et al.*, 2015). As a result, the need for strategies for smallholder farmers to face the emerging negative effect of climate change has been highlighted, such as best agronomic practices, new varieties and resilient systems (Oyekale *et al.*, 2009; Nwachukwu *et al.*, 2012; Läderach *et al.*, 2013; Jacobi *et al.*, 2015; Ruf *et al.*, 2015; Schroth *et al.*, 2016).

Extreme events related to prolonged dry periods (El Niño Southern Oscillation -ENSO) have been quantified across cacao regions globally. For example, extreme events compared to conventional historical data caused 62% loss in cacao production in Sulawesi (over an average of 28 episodes), a 27% loss in West Africa (episode 1982-83), a 19% loss in Ecuador (episode 1997-98) and 13% tree mortality with an 89% decrease in cocoa yield in Brazil (episode 2015-16) (Vos *et al.*, 1999; Keil *et al.*, 2007; Ruf *et al.*, 2015; Gateau-Rey *et al.*, 2018). Early climatic studies based on predicted maximum temperatures, have suggested that some regions in West Africa will become inappropriate for cacao farming (Läderach *et al.*, 2013; Schroth *et al.*, 2016). Although models are useful tools to assist the decision making for crop

management practices and mitigate climate risks (Medina and Laliberte, 2017), it is important to have underlying data on underlying responses of cacao trees to environmental factors. Physiological information is an essential element for improving the accuracy of plant modelling and most of the model failings would be overcome by better understanding of the main physiological responses to abiotic stress (Marin *et al.*, 2014). Recently, a study based on a land-surface model which integrated additional climatic information and experimental data obtained under controlled facilities in the UK, showed that the effect of elevated $[CO_2]$ might ameliorate the impacts of high temperatures and variation in rainfall patterns in West Africa (Black *et al.*, 2021). Furthermore, a more comprehensive picture of the interaction between combined climatic parameters such as elevated $[CO_2]$, temperature and water deficit on physiological responses of cacao could also lead to the potential for breeding programmes to develop or select more resilient genotypes which are more adapted to future climate change scenarios (Lahive *et al.*, 2019).

1.3.1 Temperature effects on cacao

Growth and development responses to temperature have been reported in cacao. Working with young cacao plants under controlled conditions, Sale (1968) observed a shorter interval between leaf flushes at a day time temperature of 30°C compared to either 26.7°C or 23.3°C while the number of leaves per flush and leaf area increased with lower day or night temperature. According to De Almeida *et al.* (1987a), monthly average temperatures above 23°C coincided with periods of a high number of flushes. In a complementary study, Cazorla *et al.* (1989) observed lower flushing rates at temperatures below 23°C in the south-east of Bahia, Brazil between June and September. Leaf temperature may also impact on leaf longevity; Miyaji *et al.* (1997) observed two periods of intensive leaf fall over the course of 17 months in 7 year old cacao plants in Bahia, Brazil, which coincided with days when the air temperature and solar radiation were higher. Evidence for genotypic variation in response to temperature was observed in cacao by Daymond and Hadley (2004) who reported different growth responses and different base temperatures of four genotypes grown under glasshouse conditions.

Exploring cacao flowering responses to temperature in Trinidad, Sale (1969) observed higher numbers of flowers per cushion per plant at day time temperatures of 26.7°C and 30°C compared with 23°C in an experiment under controlled environment conditions. Studies based on field data, showed that flowering intensity of mature cacao plants in Bahia, Brazil follows a seasonal pattern decreasing from June to September as result of lower mean temperatures below 20°C (Alvim, 1977). For the same region it was reported that a minimum daily average temperature of 23°C is required for flowering, based on data collected over several years in Brazil (De Almeida *et al.*, 1987b). Below this temperature, the authors observed a reduction of flowering about 3 to 4 weeks later.

Alvim (1977) pointed out that fruit development was affected by seasonal differences in temperature in Brazil. He observed that the duration of pod growth was shorter in warmer periods and longer at cooler times of the year. Similarly, End *et al.* (1988) also demonstrated a reduction in time to maturity with increased temperature in plants grown under greenhouse conditions. The analysis indicated that temperature had a greater effect than light intensity on time to maturity. Examining the effects of temperature on cherelle wilt (abortion of developing cacao pods) in mature cacao trees, Hadley *et al.* (1994) showed a reduction in the time from pollination to first symptoms of cherelle wilt with increasing mean daily temperatures from 60 days at 20°C to 38 days at 27°C. The percentage of wilted cherelles increased with the temperature from 9.5% at 20°C to 65% at 27°C. Daymond and Hadley (2008) showed that fruit losses were greater at higher temperatures but also differed between genotypes. They demonstrated that the rate of pod development increased with temperature and there was a negative relationship between temperature and time to pod maturation. The effects of temperature on bean quality has also been explored. Niether *et al.* (2017) comparing bean quality at the beginning and end of the dry season in Bolivia, found that the weather affected the chemical composition of beans: in the dry season, the high temperatures (and low soil water content) were associated with increased antioxidants in the beans and reduced fat content. Similar findings on bean fat content were observed by Daymond and Hadley (2008) in cocoa trees growing under controlled temperature greenhouse conditions.

Raja Harun and Hardwick (1988) determined how photosynthesis and transpiration are affected by temperature and vapour pressure deficit (VPD) in mature leaves of cacao plants. They noted an increase in VPD at each leaf temperature decreased stomatal conductance. Transpiration increased over a range of increases in VPD, but with further increases in VPD, transpiration remained constant at each leaf temperature. The authors also reported a slight decrease of around 10-15% in photosynthetic rate when temperatures increased from 20 to 30°C. However, Balasimha *et al.* (1991) reported an optimum temperature range for photosynthesis under field conditions from 31 to 33°C amongst a group of cacao accessions susceptible and tolerant to drought stress in India. DaMatta (2007) showed that the decrease in photosynthetic rate at supra-optimal temperatures could be explained by the direct effect of temperature or a rise in atmospheric VDP leading to stomatal closure, or a combined effect of these two factors. Acheampong *et al.* (2013) observed seasonal variation in photosynthesis rates and suggested that this was most likely to be correlated with changes in VPD over time from the rainy season to the dry and warmer period in Ghana.

1.3.2 CO_2 effects on cacao

Balasimha *et al.* (1991), examining the influence of environmental factors on cacao photosynthesis in India, reported a nearly linear positive relationship between net photosynthesis rate and leaf internal [CO_2] (*Ci*). Baligar *et al.* (2005) conducted a short-term experiment at two CO_2 concentrations (380 and 700 ppm) and three Photosynthetic Photon Flux Densities (PPFD: 65, 190, 1050 µmol m^{-2} s^{-1}) under controlled glasshouse conditions on cacao seedlings. Plants growing at the higher [CO_2] increased the uptake of all mineral nutrients and there tended to be an increase in measured shoot and root growth parameters. Subsequently, Baligar *et al.* (2008) working on three different genotypes found significantly increased rates of net photosynthesis and leaf internal [CO_2] (*Ci*) with an increase in [CO_2] from 85 to 680 ppm while g_s and transpiration rates (*E*) decreased by about 65%. This reduction in stomatal conductance is greater than for some perennial and annual species that reported reductions of 24% and 40% respectively (Bunce, 2004) or younger forest tree species where a 21% reduction was reported (Medlyn *et al.*, 2001). Baligar *et al.* (2008) also suggested that increasing atmospheric [CO_2] could probably improve cacao water-use efficiency.

Photosynthetic light response curves were performed on four contrasting cacao genotypes (Amelonado, CL 19/10, SCA 6 and POUND 7/B) in a greenhouse experiment to determine the response of photosynthetic parameters to instantaneous increases in [CO_2] (Lahive, 2015). All genotypes responded to CO_2 elevation with an increase in photosynthetic rate. However, the magnitude of change in response to an increase in [CO_2] of photosynthetic parameters such as light-saturated photosynthesis, quantum efficiency, light saturation point, stomatal conductance, and water use efficiency appeared to be genotype specific.

1.3.3 CO_2 and temperature effects on cacao

Naresh Kumar *et al.* (2012) explored the independent effects of CO_2 and temperature on gas exchange, chlorophyll parameters, and leaf biochemical composition (non-structural carbohydrates) of grafted cacao plants grown for two-years in an Open-Top Chamber (OTC) facility. Elevated [CO_2] (550 and 700 ppm) and elevated temperature (+2°C above ambient OTC) were compared with a control treatment comprising ambient CO_2 and temperature. The authors reported significant increases in photosynthetic rates, while transpiration was similar to the ambient treatment, leading to increases of 50 and 112% in instantaneous water use efficiency (WUE) at 500 and 700 ppm, respectively. Moreover, elevated temperature caused a slight reduction in photosynthetic rate but there was an increase of 20% in instantaneous WUE, due to lower stomatal conductance. Under both elevated CO_2 and temperature treatments an increase in the concentration of chlorophyll a compared with chlorophyll b were observed relative to controls, as well as an increase in total soluble sugars and starch in leaf tissues.

Subsequently, in a study using six-month old seedlings grown under similar OTC facilities, and a combination of elevated $[CO_2]$ (550 ppm) and temperature (+2-3°C above ambient OTC) over the course of 8 months, Hebbar *et al.* (2020) showed that increased CO_2 could minimize the severity of high temperatures on photosynthesis rate, leaf water potential and biomass accumulation. However, seasonal variation in temperature across the experimental period and the limitation of humidity control under the OTC facilities were evident. It has been noted that several environmental parameters might vary and are not under full control under OTC such as air turbulence, light intensity, air temperature and humidity (Feng *et al.*, 2018). Controlled-environment facilities such as growth chambers and glasshouses, provide long-term and stable conditions which make clear differences among treatments and allow treatment combinations to be imposed (D'Andrea and Rinaldi, 2010).

1.3.4   Drought effects on cacao

Rainfall patterns (amount, distribution and duration) are one of the most important factors affecting yield in cacao (Wood, 1985; Balasimha *et al.*, 1991; Zuidema *et al.*, 2005; De Almeida and Valle, 2007; Moser *et al.*, 2010). Alvim (1977) stated that in tropical conditions an average of 1,400 – 2,000 mm annual rainfall is adequate to support the growth in cacao trees, but less than 1, 200 mm might result in soil water deficit and reduced growth and yield. Water deficit resulting from prolonged dry periods has a negative impact on cacao which is not considered resilient to extreme weather conditions (De Almeida and Valle, 2007).

Effects on development and symptoms such as leaf fall, yellowing of leaves, wilting, small leaves, slow stem growth and seedling death are symptoms of drought (Carr and Lockwood, 2011). In early studies, Sale (1970) carrying out research on young cacao trees grown in a glasshouse over 20 months, noted that dry soil treatments caused reductions in flushing frequency, dry weight, leaf area, and leaf longevity. He also noted that flowering declined under drought but when plants were transferred to the well-watered treatment flowering returned significantly. Sale (1970b), examining air humidity as a factor related to water deficit, observed that cacao plants had small leaves and greater weight per unit area and thicker leaves when grown at high relative humidity. Additionally, high humidity alternated with low humidity resulted in greater dry weight and significant levels of flowering. Other studies reported reduction in leaf area expansion by approximately 50% in water stressed cacao plants (Deng *et al.*, 1989; Joly and Hahn, 1989a) and similar reductions in root and shoot dry weight in young cacao plants when irrigation was withheld for 12 days (Mohd Razi *et al.*, 1992). Similarly, Dos Santos *et al.* (2014) reported reductions in growth parameters such as final biomass, leaf area and leaf number of cacao progenies subjected to drought conditions in Bahia, Brazil. However, Moser *et al.* (2010) did not observe reductions in biomass production in 4 year old cacao trees grown within an agroforestry system in

response to soil moisture deficit over a period of 13 months, although the bean yield was reduced by 10%. The authors highlighted physiological adaptations such as root osmotic adjustments, high air humidity during soil moisture deficit and shading species as possible mitigation factors.

The root system plays an important role under drought conditions. The nature and extent of root systems are considered to be important factors affecting plant responses to water stress. Using a carbon isotope (^{14}C) technique, Deng *et al.* (1990) showed that the proportion of labelled assimilates exported from the source leaves was strongly affected by the cacao seedlings' water status. They suggested that water deficit increased the proportion of ^{14}C labelled to the roots enhancing the access to deeper water availability. Recently, an evaluation of morphological traits in a diallel scheme of seven cacao genotypes found differential root growth, particularly fine roots associated with drought tolerance amongst different genotypes (Dos Santos *et al.*, 2018).

Osmotic adjustment has a role in plant adaptation to drought through turgor maintenance and protection of specific cellular functions by defined solutes. In Brazil, De Almeida *et al.* (2002) reported variation amongst eight clones in response to drought in terms of osmotic adjustment associated with potassium and phosphorus accumulation in the leaf. On the other hand, no long-term sustained osmotic adjustment was reported in 4-year old cacao trees evaluated for drought tolerance in Merida, Venezuela (Rada *et al.*, 2005). The authors observed a sustained turgor pressure for an initial period of 12 days after withholding water, however the trees were not able to sustain this response over a period of 25 days without irrigation. Another study with juvenile Criollo-type cacao over two dry periods reported that the variation in the level of osmotic adjustment might be sufficient to categorize particular cacao genotypes as more tolerant than others (Araque *et al.*, 2012).

The balance between water absorption by the roots and transpiration by the leaves defines the water status of the plant. When soil dries, water becomes less available and if atmospheric conditions are maintained at a high level of evaporative demand, then the plants tend to reduce transpiration through stomatal closure in order to maintain hydration in tissues (Garnier and Berger, 1987). Consequently, stomatal closure reduces stomatal conductance during drought periods which also impacts photosynthesis. Bae *et al.* (2008) noted a magnitude of 50% drop in photosynthesis rate and stomatal conductance in cacao trees under drought in field conditions and Araque *et al.* (2012) observed stomatal closure in young cacao plants during the dry seasons, causing a 73% drop in both CO_2 assimilation and transpiration rates. Additionally, the negative effect of water deficit on photosynthesis has also been associated with damage to or low efficiency of photosynthetic apparatus and reductions in chlorophyll content and leaf nitrogen content (Tezara *et al.*, 2020; Anokye *et al.*, 2021; Osorio Zambrano *et al.*, 2021; Jaimez *et al.*, 2022).

The bulk leaf water potential (Ψ; units MPa) is a simple indicator of leaf water status; the more negative the value, the more dehydrated the leaf. Working on the water status of cacao seedlings under controlled conditions, Joly and Hahn (1989b) observed that net photosynthesis declined when leaf Ψ fell below -0.8 to -1.0 MPa. Similarly, Deng *et al.* (1989) reported that moderate stress occurred when leaf Ψ fell to between -0.8 to -1.2 MPa, whilst severe stress occurred when Ψ fell below -1.76 MPa. Balasimha *et al.* (1991) also pointed out that maintenance of photosynthetic rates in cacao can be associated with high leaf water potential (leaf Ψ) and that drought tolerance is mainly attributable to the effective regulation of stomata. In this way, variation in leaf Ψ has been proposed as a means of identifying cacao genotypes that are tolerant to water deficit. Balasimha *et al.* (1991), comparing drought tolerant and susceptible cacao genotypes, stated that those that maintained higher midday leaf Ψ values under drought treatment could be considered the most potentially tolerant. In another study, eleven three-year-old cacao genotypes from 5 countries were evaluated under drought conditions (Apshara *et al.*, 2013). The authors observed genotypic variation in the photosynthetic response to water deficit and noted resilience to this abiotic stress through reduced transpirational losses through stomata closure in order to maintain leaf Ψ. Previously, Nunes (1967) had also reported genotypic variation in drought tolerance in cacao which was attributed to differences in stomatal responses and transpiration. Working with four cacao varieties under drought conditions, De Almeida *et al.* (2016) concluded that stomata closure was an effective mechanism to preserve leaf Ψ and suggested that this should be a potential trait for breeding efforts. Recently, studies have demonstrated the correlation between leaf Ψ and physiological parameters such as photosynthesis rate, stomatal conductance and leaf transpiration and how leaf Ψ might help to identify susceptible and tolerant cacao genotypes (Tezara *et al.*, 2020; Osorio Zambrano *et al.*, 2021; Jaimez *et al.*, 2022).

Alongside physiological parameters, biochemical traits have been considered in order to understand cacao responses to drought. According to Dos Santos *et al.* (2014), drought can activate the transcription of genes related to the biosynthesis of abscisic acid (ABA) which may stimulate osmotic adjustments through stomatal closure (Balasimha, 1983) or biosynthetic pathways of other components such as polyamines (putrescine, spermidine and spermine) and proline (Balasimha, 1983; Bae *et al.*, 2008). Recent studies have included these parameters in order to identify cacao genotypes resilient to water deficit (Niether *et al.*, 2020; Dzandu *et al.*, 2021; Juby *et al.*, 2021).

1.3.5 CO_2 and drought effects on cacao

Recent research on the effects of elevated [CO_2] in combination with water stress on juvenile cocoa plants, has been conducted at the University of Reading (Lahive *et al.*, 2018). Under elevated [CO_2] (~700 ppm) light-saturated photosynthesis and quantum efficiency were enhanced, while water use

efficiency - WUE (both intrinsic and instantaneous) increased significantly compared with the plants grown under ambient [CO_2]. Leaf area, leaf fresh and dry mass and stomatal density was greater at elevated [CO_2]. The authors confirmed that elevated [CO_2] appears beneficial to photosynthesis and growth in cacao and the impact of water deficit could be mitigated by higher [CO_2]. Later, the response of different genotypes to elevated [CO_2] and water stress was studied by Lahive *et al.* (2021), using six different clones grown for 23 months under glasshouses conditions. The study showed that an increase of atmospheric [CO_2] stimulates the photosynthetic rate (at leaf and canopy-level), water use efficiency and growth parameters in mature plants similar to that seen in young seedlings. Despite this response, there was less of a growth enhancement in comparison to younger plants. It was also shown that elevated [CO_2] might alleviate some of the negative effects of water deficit in photosynthetic responses and biomass production.

The long term effect of [CO_2] concentration and water deficit treatments on reproductive parameters, including yield and bean quality of six cacao genotypes was studied by Handley (2016). He noted an increase in fluctuation of flowering intensity throughout time at elevated [CO_2] (~700ppm). An increase in husk weight and thickness, final pod size and maximum rate of growth, as well as individual bean weight was also observed at elevated [CO_2] in the second year of growth. Conversely, there was decrease in total cacao butter and unsaturated fat percentages at elevated [CO_2]. Handley (2016) also pointed out that different genotypes varied in their responses. Water stress did not affect pollen development, flowering or cacao butter content. Pod growth parameters and final pod size were negatively affected by the water deficit in the first year only. Finally, wood and leaf biomass were both reduced in response to water deficit. Overall, the study showed evidence that elevated [CO_2] may alleviate the negative impact of water deficit, this being more pronounced in some genotypes compared with others.

1.4   Justification of this research

Climate change factors such as elevated [CO_2], increasing temperature and water deficit are affecting tropical forest ecosystems, and will continue to do so for the foreseeable future. It has been shown that climate change is impacting on cacao production around the world, and a potential decline in yields has emerged as an important concern for millions of smallholders and the chocolate industry. One of the most important challenges for science and society is predicting the responses of crops to future global change scenarios. Physiological data provides the basis to construct models that can predict changes in production and the responses of cacao to future climatic variability. In addition, the challenge of climate change calls for the exploration of genetic diversity among cacao genotypes for developing new resilient varieties.

Responses to abiotic stresses in cacao, have mainly been reported on individual climate parameters such temperature, CO_2 and drought. Based on the literature review, there are gaps in the current knowledge of cacao responses to the interaction between individual climate parameters. Recent approaches using controlled environment facilities at the University of Reading have examined the interactions between [CO_2] and drought on cacao physiology (Handley, 2016; Lahive *et al.*, 2018, 2021). In order to contribute to the current knowledge and understanding about the effects of climate change on cacao, this thesis aims to examine the combined effects of elevated [CO_2], high temperatures and water deficit on growth and physiology of juvenile and mature cacao, as well as different clonal genotypes of cacao. Initially, short-term investigation into the combined effects of elevated [CO_2] and temperatures on plant growth and photosynthesis of young cacao plants are analysed (Chapter 3). Secondly, water limiting condition is incorporated to examine the growth and physiological responses of cacao seedlings subjected to [CO_2] enrichment and a broad range of temperatures (sub-optimal to supra-optimal) (Chapter 4). Thirdly, a longer-term exploration of the responsiveness of growth and photosynthesis of contrasting mature cacao genotypes to a combination of elevated [CO_2] and warming scenarios is carried out (Chapter 5). Finally, a complementary examination of the combined effects of elevated [CO_2] and temperature on reproductive components and bean yield of different mature cocoa genotypes is conducted (Chapter 6).

2 General materials and methods

2.1 Cocoa plant material

2.1.1 Chapter 3: "Temperature and [CO_2] effects on the growth and physiology of two juvenile cacao genotypes (*Theobroma cacao* L.)"

Juvenile plants of the cacao genotypes, PA 107 and SCA 6 were used for the first experiment in growth cabinets. Pods of open-pollinated PA 107 were supplied by The Cocoa Research Institute of Ghana (CRIG) and cacao seedlings were germinated at The International Cocoa Quarantine Centre (ICQC) greenhouse at the University of Reading. Subsequently, the plants were transferred to a controlled temperature glasshouse at the Crops and Environment Laboratory (CEL), the University of Reading. Plantlets of the clone SCA 6 were obtained from *in vitro* propagated plants using the somatic embryogenesis method (Guillou *et al.*, 2018) at Nestlé Research Centre in Tours, France. SCA 6 plants were delivered to the UK and maintained in the same controlled temperature glasshouse at CEL. Details on cacao plant management are given in Chapter 3.

2.1.2 Chapter 4: "The impacts of a broader range of temperature, [CO_2] and water deficit on growth and physiology of juvenile cacao plants (*Theobroma cacao* L.)"

Seedlings of the cross T 63/971 x T 60/887 were used for the second growth cabinet experiment. The T 63/971 x T 60/887 pods were provided by the Cocoa Research Institute of Ghana (CRIG) and the seedlings were raised in a controlled temperature glasshouse at CEL. Details on cacao plant management are provided in Chapter 4.

2.1.3 Chapters 5 and 6: "Combined effect of elevated [CO_2] and temperature on plant growth and physiology of six contrasting mature cacao genotypes (*Theobroma cacao* L.)", and "The effects of elevated [CO_2] and increased temperature on reproductive development and pod components of contrasting mature cacao genotypes (*Theobroma cacao* L.)"

Mature plants of six cacao genotypes, previously raised in the Cocoa Climate Change Glasshouse at the Crops and Environment Laboratory (CEL), the University of Reading, were available for the third and fourth experiment started on September 2019. The plants were originally obtained through somatic embryogenesis (Guillou *et al.*, 2018) at Nestlé Research Centre in Tours, France and delivered to the UK in Feb 2017. Cacao trees were three years old at the start of the experiment. Details of the genotypes used for all the experiments as well as their main characteristics are outlined in the Table 2.1. Information is derived from the International Cocoa Germplasm Database (ICGD).

Table 2.1 Summary of characteristics of the eight cacao genotypes used in the experiments. The table presents the main characteristics of each genotype (Turnbull and Hadley, 2022)

Genotype	Selected	Compatibility	Additional Information
SCA 6	Peru	Self-incompatible	Widely used in breeding programs for its resistance to witches' broom disease
PA 107	Peru	Self-incompatible	Used in breeding programs. Tolerant to Vascular Streak Dieback (VSD). Varied tolerance/susceptibility to *P. palmivora*
T 63/971 x T 60/887	Ghana	N/A	Seed garden material crossed in Ghana
CCN 51	Ecuador	Self-compatible	Tolerant to witches' broom disease. Diverse resistance/susceptibility to other diseases. Widely cultivated in S. America
T 85/799	Ghana	Self-compatible	Seed garden parent in Ghana. Tolerant to *P. palmivora*. Resistant/tolerant to Cocoa Swollen Shoot Virus (CSSV)
ICS 6	Trinidad	Self-compatible	Tolerant to witches broom. Diverse resistance/susceptibility to other diseases
IMC 20	Peru	Self-incompatible	Resistant to *P. palmivora*
PA 7	Peru	Self-incompatible	Seed garden parent in Ghana. Tolerant to CSSV. Diverse tolerant/susceptibility to *P. palmivora*

2.1.4 Plant culture

Juvenile plants and mature trees were grown in a substrate of sand, gravel and vermiculite (1:2:2 vol:vol:vol). For the first and second experiment, plants were raised in 5L plastic pots (Fargro Ltd, Littlehampton, UK) (Figure 2.1a). Mature trees used in the third and fourth experiment were potted into 50 L plastic pots (Fargro Ltd, Littlehampton, UK) (Figure 2.1b). Nutrition and irrigation management is described in section 2.4.
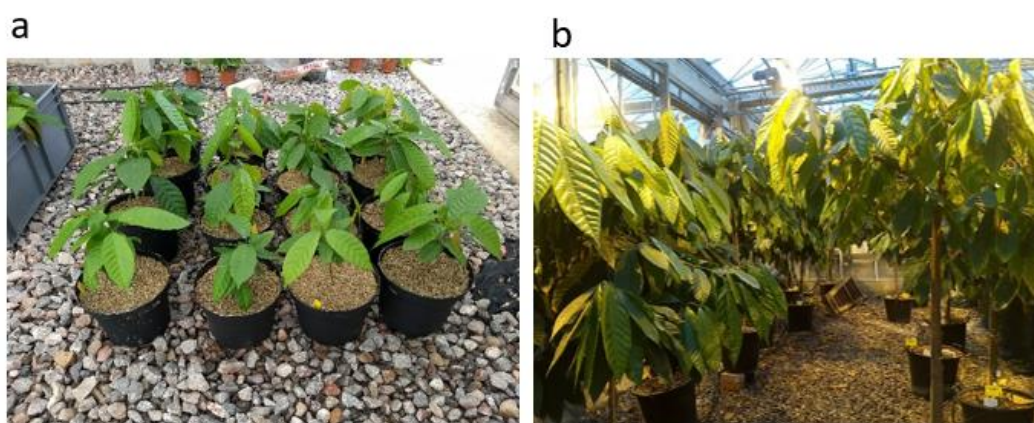
Figure 2.1 Plant material used for the first and second experiment in growth cabinets (a) and third and fourth experiment under glasshouses (b)

Yellow sticky traps were hung in the growth cabinets and glasshouses for aphid control and *Amblyseius californicus* and *Amblyseius andersoni* sachets (Bioline AgroSciences Ltd, Essex, UK) were regularly introduced for biological control for red spider mites (*Tetranychus urticae*). Additionally, pesticide applications were conducted using a portable fogger with Abamectin (5ml in 10 L) treatment when red spider mite hotspots were identified in the glasshouse experiment.

In order to achieve consistent height and a well-balanced structure, mature cacao genotypes were pruned on August 2019 before being placed in the different climatic treatments. Additional maintenance pruning was carried out on December 2019 and June 2020. The main criteria for pruning was to limit tree height to 3 meters, which is the height of the shade screening and some new lateral branches and shoots were removed to avoid overlapping. The total pruned dry weight was measured and recorded for the final biomass analysis. In addition, senesced leaves were collected regularly and dry biomass was also recorded in each glasshouse. Finally, chupons growing from the base of the trunk were removed routinely.

2.2   Growth cabinet facilities

The first and second experiment were carried out in twelve Fitotron® High Specification Plant Growth Chambers with a 1.5 m^2 growth area and 2,000 L growth volume (model HGC 1514; Weiss Gallenkamp, UK) located in a growth cabinets hall at CEL (Figure 2.2). Temperature, relative humidity, lighting and CO_2 were monitored using SpecView SCADA control software (SpecView Ltd, East Sussex, UK). The cabinets were designed to maintain temperatures between -10 °C and +45 °C, humidity ranging from 40% to 85% RH, light intensity up to 1000 µmol m^{-2} s^{-1} at 500 mm from 52 cool white fluorescent lamps (Philips TL5 HO 54W 840, Holland) and CO_2concentration between 400 and 3,500 ppm. Details of temperature regimes and humidity for each experiment are presented in Chapters 3 and 4.

Figure 2.2 The growth cabinets used for the experiment with juvenile cacao plants, located at Crops and Environment Laboratory, School of Agriculture, Policy and Development, University of Reading.

2.3   Glasshouse facilities

The third and fourth experiments were carried out in a six-compartment glasshouse facility (each compartment measured 10m x 6m x 3.8 m), specifically-built to grow cacao plants under climate change conditions, at the CEL (Figure 2.3). The temperature, supplementary lighting and carbon dioxide concentration ($[CO_2]$) were controlled by a TomTech T200 Horticultural Control Computer (Tomtech UK Ltd, Spalding, Lincolnshire, UK).

Figure 2.3 The six compartment cocoa climate change glasshouse used for the experiment with cacao mature plants located at Crops and Environment Laboratory, School of Agriculture, Policy and Development, University of Reading

2.3.1 Temperature control

In each glasshouse the heating was provided by a Benson PV100-1, 29.4kW tubular gas heater (Ambirad Ltd., West Midlands, UK). Due to a breakdown in the gas heater located in the glasshouse 1 on April 2020, a new replacement RENZOR PVE30 gas fired air hearer (Nortek Global HVAC Ltd, West Midlands, UK) was installed in June 2020. A temperature sensor that was encased and hanging from the roof and located in the middle of each glasshouse, provided the current temperature to the control computer. The heaters turned on when the temperature declined below the heating set point. When the temperatures exceeded the venting set point, automatic vents in the roof were opened allowing air to circulate in the glasshouse. Details of temperature regimes for each experiment are described in Chapters 5 and 6.

2.3.2 Lighting control

An supplementary light system with six 400W high pressure sodium lamps (Philips SON-T, Holland) per compartment and connected to a roof mounted light sensor, provided supplementary illumination when natural light declined below 148 µmol m^{-2} s^{-1}. Supplementary illumination was provided between 06:00 and 18:00 in winter months. When the light levels exceeded 648 m^{-2} s^{-1} a 50% shade screen was closed in order to regulate the excess of the light in the glasshouse. Moreover, to avoid heat loss in the glasshouses the shade screening was also closed during the night time.

2.3.3 Carbon dioxide control

A wall-mounted infrared gas analyser (CO2 Gascard II, Edinburgh Sensors Ltd, Livingstone, UK) attached to the TomTech T200 control computer monitored [CO$_2$] in each glasshouse compartment. Three glasshouses were maintained at ambient concentrations (ambient [CO$_2$]) and three were set to 700 ppm

(elevated [CO_2]) allowing a hysteresis of 50 ppm (i.e. when the [CO_2] fell below 650 ppm, the system was set to switch on until 750 ppm was reached). In three glasshouses, flue gases from the glasshouse heater provided clean CO_2 which was distributed through two indoor drilled PVC pipes (65 mm diameter) around the perimeter walls of each glasshouse compartment and mounted 65 cm above the ground. To improve insulation and prevent CO_2 leakage from elevated to ambient glasshouses, expanded foam filler was used to fill any gaps on the partitioning glasshouses walls. Air fans were mounted 2.5 m above ground level in all the glasshouse compartments to improve air circulation. As [CO_2] elevation required heater ignition in the elevated CO_2 glasshouses, a two way vent system (facing outwards/inwards) was attached to the hot air outlet on the top of the gas heater so that hot air from the heater could be vented outside the greenhouse if the greenhouse temperature exceeded the set point. Details of [CO_2] levels reached at each experiment are described in Chapters 5 and 6.

2.3.4 Humidity system

A misting system at ground level was installed to enable high humidity to be maintained in each glasshouse. An electronic timer (TES7-MP, Luceco PLC, London, UK) switched on the misting system ten times each day from 8:45 to 17:45 for 6 minutes. Sprayer nozzles (mini-sprayers 180° 40 L h-1, Palaplast, Sindos, Greece) were placed at 1.6 m intervals along two 16 mm PVC pipes along the length of each glasshouse compartment. A water pump (Jet 90 pump, Stuart Turner Ltd, Oxfordshire, UK) fed water to the nozzles which was fed by a 120 L plastic tank containing pre-treated tap water. The T200 control computer recorded humidity in each compartment. The average humidity reached for each experiment is presented in Chapters 5 and 6.

2.4 Fertigation system

2.4.1 Growth cabinets

A modified Long-Ashton nutrient solution (Table 2.2) adjusted for use on cocoa at the University of Reading (End, 1990), was delivered to each plant through a pressure-compensated dripper (Netafim Ltd., Tel Aviv, Israel) held in place by a stake inserted into the substrate. Each dripper provided the plants with water and essential nutrients. The plants were irrigated for five minutes at 6:00, 11:00, 15:00 and 18:00 using a HERON Ti-40 timer system (Heron Electric, West Sussex, UK). The nutrients were delivered through the irrigation system from two 35 L plastic tanks containing concentrated (50x) nutrients solutions (A and B) to an 84 L mixing tank. A third 35 L stock tank containing a mixture of dilute nitric and phosphoric acid, was used for the pH control in the mixing tank. For all the experiments in the growth cabinets, the concentration of nutrients in the nutrient solution was controlled by an EC/pH HANNA controller (model HI9913, Hanna instruments Ltd, UK) connected to an EC probe (HANNAH

conductivity sensor, model HI3001, Hanna instruments Ltd, UK) and pH electrode (HANNA pH electrode, model HI1002/3, Hanna instruments Ltd, UK) immersed in the nutrient solution in the mixing tank. Nutrient solutions were maintained at a conductivity and pH of 2.0 mS cm^{-1} and 5.7-5.8 respectively. When the EC fell below the set point or pH rose above the pH range, three dosing pumps (HANNA dosing pumps, model: BL, Hanna instruments Ltd, UK 7) provided nutrients from the stocks tanks A and B or acid into the mixing tank.

Table 2.2 Composition of the cocoa nutrient solution applied as soluble fertilizers. Calcium is not incorporated due to sufficient concentration in the tap water

Nutrient solution	Formula	Amount per L water
Solution A		
Potassium Nitrate	KNO_3	0.43 g
Ammonium Nitrate	NH_4NO_3	0.39 L
Solution B		
Potassium sulphate	K_2SO_4	0.12 g
Magnesium Sulphate	$MgSO_4$	0.24 g
Potassium Dihydrogen Phosphate	KH_2PO_4	0.15 g
EDTA		0.03 g
Nitric Acid	HNO_3	0.04 L
Micronutrients		
Boric Acid	H_3BO_3	0.01 g
Manganese sulphate	$MnSO_4$	0.001 g
Zinc Sulphate	$ZnSO_4$	0.02 g
Ammonium Molybdate	$(NH_4)_6Mo_7O_{24}$	0.001 g
Copper Sulphate	$CuSO_4$	0.001 g

A submersible water pump (Hozelock Cascade 700 Fountain and waterfall pump, Hozelock Ltd., Birmingham, UK) ensured mixing of nutrients in the mixing tank. Dilute nutrient solution was pumped to the growth cabinets through 22 and 16 mm PVC pipe through twelve solenoid valves (Type 200, NaanDanJain Ltd., Israel) via a water pump (RG250-2 pump, Stuart Turner Ltd, Oxfordshire, UK) which was automatically controlled by the HERON Ti-40 timer system.

2.4.2 Glasshouses

In the glasshouse, cacao plants were fed daily with the same modified Long-Ashton nutrient solution (Menzel, 2021). Nutrient A and B stock solutions were stored in 227 L plastic tanks and the acid was stored in an 80 L tank (Table 2.2). These were pumped to 227 L volume mixing tank with water. As with the nutrient solution for the growth cabinets, the conductivity and pH of the nutrient solution was controlled via a conductivity (EC) controller and probe (HANNA EC controller, model HI943500 and HANNA conductivity sensor, model HI7638, Hanna instruments Ltd, UK), and pH controller and sensor (HANNAH pH controller, model HI8710 and HANNAH pH electrode, model HI1230, Hanna instruments Ltd, UK) respectively. The diluted nutrient solution in the mixing tank was maintained at pH between 5.7-5.8 and an electrical conductivity about 2.0 mS cm^{-1}. When probes registered pH levels above the range or below the EC setting points, three dosing pumps (HANNA dosing pumps, model: BL15, Hanna instruments Ltd, UK) were switched on to inject the nutrient solution stock A, B and acid stock into the mixing tank. A submersible water pump (Hozelock cascade 700 fountain and waterfall pump, Hozelock Ltd., Birmingham, UK) maintained a homogeneous nutrient solution in the mixing tank.

The nutrient solution was distributed to the plants through 16mm PVC pipes from the mixing tank using a water pump (Jet 40 pump, Stuart Turner Ltd, Oxfordshire, UK) and four solenoid valves (Bermad 200 series, Hungerford, Berkshire, UK). Irrigation regimes were controlled by a HERON MCI-96 timer system (Heron Electric, West Sussex, UK) set to provide 6 irrigations per day for 8 minutes. Dilute nutrient solution was applied to each plant via Netafim pressure compensated drippers (Netafim Ltd., Tel Aviv, Israel) which were held in place by stakes in the substrate.

3 Temperature and [CO$_2$] effects on the growth and physiology of two juvenile cacao genotypes (*Theobroma cacao* L.)

3.1 Introduction

Cacao, *Theobroma cacao* L. is an important commodity crop for the production of chocolate, cosmetics, beverages, and other derivative products (Lima *et al.*, 2011). An estimated 4,843,000 tonnes of cocoa beans were produced in 2020/2021, mainly by smallholder farmers in tropical regions of Africa, Asia and America (ICCO (International Cocoa Organization), 2021). It is known that the concentration of atmospheric carbon dioxide ([CO$_2$]) has been increasing since the beginning of the Industrial Revolution and is projected to double its current concentration by the end of the century under an intermediate scenario of projected greenhouse gas emissions (Representative Concentration Pathway (RCP) 6.0) according to the Intergovernmental Panel on Climate Change (IPCC, 2014). Cumulative emissions of CO$_2$ and other greenhouses gases are resulting in an increase in global temperatures leading to changes in weather patterns which may impact plant development. Elevated [CO$_2$] has positive effects on plant growth (expressed as greater biomass) as a result of high rates of photosynthesis and higher water content due to partial stomata closure, as well as greater light use efficiency (Conroy *et al.*, 1990; Drake *et al.*, 1997; Ainsworth and Long, 2005; Leakey *et al.*, 2009). In contrast, warmer temperatures can negatively affect plant growth and can accelerate development rate, thereby impacting final productivity (Hatfield and Prueger, 2015). Other impacts of high temperatures include reduced photosynthetic efficiency due to reduced Rubisco activity, increased photorespiration, and stomatal closure due to increased vapour pressure deficit (VPD) (Krause *et al.*, 2015; Slot and Winter, 2016).

The physiology and development of cacao is sensitive to changes in temperature (Raja Harun and Hardwick, 1988; Hadley *et al.*, 1994b; Hebbar *et al.*, 2020). Sale (1969b) showed the effects of temperature on plant growth in cacao. Using young plants, the author observed that elevated average day temperatures of 30 °C increased the number of flushes per plant as a result of the loss of apical dominance, while the number of expanded leaves per flush and mean area per leaf increased as the temperature decreased from 30°C to 23.3 °C. In addition, shoot growth rate was higher at higher temperatures (Sale, 1969b). A study conducted by Daymond and Hadley (2004) in semi-controlled conditions with four cacao genotypes (var. Amelonado, AMAZ 15–15, SCA 6 and SPEC 54/1), and three simulated temperature conditions (Bahia, Brazil; Tafo, Ghana; and Lower Perak, Malaysia), demonstrated significant increases in stem-cross sectional area between genotypes and the highest growth rate under the warmest conditions. In this study, some genotypes appeared to be more responsive to temperature changes than others. Raja Harun and Hardwick (1988) studying the effects of temperature and vapour pressure deficit (VPD) on cacao, reported that temperatures ranging from 20 –

30°C did not markedly affect photosynthetic rate, but values decreased above 30°C; however the authors suggested that this response was an indirect effect of increased VPD that led to stomatal closure. In a recent study, Hebbar *et al.* (2020) observed a reduction of 30% in photosynthesis and 21% in biomass accumulation in young cocoa plants grown at 3°C above the control treatment which had a day/night temperature of ~32.3/23.9°C.

Increasing $[CO_2]$ (above 380 ppm) in the short-term could enhance the growth and yield of several C_3 crops since the current $[CO_2]$ restricts maximum photosynthesis (Kimball *et al.*, 2002). Elevated $[CO_2]$ may reduce stomatal conductance and transpiration, and improve water use efficiency, while simultaneously enhancing photosynthesis and plant growth (Drake *et al.*, 1997). Studies examining the effects of elevated $[CO_2]$ on young cacao plants have demonstrated positive responses (Baligar *et al.*, 2005, 2008, 2021a, 2021b; Lahive *et al.*, 2018). Cacao seedlings grown at elevated $[CO_2]$ (700 ppm) exhibited enhanced mineral nutrient uptake and increased shoot and root growth compared to ambient $[CO_2]$ (380 ppm) (Baligar *et al.*, 2005). In a complementary study, Baligar *et al.* (2008) reported an improvement in photosynthesis by 33% when the $[CO_2]$ was raised from 85 to 680 ppm, yet a minimal response when increased from 680 to 850 ppm. The authors also noted that elevated $[CO_2]$ led to a decrease in stomata conductance by about 65%. A study by Lahive *et al.* (2018) using the Amelonado variety demonstrated that elevated $[CO_2]$ enhanced light-saturated photosynthesis rate, led to the improvement of water use efficiency (WUE) due to higher photosynthesis rate rather than decreases in stomata conductance, and increased the leaf area and the leaf carbon-nitrogen ratio. Baligar *et al.* (2021a) reported increases in growth parameters (dry biomass, root length, height, leaf area, specific leaf area, relative growth rate and net assimilation rate) and nutrient uptake with intraspecific differences among seven young cacao genotypes subjected to elevated $[CO_2]$ under controlled greenhouse conditions.

Combined and interactive effect of elevated $[CO_2]$ and temperature on plant growth, photosynthesis and yield have been investigated in several crops. Elevated $[CO_2]$ may significantly mitigate warming conditions, particularly in some C_3 crops (Lee, 2011). DaMatta *et al.* (2018) reported that photosynthetic impairments in *Coffee arabica* L. and *Coffee canephora* can be attenuated by $[CO_2]$ enrichment. However, Kumari *et al.*, (2019b) demonstrated that depending on the cultivar, the beneficial direct improvement on growth and yield from elevated $[CO_2]$ can be counteracted by elevated temperatures in pea plants (*Pisum sativum* L.). Despite the importance of cocoa, there is a little information about how young plants respond to the combined effects of warming and elevated $[CO_2]$. This study aimed to determine under controlled conditions (controlled environment (CE) growth chambers) how elevated temperature and $[CO_2]$ affect the growth and physiology of juvenile cacao genotypes. The hypotheses tested were: a) growth and photosynthesis are negatively affected by temperature increases of +2.5 and

+5°C above the average current temperatures in West Africa where cocoa is cultivated; b) growth and photosynthesis responses are enhanced at elevated $[CO_2]$; c) there is genotypic variation in the response of growth and photosynthesis to elevated temperature and $[CO_2]$; d) elevated $[CO_2]$ may ameliorate the possible negative effects of elevated temperature on juvenile cacao plant physiology.

3.2   Materials and Methods

3.2.1   Plant material and experimental setup.

Two cacao genotypes (SCA 6 and PA 107) were used for this experiment. Open pollinated PA 107 seedlings were provided by The Cocoa Research Institute of Ghana and raised from 11-06-18 to 02-10-18 in the International Cocoa Quarantine Centre (ICQC) greenhouses at the University of Reading in 1 L pots containing a mixture of sand, gravel, vermiculite (1:2:2 vol:vol:vol). The plants were maintained under day/night minimum temperature of 25 and 20 °C respectively, at ambient $[CO_2]$ and were irrigated six times daily with a cocoa nutrient solution, which is a modified Long Ashton solution, maintained at a pH of 5.7 and an electrical conductivity (EC) of 2.0 mS cm^{-1} (End, 1990). On 03-10-18 the plants were transferred temporarily to a controlled temperature glasshouse at the Crop and Environment Laboratory (CEL) at the University of Reading and transplanted into 5L pots filled with the same mix of substrate and irrigation regime. The environmental conditions in the glasshouse were set to range from 19°C (minimum temperature) to 32°C (maximum temperature); light intensity varied between 148 µmol m^{-2} s^{-1} and 648 µmol m^{-2} s^{-1} photosynthetically active radiation (PAR). Supplementary lighting (using 400W high pressure sodium lamps) was used to extend the day length to 12 hours and increase ambient light levels and shade screens used when light was excessive.

The SCA 6 plants were raised from *in vitro* propagation using the somatic embryogenesis method (Guillou *et al.*, 2018) at Nestlé Research Centre in Tours, France, and planted in an acclimatization greenhouse on 25-07-18 in 40*40 mm coco-peat pellets (Preforma Plugs, Jiffy Products International, Lindtsedijk, Netherlands). After two months of acclimation, the plants were transplanted into 0.4 L pots containing wood-peat fibre growing substrate (Green Fibre 5-665, Klasmann-Deilmann, Geeste, Germany). During the acclimatization process, the plants were irrigated with water by hand between two or three times per week and supplementary soluble nutrients (12-17-29 seedling, Master Plant-Prod Inc., Brampton, Canada) at 2g/L dose were added when necessary. During the acclimatization period in France, the greenhouse condition was set up with day/night temperatures of 25-28/25-27 °C, 68-70% RH, ambient $[CO_2]$ and a 12 hour light regime was maintained using supplementary lighting when natural light was below 690 µmol m^{-2} s^{-1}. On 08-08-18, the SCA 6 plants were transferred to the UK and maintained in the temperature controlled glasshouse at the CEL at the University of Reading as described previously. They were transplanted on 15-08-18 into 5L pots filled with the same sand, gravel

and vermiculite substrate, irrigation regimes and environmental conditions as described for the PA 107 seedlings.

On 10-10-18, plants of both SCA 6 and PA 107 genotypes were transferred into twelve growth cabinets with a growth area of 1.5 m^2 and 2,000 L growth volume (model HGC 1514; Weiss Gallenkamp, UK). Each cabinet was divided in two sections in order to allocate both genotypes randomly. Nine plants per genotype were placed randomly in each half of the cabinet and were repositioned fortnightly within each cabinet over the 87 days of the experiment to minimize any environmental variation associated with specific positions within the cabinet. An automatic drip irrigation system irrigated the plants four times per day (6:00, 11:00, 15:00 and 18:00) for 5 min at each irrigation, using the cocoa nutrient solution described above. The plants were exposed to a 12 hour photoperiod and the light intensity at canopy level was maintained at 450 - 550 µmol m^{-2} s^{-1} PAR. The PAR at canopy height was tested regularly with a portable light meter (SKR 100, Skye Instruments Ltd, Llandrindod Wells, UK). As the plants grew taller, the height of the shelves in each cabinet were adjusted to maintain a constant distance between the top of the plant and the light source and therefore a similar light intensity as the plants grew.

The growth cabinets were set to provide six treatments in a factorial combination comprising two CO_2 concentrations (ambient (a target of 400ppm) and elevated (a target of 700ppm)) and three day/night temperature regimes T1 (31/22°C, control temperature), T2 (33.5/24.5°C, control temperature + 2.5°C), and T3 (36/27°C, control temperature + 5.0°C). Each treatment was replicated in two different cabinets (Figure 3.1). The temperature regimes in the growth cabinet were set to follow a daily sine wave temperature profile; the maximum and minimum in the control temperature was 31°C (from 13:00 to 15:00) and 22°C (from 03:00 to 7:00), simulating the cacao-growing region in Ghana (data obtained from the Ghana Meteorological Service). The relative humidity (RH, %) in each cabinet was varied depending on the temperature in order to maintain a VDP of 0.9 kPa across the treatments to avoid confounding effects of increasing VPD with temperature. Figure 3.2 shows the daily average temperature, relative humidity, and [CO_2] recorded throughout the experimental period. Regarding [CO_2], good control was achieved for elevated [CO_2] but the actual [CO_2] at 400 ppm was slightly higher than the target (Figure 3.2).

Treatment	[CO$_2$] (ppm)	Temperature (°C)	Genotypes
T1	400	Tc	SCA 6 - PA 107
T2	700	Tc	SCA 6 - PA 107
T3	400	Tc+2.5°C	SCA 6 - PA 107
T4	700	Tc+2.5°C	SCA 6 - PA 107
T5	400	Tc+5.0°C	SCA 6 - PA 107
T6	700	Tc+5.0°C	SCA 6 - PA 107
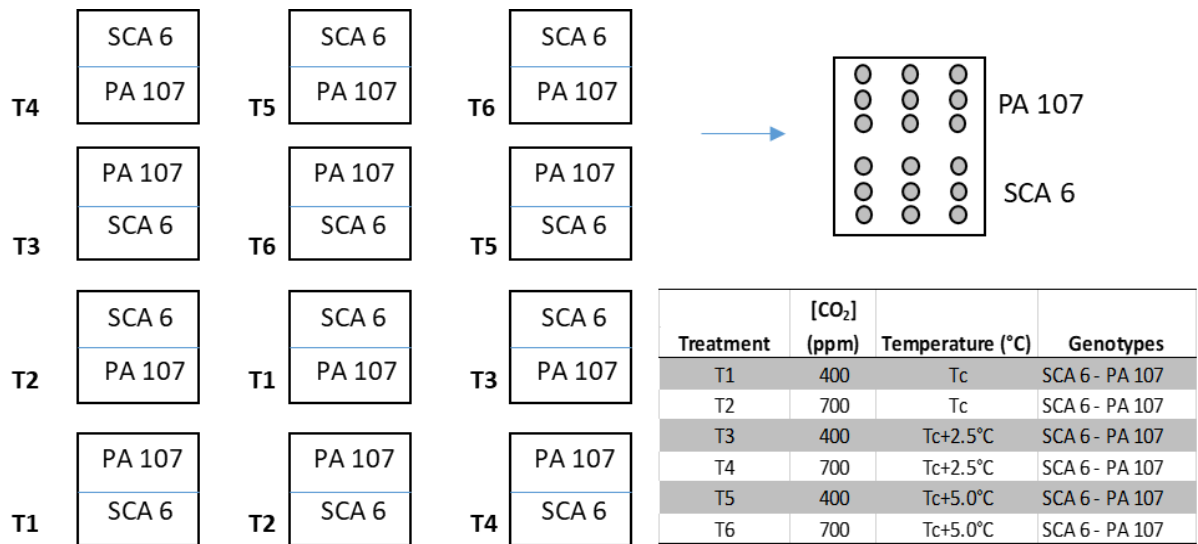
Figure 3.1 Arrangement of climatic treatments (T = [CO$_2$] x Temperature) and juvenile cacao genotypes across 12 growth cabinets used for the experiment, located at the Crops and Environment Laboratory, School of Agriculture, Policy and Development, University of Reading. Each box represents a growth cabinet
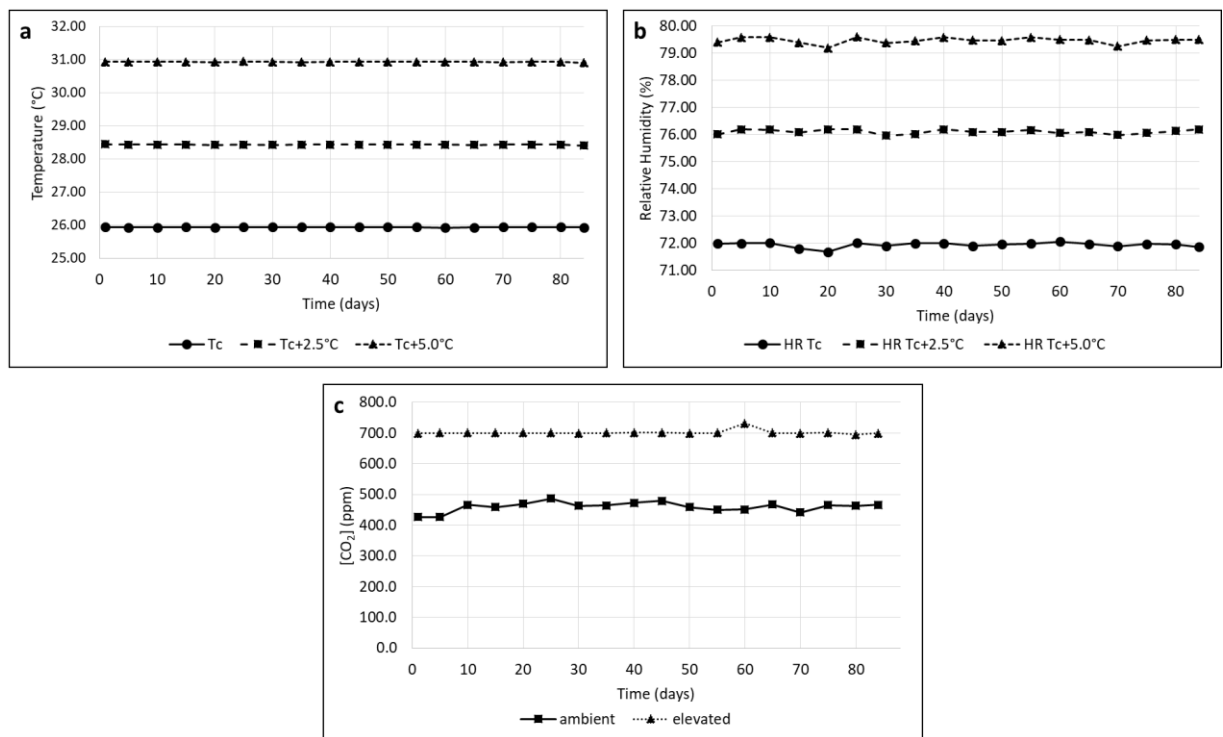
Figure 3.2 Daily average temperature (°C) (a), relative humidity (%) (b), and [CO$_2$] (ppm) (c) logged throughout 87 days of experimental period. Average values: Tc (25.9 (±0.1)°C), Tc+2.5°C (28.4 (±0.1)°C), Tc+5.0°C (30.9 (±0.1)°C); [CO$_2$] values: ambient (459.7 (±3.8) ppm) and elevated (701.2 (±1.8)ppm); Relative humidity values: HR Tc (71.9 (±0.1)%),HR Tc+2.5°C (76.1 (±0.1)%), and HR Tc+5.0°C (79.4 (±0.1)%).

3.2.2   Plant growth measurements

Non-destructive observations. Plant height (cm) and stem diameter (mm) were recorded at 6, 20, 33, 47, 65, and 81 days on three tagged plants per genotype in each growth cabinet (i.e. six plants per treatment combination) for the duration of the experimental period. Height was measured from the surface of the substrate to the shoot apex using a measuring tape. Stem diameter was recorded at 5 cm above the substrate using a digital calliper. Additionally, after leaf emergence, a new leaf from three random plants per genotype in each cabinet was labelled and its leaf length (cm) and chlorophyll content ($\mu g\ cm^{-2}$) were measured twice per week. Leaf length was recorded for 30 days and chlorophyll content for 45 days. Leaf length was recorded using a measuring tape. Chlorophyll content was measured using a CL-01 portable chlorophyll meter (*Hansatech Instruments Ltd.*, Norfolk, UK). The readings were converted to chlorophyll content ($\mu g\ cm^{-2}$) using the linear regression for cacao: c = (1.945 × chlorophyll meter reading) + 11.392), reported by Daymond *et al.* (2011).

Flushing interval (days) and the number of expanded leaves per flush were recorded three times per week on three tagged plants of both genotypes in each cabinet. Flushing interval was measured as the number of days between the unrolling of the last leaf of Flush 1 and unrolling of the first leaf of the subsequent flush (Lahive, 2015). Stomatal density (number of stomata mm^{-2}) was determined before the last destructive harvest. Three plants per genotype in each cabinet were selected and one leaf epidermal imprint per plant was taken from the abaxial surface using clear nail varnish and adhesive cellophane tape. The impressions were examined and digital images obtained using a *Leitz Dialux 20* light microscope with a *Leica DFC450* digital camera attached with three images per imprint obtained using *Leica Application suite* version 4.6.2 (*Leica Microsystems*, Wetzlar, Germany). *ImageJ* version 2.2 analysis software (Rueden *et al.*, 2017) was used for image processing and to count stomata per unit area at 400x magnification.

Destructive observations. Plant destructive harvests were performed at the beginning (H0), after 34 (H1), 50 (H2), 65 (H3) and 88 (H4) days of exposure to the different treatment combinations. Plants harvested at H0 were representative of plants going into the treatment combinations. Three plants per genotype were harvested at H1 and H2 in each treatment and six plants per genotype were harvested at H3 and H4 in each treatment. At each harvest, the plants were cut at the base of the stem, total leaf number and fresh weight of roots, stems and leaves (g) were recorded using an electronic balance (KERN, model PCB 250-3, *KERN & SOHN*, Balingen, Germany). Dry weights were recorded after samples were transferred into a ventilated drying oven at 70°C for at least 48 hours until they reached a constant weight. The leaf area (cm^{2}) of fresh samples was measured using a WD3 WinDIAS leaf image analysis system (*Delta-T Devices Ltd,* Cambridge, UK). Dried subsamples of leaves from each of the five harvests

were ground to a fine powder for laboratory determinations of carbon and nitrogen content using a *LECO CNH628* Series Elemental Analyser (*LECO Corporation*, Michigan, US).

3.2.3 Plant physiology measurements

Gas exchange parameters. Measurements of net photosynthetic rate (*Pn*, µmol m^{-2} s^{-1}), transpiration rate (*E*, mmol m^{-2} s^{-1}) and stomatal conductance (*g$_s$*, mol m^{-2} s^{-1}) were made using a portable infrared gas analyser fitted with an artificial light attachment and an internal CO_2 source (*LC pro*-SD, *ADC BioScientific*, Great Amwell, Herts., UK) on the youngest fully expanded and hardened leaf from three random plants per genotype in each cabinet. Three sets of measurements were performed 30, 56 and 80 days after the beginning of the treatments between 09:00 and 13:00 on each day. Measurements were made at 696 µmol m^{-2} s^{-1}, which can be considered saturating for cacao (Baligar *et al.*, 2008; Lahive *et al.*, 2018), the [CO_2] in the leaf chamber was set to the growth CO_2 concentration, i.e., ~ 400 and 700 ppm for ambient and elevated CO_2 treatments respectively, and the temperature in the leaf chamber was set to correspond to the maximum temperature for each growth cabinet. Intrinsic Water Use Efficiency (*iWUE*, µmol mol^{-1}) was calculated as *Pn /g$_s$*.

Chlorophyll fluorescence parameters. The measurements were taken in conjunction with the gas exchange observations. The maximum quantum efficiency of photosystem II (measured as *F$_v$/F$_m$*, ratio) and the performance index (PI) was recorded on the same leaves as used for gas exchange measurements using a Handy PEA chlorophyll fluorimeter (*Hansatech Instruments Ltd*, Norfolk, UK). The leaves were dark adapted using specialised clips for at least 30 minutes before being measured.

3.2.4 Statistical analysis

All analyses were carried out using the open-source statistical software R version 4.0.4 (R Core Team, 2021). The experiment was considered to be a completely randomized split plot design with three factors, with the combination of [CO_2] and temperature (growth cabinets) as the main plot and genotypes as sub plots. Homogeneity of variances and normality of distributions were tested for each variable before statistical analyses. Additionally, to test whether there was a cabinet effect, t-tests were performed between cabinets with the same treatment combinations. In all analyses, test results were considered significant at $P < 0.05$. Bonferroni post hoc test was used to compare group means where ANOVA determined significant effects.

A Repeated measures (REM) ANOVA was performed to evaluate the effects of [CO_2], temperature and cacao genotypes over time on plant height, stem diameter, and chlorophyll content responses. For leaf length, a non-linear regression analysis was used to describe the growth increase over the time. A

four parameter generalised logistic was fitted using the *drm* function from R package *drc* (Ritz *et al.*, 2015) according to the equation 3.1

$$\frac{a+d}{1+\exp(-b(T-c))}$$ 3.1

Where T is time in days, *a* is the upper asymptote, *d* is the lower asymptote, *c* is the time (T) value with a response half-way between *a* and *d*, while *b* is the correspondent slope around the inflection point. The generalised logistic regression was performed across the treatments and the biological parameters, maximum leaf length (cm), maximum leaf growth rate (cm day^{-1}), time to maximum leaf length growth rate (days), were obtained (*a*, *b*, and *c* respectively in equation 3.1). Time to reach the 95% of maximum leaf growth (days) was calculated from the equation at each observation. Subsequently, effects of genotype, temperature and [CO_2] on the four parameters were performed using three-way ANOVA.

For flushing interval, number of leaves per flush and stomatal density a three-way ANOVA was used to test the main and interactive effects of [CO_2], temperature and genotypes. For the gas exchange parameters (*Pn*, *E*, *g$_s$*, and iWUE) and *F$_v$/F$_m$* and *PI*, a four-way ANOVA was carried out with the main factors [CO_2], temperature and cacao genotypes measured at the three different times across the experimental period.

Functional Growth Analysis

Polynomial regression analysis was carried out on the harvest data of total dry weights and leaf area of individual plants across time. Comparing successive harvests in any treatment combination, smoothed mean values for various growth indices can be estimated (Hughes and Freeman, 1967). Natural log transformed data was fitted to polynomial equations in the form:

In DWt = b_0 + b_1t + b_2t^2 +.... + b_nt^n 3.2

In LA = b_0 + b_1t + b_2t^2 +.... + b_nt^n 3.3

Where *DWt* is the total plant dry weight (g), *LA* is the leaf area (cm^2), *t* is time of harvest, in days, and *b$_n$* is the regression coefficients for polynomial degree n. The relative growth rate (RGR, g.g^{-1}.day^{-1}) was derived directly by differentiation from equation (3.2):

d(ln DWt)/dt = 1/DWt . dDWt/dt 3.4

The leaf area ratio (LAR, cm^2.g^{-1}) was estimated as

Anti ln (ln *LA* – ln *DWt*) 3.5

Finally the net assimilation rate (NAR, $g.cm^{-2}.day^{-1}$) which is the net dry weight gain expressed per unit leaf area, was calculated as follows

$$[d(\ln DW)/dt]/[\text{ Anti ln (ln } LA - \ln DWt)] \qquad\qquad 3.4 / 3.5$$

3.3 Results

3.3.1 Growth responses

Plant height. Overall, height of both genotypes increased approximately linearly over the experimental period ($P < 0.001$) and, averaged over both genotypes, ranged from 37.9 (±1.4) at 20 days to 112.1 (±2.5) cm after 81 days (Figure 3.3). A significant positive effect of increased temperature was observed after 33 days and for elevated [CO_2] after 47 days ($P < 0.05$). Prior to these time points no significant effects of temperature or [CO_2] were observed. At the final time point (81 days), there was no significant effect of genotypes on plant height, however, there was a significant positive effect of [CO_2] ($P < 0.05$) and temperature ($P < 0.01$). The overall height of the two genotypes increased from 105.8 (± 3.2) cm at ambient [CO_2] to 118.3 (±3.7) cm at elevated [CO_2]. Similarly, there was an increase in the height from 98.5 (±3.9) at 31/22°C, to 114 (±3.7) at 33.5/24.5°C and 123.6 (±4.1) cm, at 36/27°C. There was no interaction between the treatments.
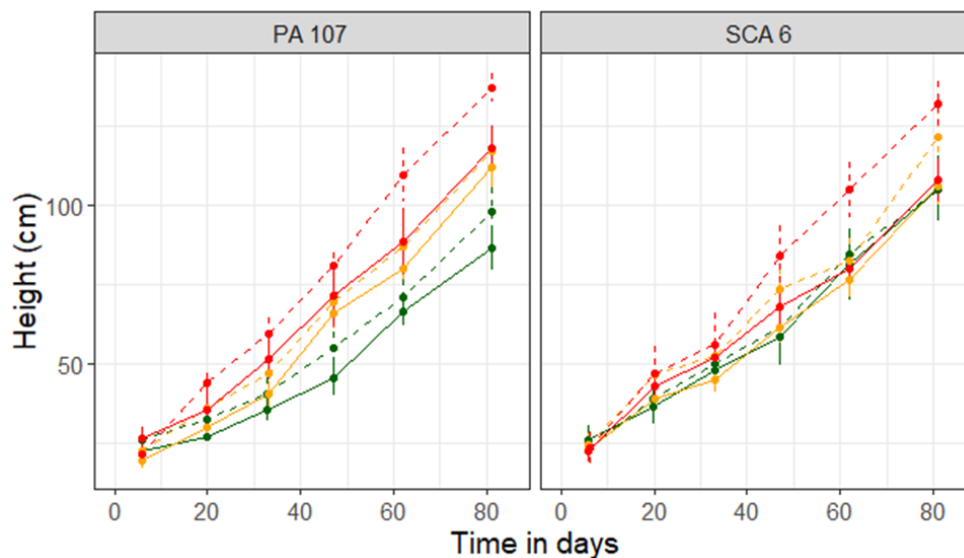
Figure 3.3 Effect of [CO_2] and temperature on plant height of two juvenile cacao genotypes over time. Error bars show the standard error of the mean (n=6). Temperature treatments are 31/22°C (green), 33.5/24.5°C (orange) and 36/27°C (red). [CO_2] treatments are ambient (solid line) and elevated (dashed line).

Stem diameter. On average, stem diameter more than doubled during the experimental period for both genotypes ($P < 0.001$) from 6.7 (±0.2) mm at 20 days to 14.9 (±0.3) mm at 81 days. Stem diameter of PA 107 was significantly higher compared to SCA 6 ($P < 0.01$) from 33 days (Figure 3.4). At the final time

point (81 days), there was a 9% increase in stem diameter of plants grown at 700 ppm compared to those grown at 400 ppm ($P < 0.05$). A significant genotype and temperature interaction ($P< 0.05$) was observed at the final time point (81 days). For PA 107, the stem diameter increased by 12% with a temperature increase from 31/22 to 33.5/24.5°C (14.8 (±0.6) to 16.6 (±0.6) cm) but remained similar at 36/27°C (16.6 (±0.8) cm). Conversely, for SCA 6 the stem diameter decreased 11% with temperature increases from 31/22 to 33.5/24.5°C (15.1 (±0.7) to 13.4 (±0.6) cm and remained similar at 36/27°C (13.1 (±0.7) cm).
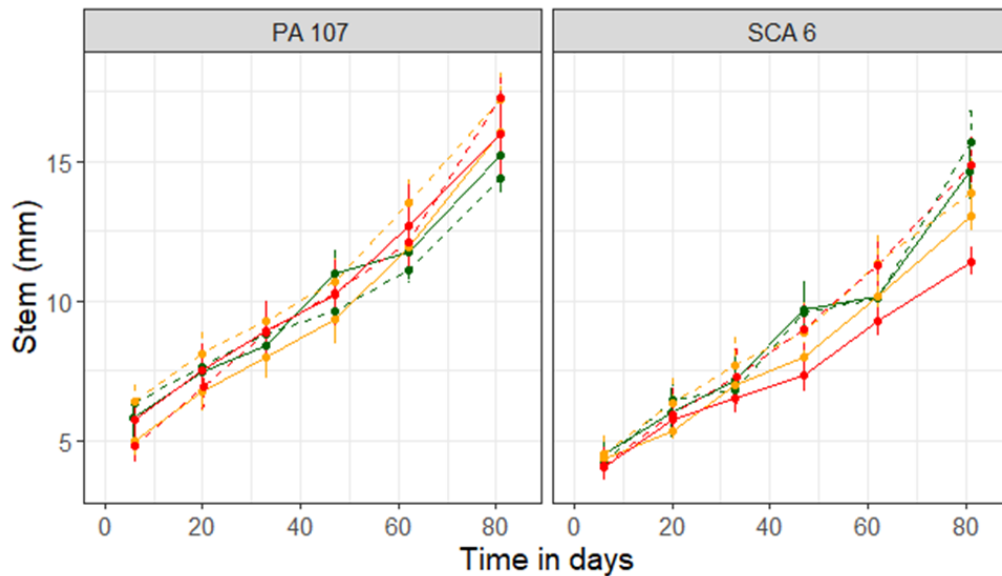
Figure 3.4 Effect of [CO_2] and temperature on stem diameter of two juvenile cacao genotypes over time. Error bars show the standard error of the mean (n=6). Temperature treatments are 31/22°C (green), 33.5/24.5°C (orange) and 36/27°C (red). [CO_2] treatments are ambient (solid line) and elevated (dashed line).

Increase in leaf length: The increase in leaf length for each treatment combination for genotypes PA 107 and SCA 6 are shown in Figures 3.5 and 3.6, respectively, and on maximum leaf length (logistic regression parameter "*d*") in Figure 3.7. A significant interaction between genotype and temperature was observed ($P < 0.001$). For SCA 6 final leaf length decreased with increasing temperature whilst for PA 107 final leaf length remained approximately constant with increasing temperature. The effect of the [CO_2] on leaf length was inconsistent between genotypes and temperature. For example, at 36/27°C, leaf length was higher at elevated [CO_2] for PA 107, whereas for SCA 6 it was higher at ambient [CO_2].
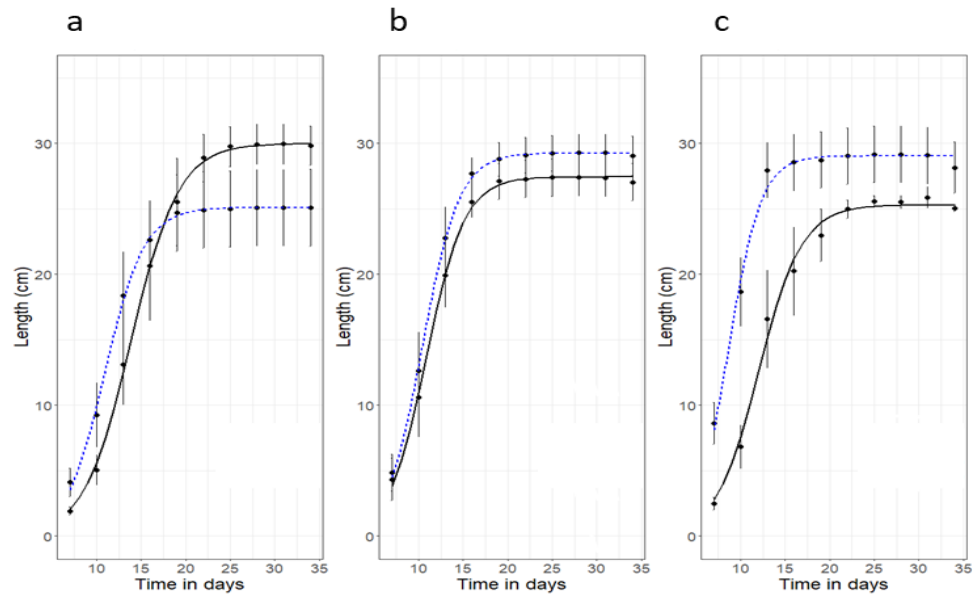
Figure 3.5 Increase in leaf length for PA 107 juvenile cacao plants grown under two [CO_2] and three temperature regimes. Curves based on 4 parameter generalised logistic equations applied to each treatment combination (n=6). Temperature treatments are 31/22°C (a), 33.5/24.5°C (b) and 36/27°C (c). [CO_2] treatments are ambient (solid line) and elevated (dashed line).
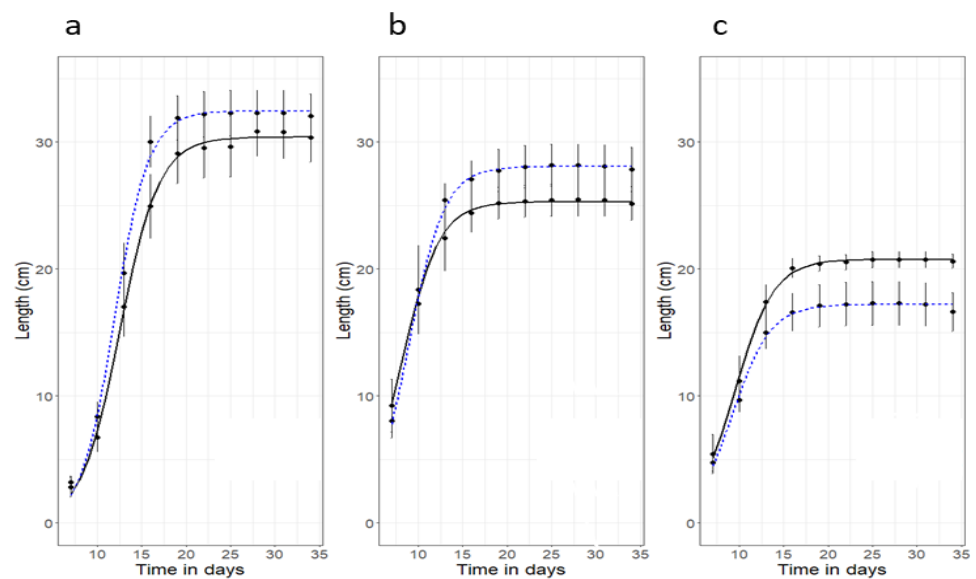
Figure 3.6 Increase in leaf length for SCA 6 juvenile cacao plants grown under two [CO_2] and three temperature regimes. Curves are based on 4 parameter generalised logistic equations applied to each treatment combination (n=6). Temperature treatments are 31/22°C (a), 33.5/24.5°C (b) and 36/27°C (c). [CO_2] treatments are ambient (solid line) and elevated (dashed line).
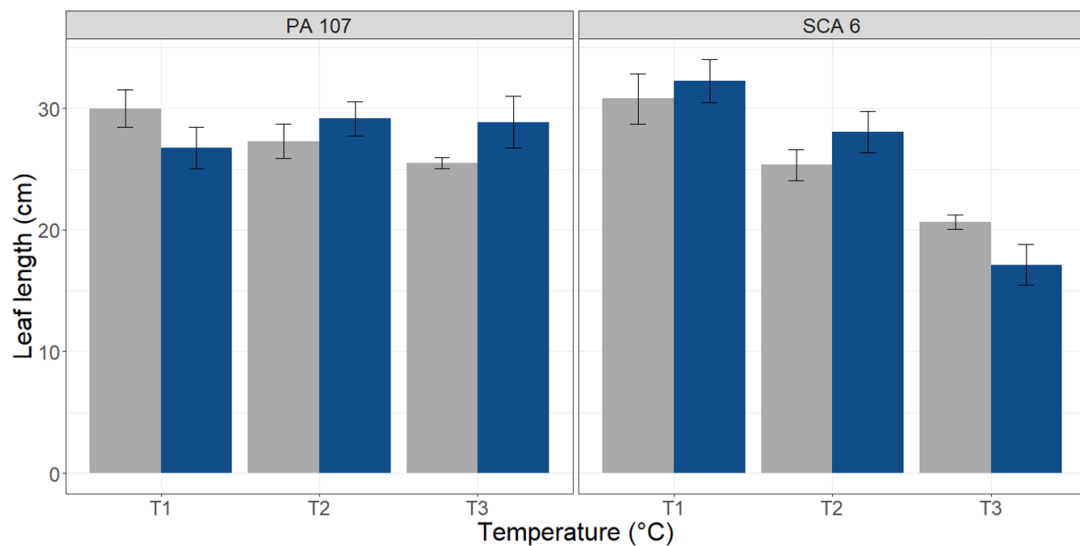
Figure 3.7 Effect of [CO_2] and temperature on maximum leaf length of two juvenile cacao genotypes. Error bars show the standard error of the mean (n=6). [CO_2] treatments are ambient (grey bar) and elevated (blue bar). Temperature treatments are 31/22°C (T1), 36/27°C (T2) and 38.5/29.5°C (T3).

Maximum leaf growth rate. Figure 3.8 shows the effect of the treatments on maximum leaf growth rate (parameter "*b*" estimated from the logistic regression) for genotypes PA 107 and SCA 6. There was a significant interaction between genotypes and the temperature regimes ($P < 0.05$) such that for PA 107, the maximum leaf growth rate increased by approximately 42% with an increase in temperature from 31/22°C to 36/27°C (0.61 (±0.04) and 0.87(±0.06) cm day^{-1} respectively). In contrast, there were no significant differences in maximum leaf growth rate between temperatures for SCA 6. There were no differences in maximum leaf growth rate between CO_2 concentrations.
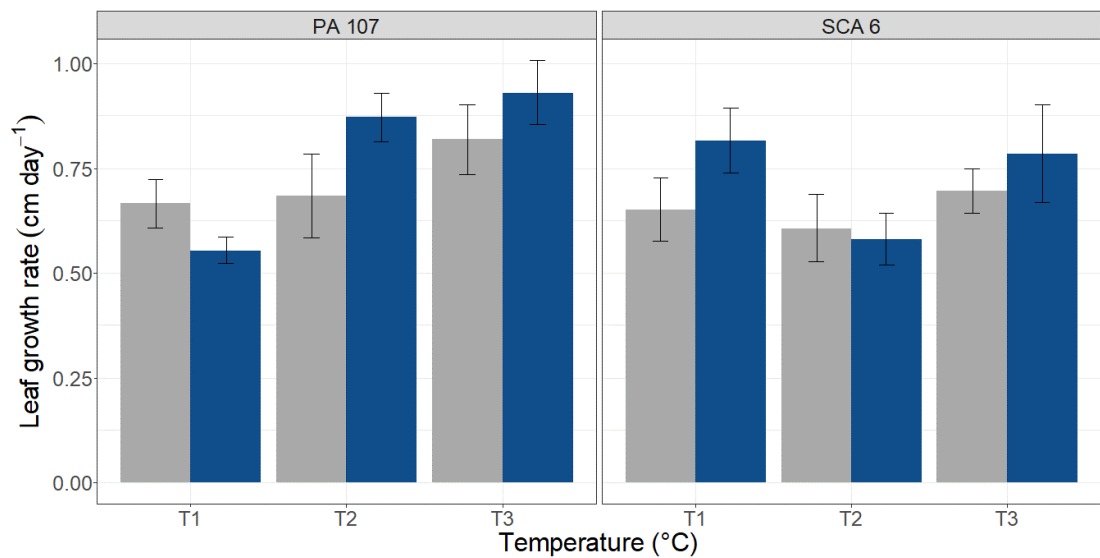
Figure 3.8 Effect of [CO_2] and temperature on maximum leaf growth rate of two juvenile cacao genotypes. Error bars show the standard error of the mean (n=6). [CO_2] treatments are ambient (grey bar) and elevated (blue bar). Temperature treatments are 31/22°C (T1), 36/27°C (T2) and 38.5/29.5°C (T3).

Time to maximum growth rate. Treatment effects on the time taken to reach the maximum growth rate (determined by the time to reach the steepest gradient from the logistic regression) for PA 107 and SCA 6 are shown in Figure 3.9. In general, time to maximum growth rate differed between temperature regimes ($P < 0.05$) decreasing by 15% from 31/22°C to 33.5/24.5°C whereas there were no significant differences between 36/27°C and 33.5/24.5°C. A significant interaction between genotypes and [CO_2] was observed ($P < 0.05$). For PA 107, time taken to reach the maximum growth rate decreased on average from 13.1 (±0.7) days to 10.1 (±0.5) days in plants grown in ambient [CO_2] (400 ppm) and elevated [CO_2] (700 ppm) respectively whereas there were no significant differences between CO_2 concentrations for SCA 6.
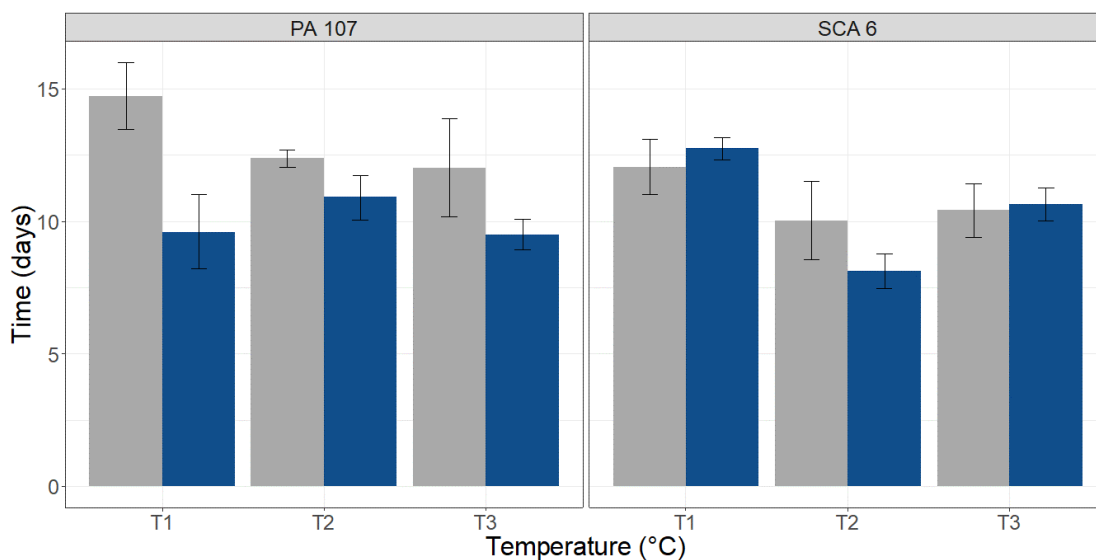
Figure 3.9 Effect of [CO_2] and temperature on time to reach the maximum growth rate of two juvenile cacao genotypes. Error bars show the standard error of the mean (n=6). [CO_2] treatments are ambient (grey bar) and elevated (blue bar). Temperature treatments are 31/22°C (T1), 36/27°C (T2) and 38.5/29.5°C (T3).

Time to reach 95% of the maximum leaf length. Treatment effects on number of days to reach 95% of the maximum leaf length (calculated from Equation 3.1) for both genotypes are presented in Figure 3.10. Overall, the time to reach 95% of the maximum leaf length decreased by 17% from 31/22°C to 33.5°C whereas at 36/27°C there was no difference in comparison to 33.5/24.5°C ($P < 0.001$). A significant interaction between [CO_2] and genotypes was observed ($P < 0.05$), such that for PA 107 time to reach 95% of the maximum leaf length decreased significantly from 16.9 (±0.9) at ambient [CO_2] to 13.6 (±0.7) days at elevated [CO_2] whereas for SCA 6 there were no significant differences between CO_2 concentration.
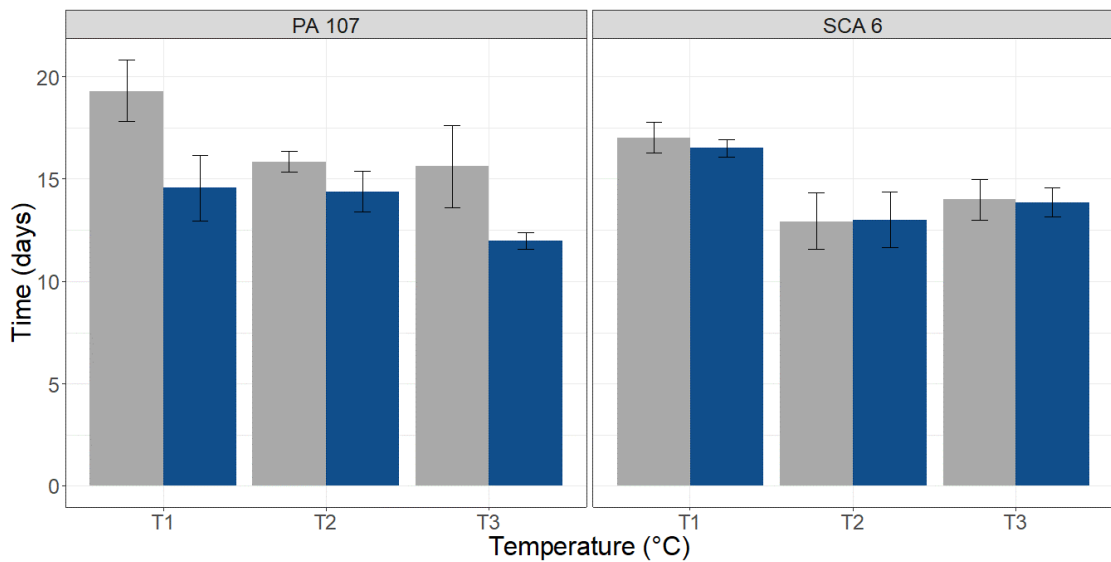
Figure 3.10 Effect of [CO_2] and temperature on time to reach 95% of the maximum leaf length of two juvenile cacao genotypes. Error bars show the standard error of the mean (n=6). [CO_2] treatments are ambient (grey bar) and elevated (blue bar). Temperature treatments are 31/22°C (T1), 36/27°C (T2) and 38.5/29.5°C (T3).

Chlorophyll Content. Leaf chlorophyll content increased over the experimental period ($P < 0.001$) on average from 13.1(±0.1) µg cm^{-2} at 10 days to 36.2 (±1.1) µg cm^{-2} at 46 days (Figure 3.11). Overall, leaf chlorophyll content was significantly higher for PA 107 compared with SCA 6 ($P < 0.001$) (27.5 (± 0.5) and 23.9 (±0.3) µg cm^{-2}, respectively). This difference was statistically different from day 31 ($P < 0.05$). There were no differences between temperature regimes, [CO_2] or their interaction on chlorophyll content.
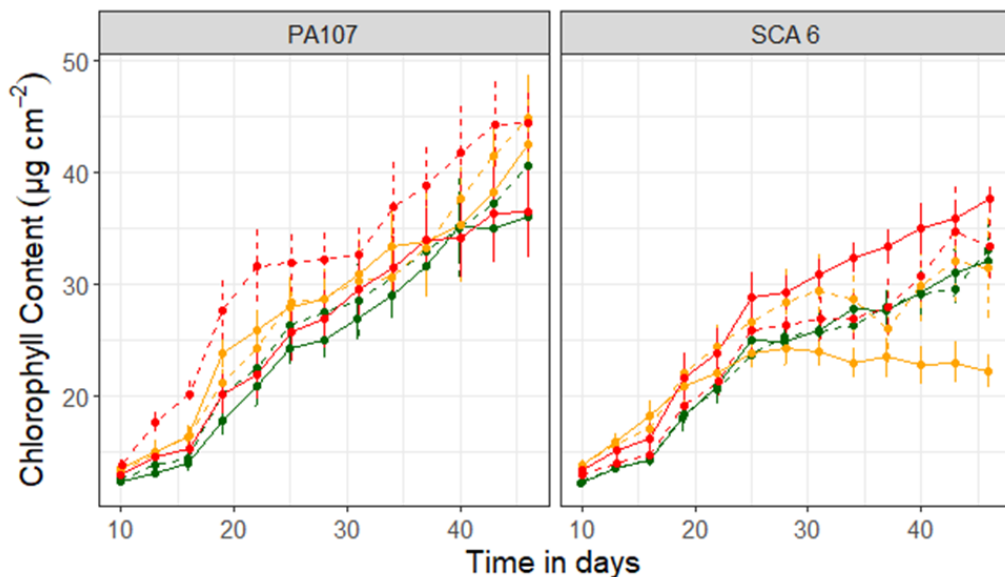
Figure 3.11 Effect of [CO_2] and temperature on chlorophyll content of two juvenile cacao genotypes over 46 days. Error bars show the standard error of the mean (n=6). Temperature treatments are 31/22°C (green), 33.5/24.5°C (orange) and 36/27°C (red). [CO_2] treatments are ambient (solid line) and elevated (dashed line).

Flushing interval. Flushing interval was not affected by the [CO_2] treatment. However, the interval between flushes was significantly reduced with increasing temperature ($P < 0.001$) in both genotypes (Figure 3.12a). Flushing interval decreased from 32 (±0.2) to 27 (±1) and 25 (±0.1) days at 31/22°C, 33.5/24.5°C and 36/27°C, respectively. The flushing interval was also different between genotypes ($P < 0.001$) being two days longer for SCA 6 in comparison to PA 107. There were no significant interactions among the main factors [CO_2], temperature and genotypes.

Number of leaves per flush. [CO_2] treatments did not affect the numbers of leaves per flush in both genotypes. However, there were significant differences between genotypes and between temperature regimes ($P < 0.01$, $P < 0.001$ respectively). PA 107 had fewer leaves per flush (17 (± 1)) compared to SCA 6 (20 (±1)) (Figure 3.12b). Furthermore, an increase in average leaf number per flush was observed with an increase in temperature ranging from 16(±1) at 31/22°C to 20(±1) leaves at 33.5/24.5°C and 36/27°C. No interactions between [CO_2], temperature and genotypes were detected.

Stomata density. Differences between genotypes and significant effects of [CO_2] and temperature on stomata density were detected ($P < 0.001$) (Figure 3.12c). Lower stomata per mm^2 were observed for PA 107 (994 (±20) in comparison to SCA 6 (1310 (±26)). There was also a reduction in stomatal density ranging from 1205 (±34) mm^{-2} to 1099 (±34) mm^{-2} when the genotypes were grown at elevated [CO_2]. Stomata density did not significantly differ between 31/22°C (1066 (±35.1) mm^{-2}) and 36/27°C (1121 (±37.5) mm^{-2}). However, at 33.5/24.5°C stomata density was significantly higher in both genotypes (1269 (±45) mm^{-2}). There were no significant interactions among the main factors [CO_2], temperature and genotype.
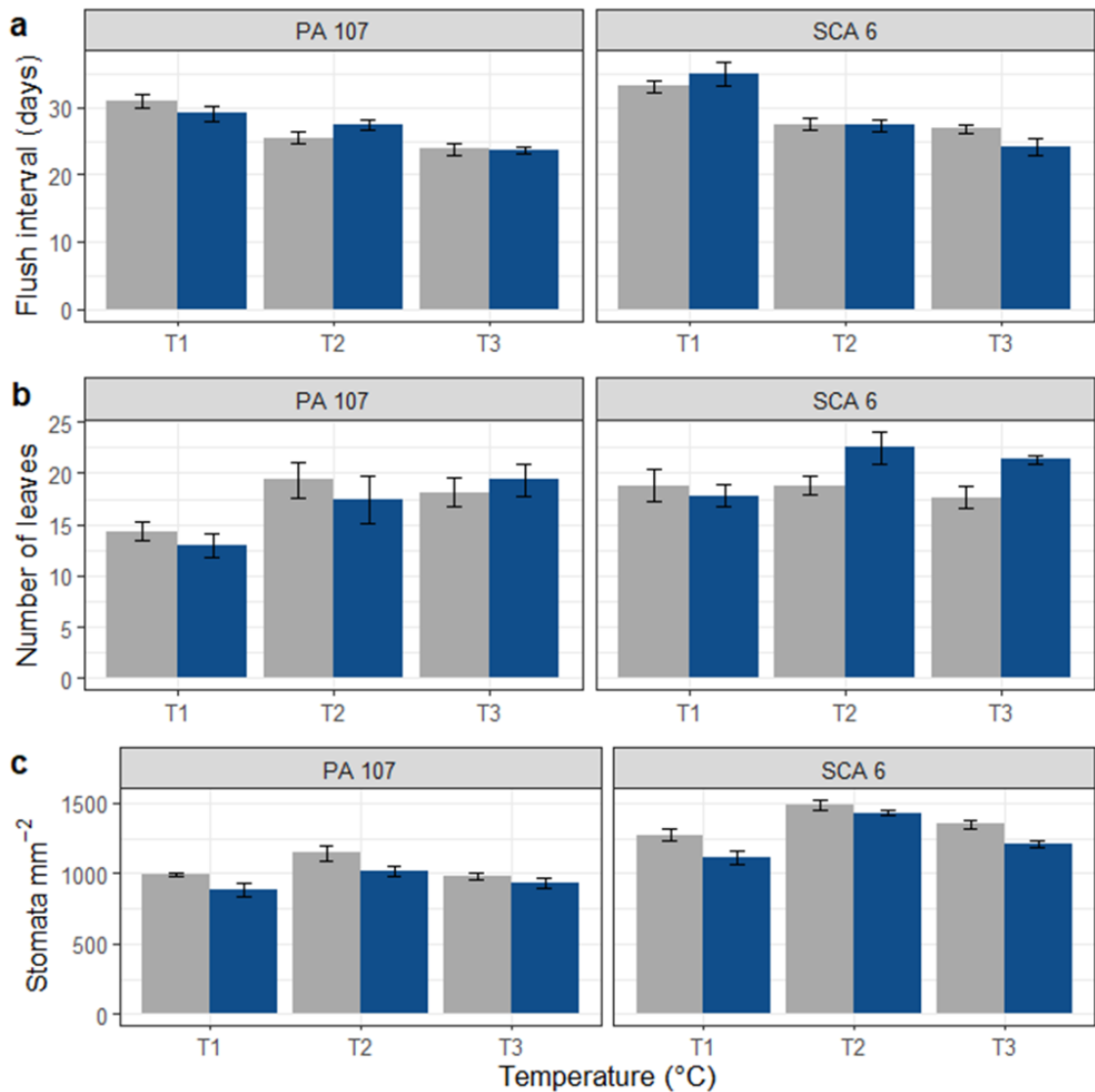
Figure 3.12 Effect of [CO_2] and temperature on Flushing interval (a), number of leaves per flush (b) and stomata density (c) of two juvenile cacao genotypes. Error bars show the standard error of the mean (n=6). [CO_2] treatments are ambient (grey bar) and elevated (blue bar). Temperature treatments are 31/22°C (T1), 36/27°C (T2) and 38.5/29.5°C (T3).

3.3.2 Gas exchange responses

Transpiration rate. Transpiration rate did not differ significantly across time (Figure 3.13). Transpiration rate increased significantly with an increase in temperature (P < 0.001), increasing by 67% from 31/22°C to 33.5/24.5°C (0.93 (±0.05) and 1.55 (±0.07) mmol m^{-2} s^{-1}) and 140% from 31/22°C to 36/27°C (0.93 (±0.05) and 2.23 (±0.07) mmol m^{-2} s^{-1}) respectively. A significant interaction between [CO_2] and temperature was observed (*P* < 0.01) with differences between [CO_2] observed only at 33.5/24.5°C where transpiration rate was lower at 700 ppm compared to 400 ppm (1.42(±0.08) mmol m^{-2} s^{-1} and 1.68 (±0.11) mmol m^{-2} s^{-1} respectively). There was also a significant interaction between genotypes and time (*P* < 0.01). Differences were observed between genotypes at 27 days being higher for PA 107

compared with SCA 6 (1.56 (±0.14) and 1.24 (±0.08) mmol m^{-2} s^{-1} respectively), whereas there was no differences between the genotypes at 55 and 72 days.
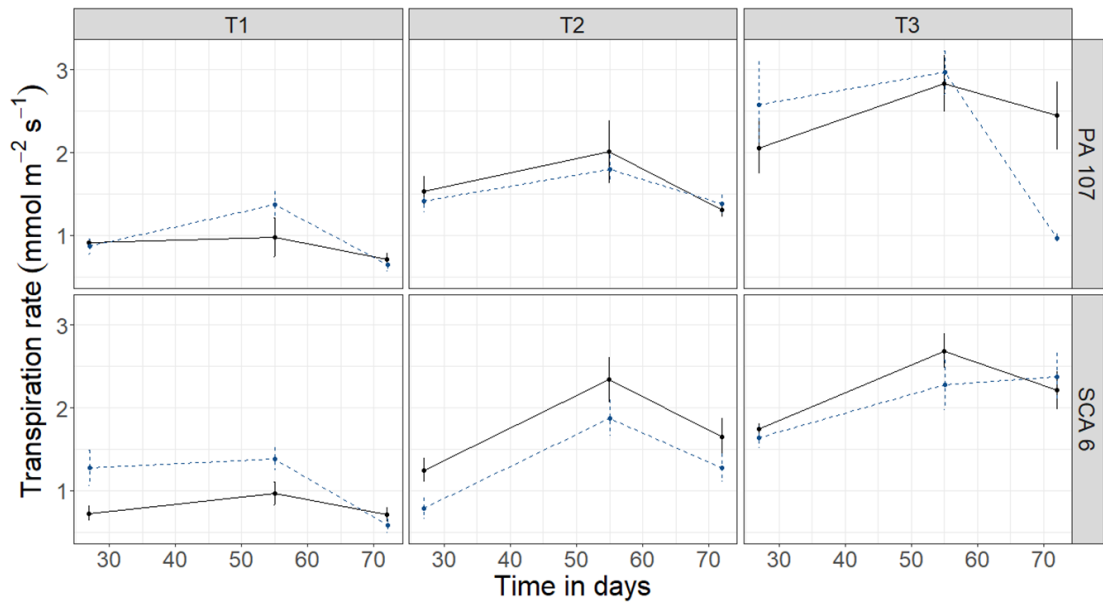
Figure 3.13 Effect of [CO_2] and temperature on transpiration rate of two juvenile cacao genotypes at 27, 55 and 72 days after the start of the experiment. Error bars show the standard error of the mean (n=6). [CO_2] treatments are ambient (solid line) and elevated (dashed line). Temperature treatments are 31/22°C (T1), 36/27°C (T2) and 38.5/29.5°C (T3).

Stomatal conductance. Overall, there were no significant differences across time in stomatal conductance (Figure 3.14). Stomatal conductance differed significantly between temperatures ($P < 0.001$) increasing by 42 % from 31/22°C to 33.5/24.5°C and 81 % from 31/22°C to 36/27°C. There was a significant interaction between [CO_2] and temperature ($P < 0.01$). At 33.5/24.5°C, the stomatal conductance was higher at ambient [CO_2] (0.051 ±0.004 mol m^{-2} s^{-1}) compared with elevated [CO_2] ppm (0.043 ±0.003 mol m^{-2} s^{-1}) whereas no significant differences between [CO_2] levels at 31/22°C and 36/27°C were observed. An additional interaction between genotype and time was observed ($P < 0.01$). No differences were observed between genotypes at 55 and 72 days. However, at 27 days PA 107 showed a higher stomatal conductance of 0.047 (±0.005) mol m^{-2} s^{-1} compared to SCA 6 with 0.036 (±0.002) mol m^{-2} s^{1}.
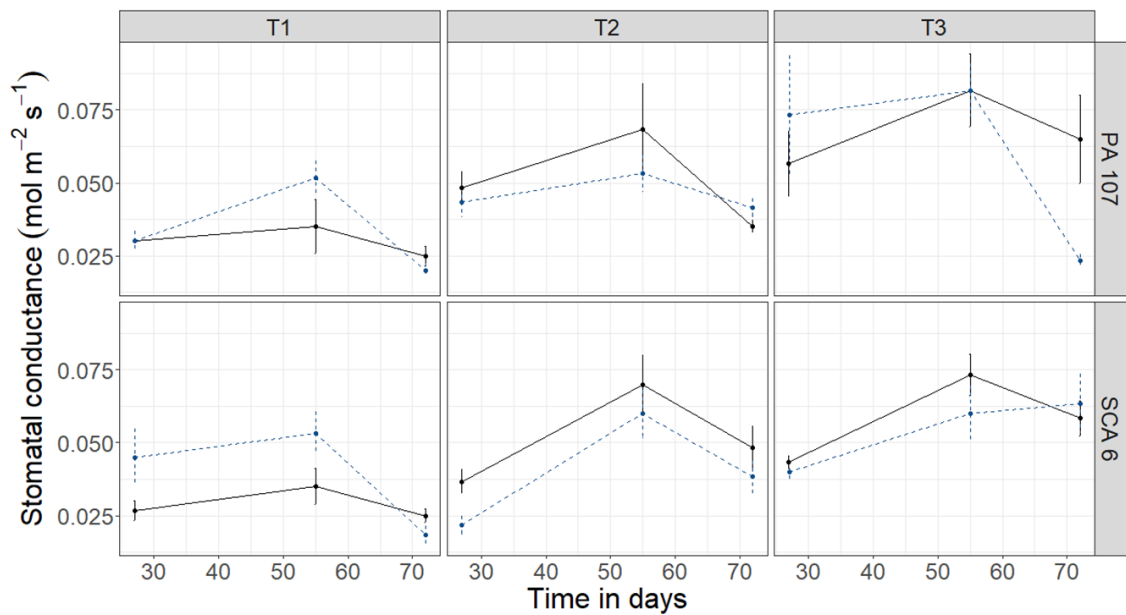
Figure 3.14 Effect of [CO_2] and temperature on stomatal conductance of two juvenile cacao genotypes at 27, 55 and 72 days after the start of the experiment. Error bars show the standard error of the mean (n=6). [CO_2] treatments are ambient (solid line) and elevated (dashed line). Temperature treatments are 31/22°C (T1), 36/27°C (T2) and 38.5/29.5°C (T3).

Net photosynthesis rate. Net light-saturated photosynthetic rate (*Pn*) varied significantly across time ($P < 0.001$; Figure 3.15) ranging from 3.04 ($\pm$0.19) µmol m^{-2} s^{-1} at 27 days to 4.87 ($\pm$0.18) µmol m^{-2} s^{-1} at 55 days and 3.42 ($\pm$0.15) µmol m^{-2} s^{-1} at the end of the experimental period. A significant increase in *Pn* was generally observed with an increase in temperature at each observation ($P < 0.01$). For plants grown at 31/22°C and 36/27°C, the increases observed were 20%, 32%, and 63% at 27, 55, and 72 days, respectively. Elevated [CO_2] had a positive effect on *Pn* in both genotypes across the experimental period ($P < 0.01$). The increase was 22%, 52% and 49% greater in plants grown at 700 ppm of [CO_2] at 27, 55, and 72 days. Genotypes differed only at 27 days ($P < 0.01$) where *Pn* was higher in PA 107 (3.53 ($\pm$0.26) µmol m^{-2} s^{-1}) than SCA 6 (2.55 ($\pm$0.26) µmol m^{-2} s^{-1}). There was no significant interaction between the treatments.
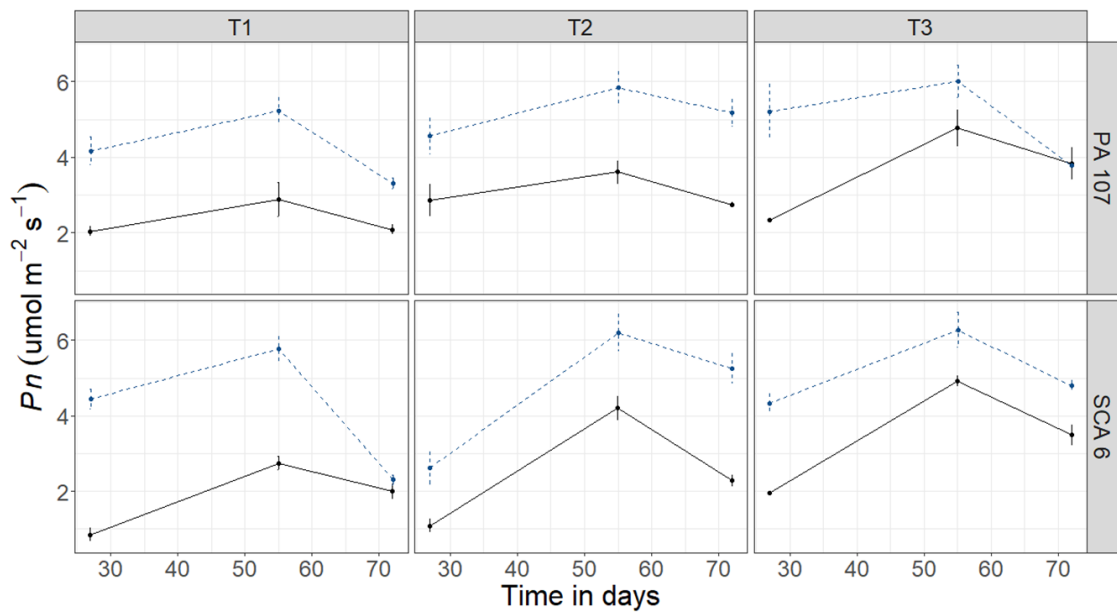
Figure 3.15 Effect of [CO_2] and temperature on net light-saturated photosynthesis rate of two juvenile cacao genotypes at 27, 55 and 72 days after the start of the experiment. Error bars show the standard error of the mean (n=6). [CO_2] treatments are ambient (solid line) and elevated (dashed line). Temperature treatments are 31/22°C (T1), 36/27°C (T2) and 38.5/29.5°C (T3).

Intrinsic water use efficiency. Intrinsic water use efficiency (*iWUE*) did not differ significantly between day 27 and 55; the mean values at these time points were: 81.27 (±5.13) µmol mol^{-1} and 89.60 (±3.63) µmol mol^{-1}, respectively. However, at 72 days *iWUE* increased significantly to 107.26 (±6.26) µmol mol^{-1} ($P < 0.001$; Figure 3.16) being largely related to an effect of elevated [CO_2]. There was a significant and positive effect of elevated [CO_2] on *iWUE* at each time point and for both genotypes ($P<0.001$) ranging from 64.68(±2.57) µmol mol^{-1} at 400 ppm to 120.74 (±3.98) µmol mol^{-1} at 700 ppm. In general, *iWUE* differed statistically between genotypes ($P < 0.05$) being, on average, 96.98 (±4.34) µmol mol^{-1} for PA 107 and 88.44 (±4.14) µmol mol^{-1} for SCA 6. In addition, there was a significant interaction between genotypes and temperatures ($P < 0.05$) at 27 days. At this time, the intrinsic water use efficiency of PA 107, declined with increasing temperature ranging from 105.87 (±12.84) µmol mol^{-1} at 31/22°C to 66.43 (±8.68) µmol mol^{-1} at 36/27°C whereas it remained stable in SCA 6 across the temperature regimes.
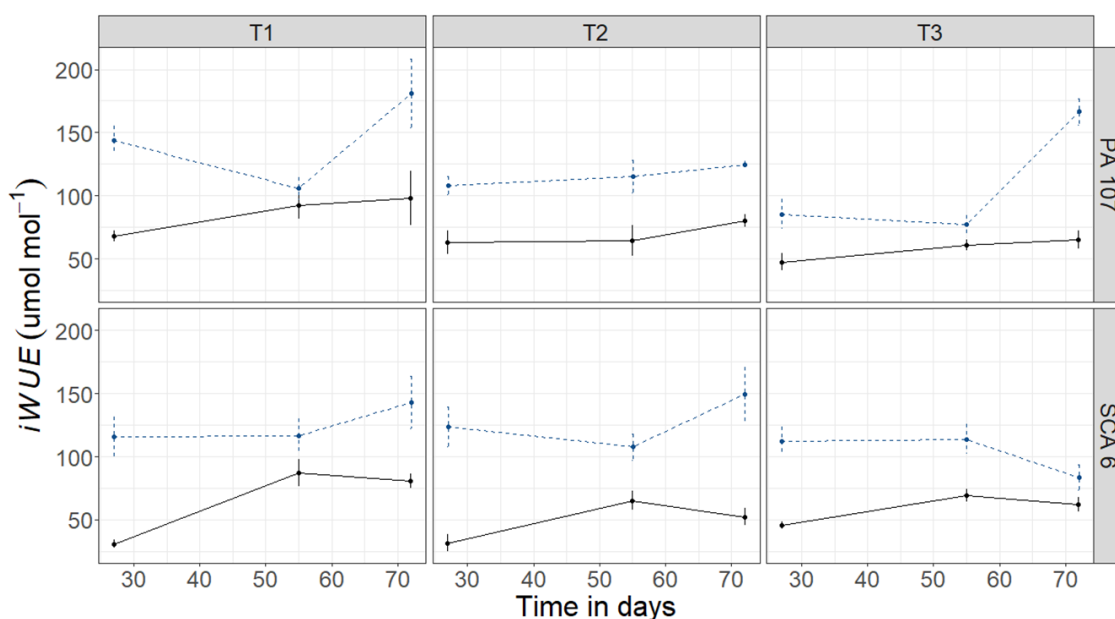
Figure 3.16 Effect of [CO_2] and temperature on intrinsic water use efficiency of two juvenile cacao genotypes at 27, 55 and 72 days after the start of the experiment. Error bars show the standard error of the mean (n=6). [CO_2] treatments are ambient (solid line) and elevated (dashed line). Temperature treatments are 31/22°C (T1), 36/27°C (T2) and 38.5/29.5°C (T3).

3.3.3 Chlorophyll fluorescence parameters

Maximum quantum efficiency of photosystem II (F_v/F_m). There was a significant overall increase ($P < 0.001$) of F_v/F_m from 0.70 (±0.01) at 27 days to 0.76 (±0.01) at 55 days. However, F_v/F_m did not differ from 55 to 72 days (Figure 3.17). A slightly higher F_v/F_m ratio was observed for PA 107 compared with SCA 6 (0.74 (±0.01) and 0.72 (±0.01), respectively) ($P < 0.05$). There was also a significant interaction between [CO_2] and time for F_v/F_m ratio ($P < 0.001$) being significantly higher at ambient [CO_2] at 27 days (0.72 (±0.01) compared with elevated [CO_2] (0.66 (±0.01)). However, F_v/F_m ratio did not differ between [CO_2] at 55 and 72 days. No difference was observed in F_v/F_m across the temperature regimes.

Performance index (PI). Across the experimental period, *PI* increased significantly ($P < 0.001$) from 27 days to 55 days from 0.57 (±0.06) to 1.14 (±0.09) but there was no significant difference after 72 days (1.14 ±0.09; Figure 3.18). Differences between genotypes were significant ($P < 0.05$). *PI* index was greater in PA 107 compared to SCA 6 (1.07 (±0.07) and 0.85 (±0.07) respectively). There were no significant effects of [CO_2] or temperature on *PI*.
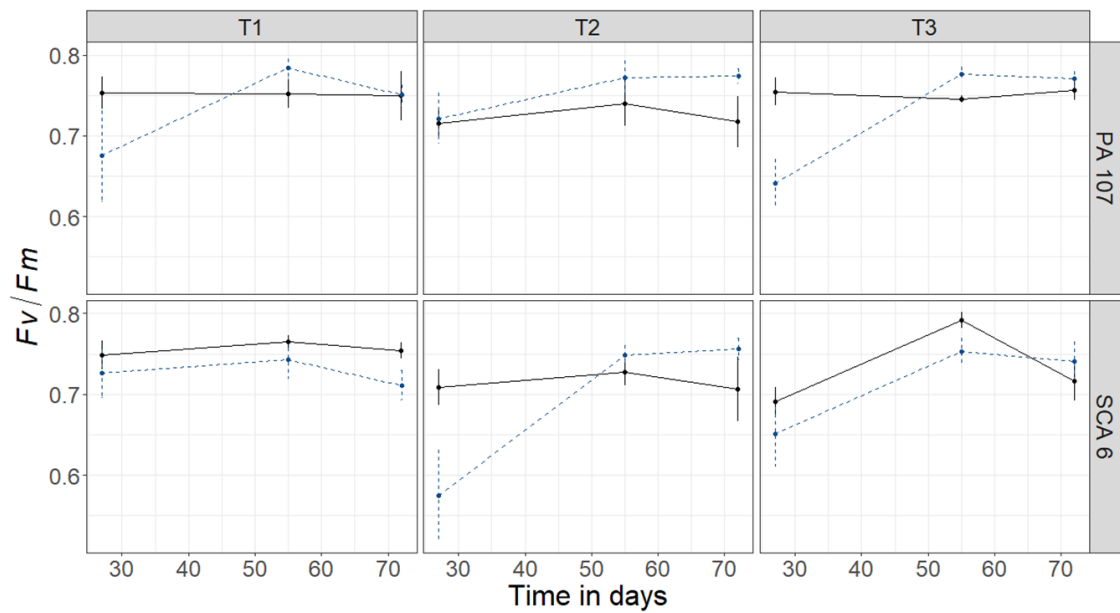
Figure 3.17 Effect of $[CO_2]$ and temperature on F_v/F_m ratio of two juvenile cacao genotypes at 27, 55 and 72 days after the start of the experiment. Error bars show the standard error of the mean (n=6).). $[CO_2]$ treatments are ambient (solid line) and elevated (dashed line). Temperature treatments are 31/22°C (T1), 36/27°C (T2) and 38.5/29.5°C (T3).
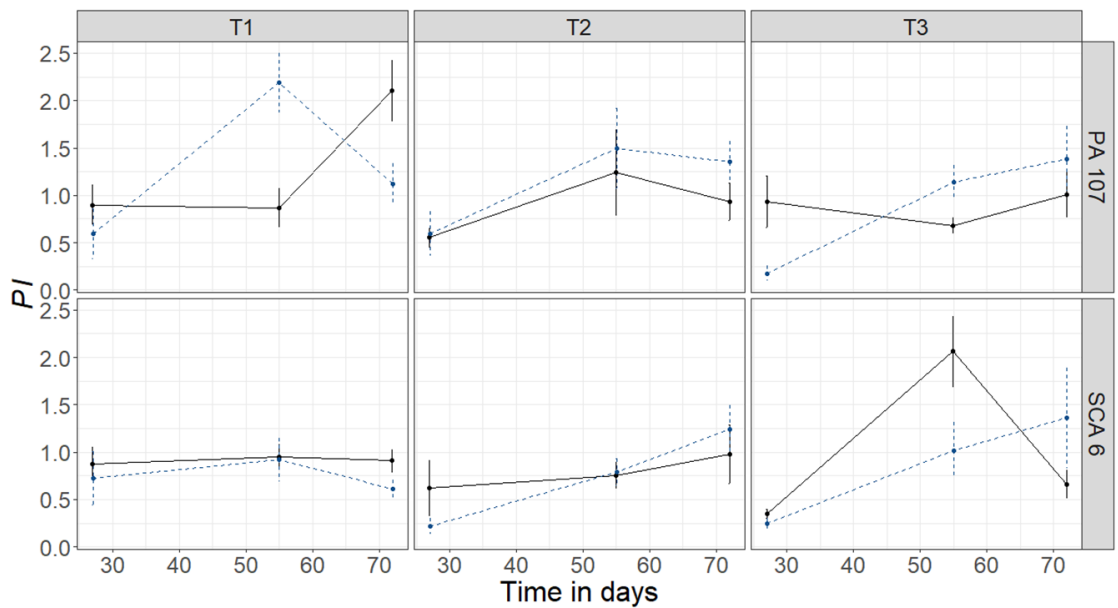
Figure 3.18 Effect of $[CO_2]$ and temperature on PI index of two juvenile cacao genotypes at 27, 55 and 72 days after the start of the experiment. Error bars show the standard error of the mean (n=6).). $[CO_2]$ treatments are ambient (solid line) and elevated (dashed line). Temperature treatments are 31/22°C (T1), 36/27°C (T2) and 38.5/29.5°C (T3).

3.3.4 Functional plant growth analysis

Total plant dry weight. Regression equations fitted to log-transformed total plant dry weight as a function of time are presented in Table 3.1 for both genotypes. The fitted functions are displayed in Figure 3.19. At the end of the experimental period, total plant dry weight was significantly higher for PA 107 than SCA 6. For PA 107, at ambient $[CO_2]$ the increase in total dry weight was similar for each temperature regime, whereas in SCA 6 total plant dry weight decreased with increasing temperature. At elevated $[CO_2]$, total plant dry weight increased with increases in temperature in PA 107 whilst no changes were observed across the temperature treatments in SCA 6. Total plant dry weight increased with increasing $[CO_2]$ for both genotypes, and seemed to compensate the negative effect of the high temperature in SCA 6.

Table 3.1 Regression equations (t in days) of total plant dry weight (*DW*) and leaf area (*LA*) for PA 107 and SCA 6 juvenile cacao plants grown under two $[CO_2]$ and three temperature regimes over 88 days.

Temp (°C)	$[CO_2]$ (pm)	$\mathrm{Log_e}$ *DW* (*DW* in g)	$\mathrm{Log_e}$ *LA* (*LA* in cm^2)
		PA 107	
	400	$1.6244+0.0270*t-1.385E\text{-}07*t^2$	$6.9251+0.0156*t+5.989e\text{-}05*t^2$
31/22	700	$1.6244+0.0414*t-1.234e\text{-}04*t^2$	$6.9363+0.0255*t-3.096e\text{-}05*t^2$
	400	$1.6244+0.0335*t-1.364E\text{-}05*t^2$	$6.9105+0.0291*t-7.463e\text{-}04*t^2$
33.5/24.5	700	$1.6244+0.0481*t-1.648e\text{-}04*t^2$	$6.9001+0.0402*t-1.606e\text{-}04*t^2$
	400	$1.6244+0.0327*t-3.916E\text{-}05*t^2$	$6.9051+0.2411*t-4.018e\text{-}05*t^2$
36/27	700	$1.6244+0.0544*t-2.194e\text{-}04*t^2$	$6.9225+0.0389*t-1.690e\text{-}04*t^2$
		SCA 6	
	400	$1.4631+0.0359*t-9.151e\text{-}05*t^2$	$6.6740+0.0331*t-1.532e\text{-}04*t^2$
31/22	700	$1.4631+0.0337*t-4.671e\text{-}05*t^2$	$6.6447+0.0336*t-1.587e\text{-}04*t^2$
	400	$1.4631+0.0301*t-4.134e\text{-}05*t^2$	$6.6541+0.0261*t-9.009e\text{-}05*t^2$
33.5/24.5	700	$1.4631+0.0362*t-7.115e\text{-}05*t^2$	$6.6083+0.0280*t-5.507e\text{-}05*t^2$
	400	$1.4631+0.0324*t-1.206e\text{-}04*t^2$	$6.6621+0.0365*t-2.674e\text{-}04*t^2$
36/27	700	$1.4631+0.0395*t-1.189e\text{-}04*t^2$	$6.6063+0.0308*t-1.295e\text{-}04*t^2$
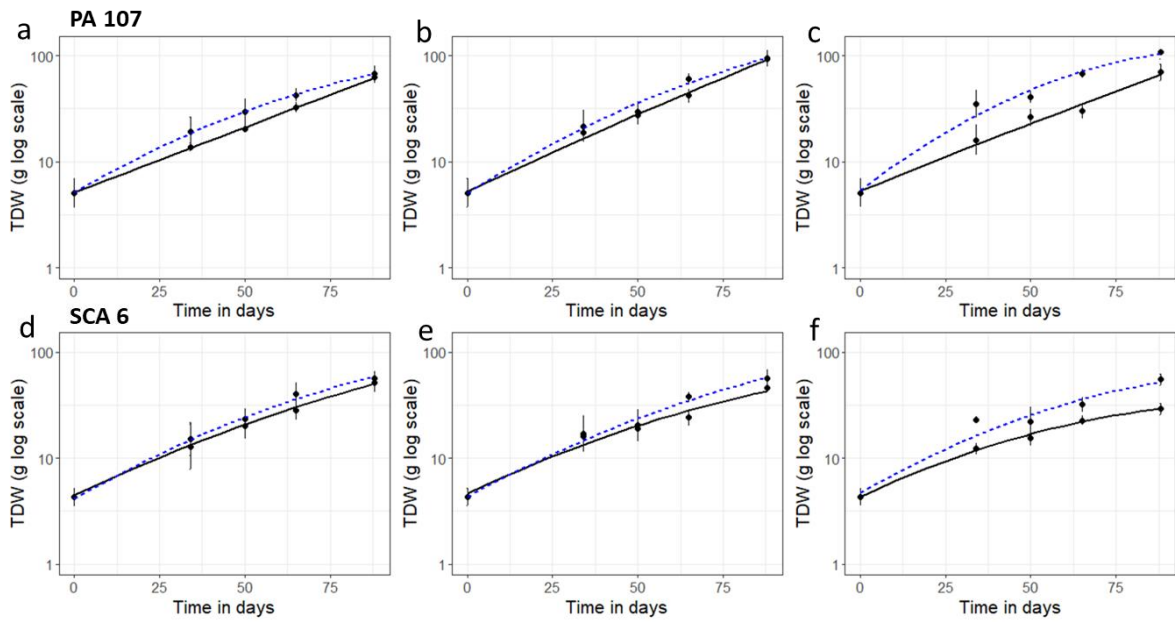
Figure 3.19 Progress curves of total plant dry weight (TDW) for PA 107 and SCA 6. The lines are the quadratic curves fitted to all the observations in each treatment combination. Points are the observed means. Error bars show the standard error of the mean (n=3 at 0, 34 and 50 days; n=6 at 65 and 88 days). Temperature treatments are 31/22°C (a), (d), 33.5/24.5°C (b),(e) and 36/27°C (c),(f). [CO_2] treatments are ambient (solid line) and elevated (dashed line).

Total leaf area. Fitted curves of total leaf area over time based on the equations in Table 3.1 are shown in Figure 3.20. The final leaf area was much higher for PA 107 than SCA 6. The effects of temperature and [CO_2] differed between genotypes. At ambient [CO_2], the negative effect of increased temperature was more evident in SCA 6 compared with PA 107. Leaf area decreased with temperature increases in SCA 6 while in PA 107 it remained similar. The effect of elevated [CO_2] showed a marked increase in total leaf area across the temperature regimes for PA 107 whereas for SCA 6 elevated [CO_2] compensated for the negative effect of the higher temperatures. Here, the response to [CO_2] enrichment seemed to be earlier in PA 107 compared to SCA 6.
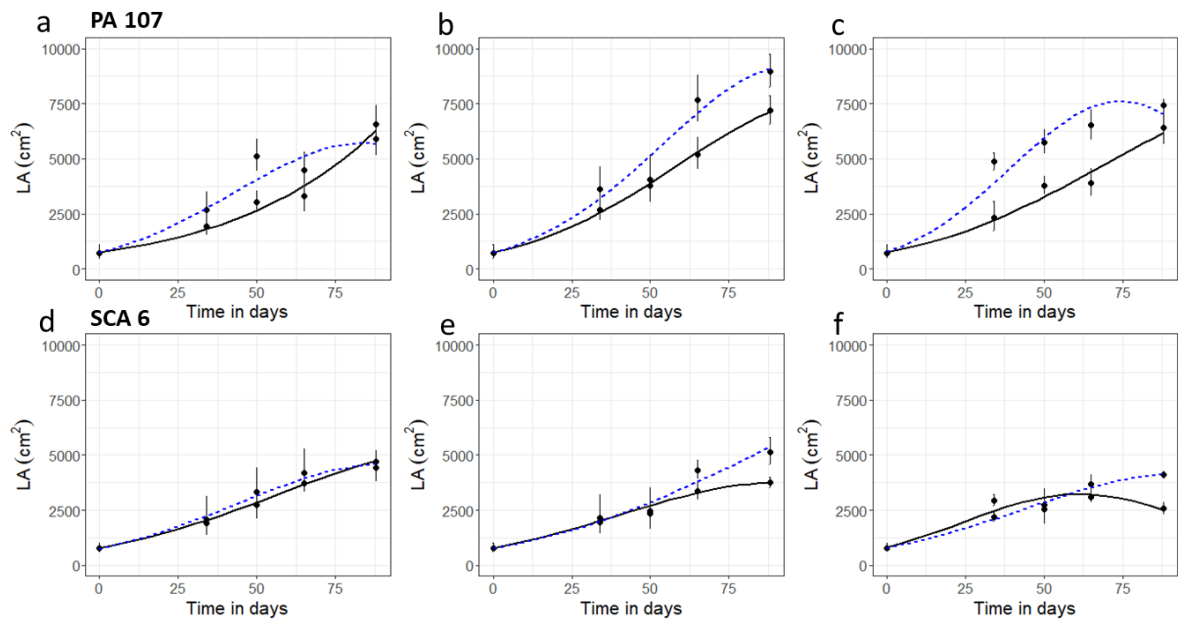
Figure 3.20 Progress curves of total leaf area (LA) for PA 107 and SCA 6. The lines are the quadratic curves fitted to all the observations in each treatment combination. Points are the observed means. Error bars show the standard error of the mean (n=3 at 0, 34 and 50 days; n=6 at 65 and 88 days). Temperature treatments are 31/22°C (a),(d), 33.5/24.5°C (b),(e) and 36/27°C (c),(f). [CO_2] treatments are ambient (solid line) and elevated (dashed line).

Relative growth rate (RGR). Relative growth rate decreased with time and differed between genotypes, [CO_2] and the temperature regimes (Figure 3.21). For PA 107, at ambient [CO_2] the relative growth rate decreased slightly over time and little change was observed with an increase in temperature. In contrast, RGR was higher at elevated CO_2 than ambient CO_2 and initial RGR increased with higher temperature but fell more rapidly over time. For SCA 6, RGR was generally higher at elevated [CO_2] compared to ambient [CO_2] there was little difference in RGR across the temperature regimes with the lowest RGR observed at 36/27°C by the end of the experimental period.
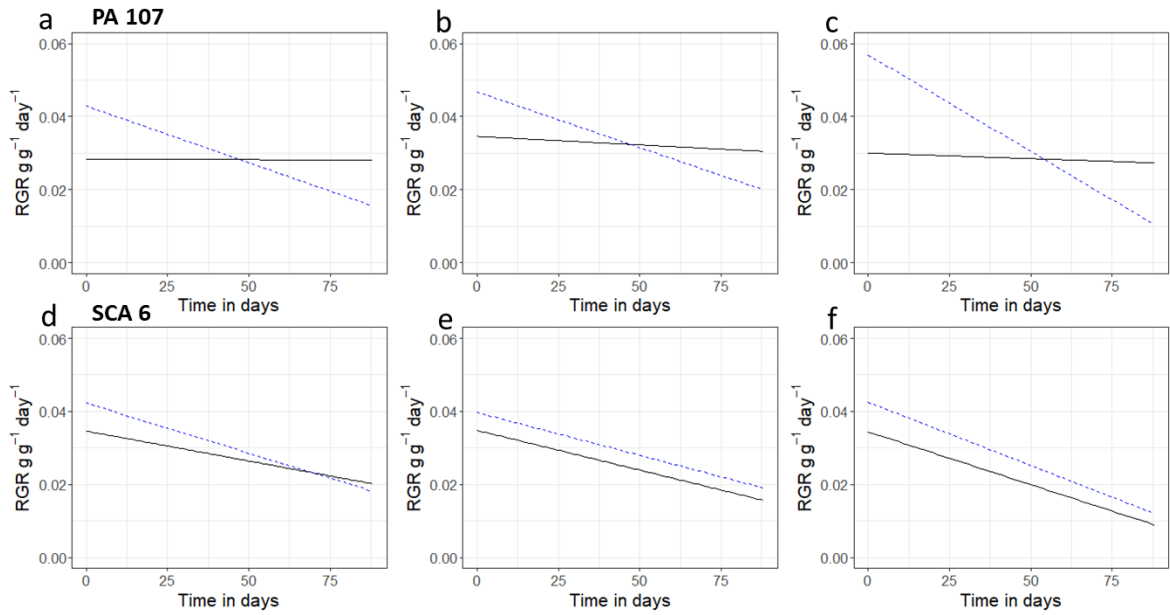
Figure 3.21 Progress curves of relative growth rate (RGR) derived from Log_e DW differentiation for PA 107 and SCA 6 juvenile cacao plants grown under two [CO_2] and three temperature regimes. Lines are calculated according to equation 3.4. Temperature treatments are 31/22°C (a),(d), 33.5/24.5°C (b),(e) and 36/27°C (c),(f). [CO_2] treatments are ambient (solid line) and elevated (dashed line).

Leaf area ratio (LAR). Leaf area ratio (calculated from equation 3.5) decreased over time for both PA 107 and SCA 6 (Figure 3.22). LAR decreased over time due to the more rapid increases in total dry biomass relative to changes in total leaf area. There were no significant differences in LAR under ambient and elevated [CO_2] in PA 107 grown at 33.5/24.5°C and SCA 6 grown at 31/22°C and 33.5/24.5°C. However, at the remaining temperatures LAR was higher at ambient [CO_2] compared to the elevated [CO_2]. Here, higher total biomass observed at elevated [CO_2] may have explained the effect on LAR.
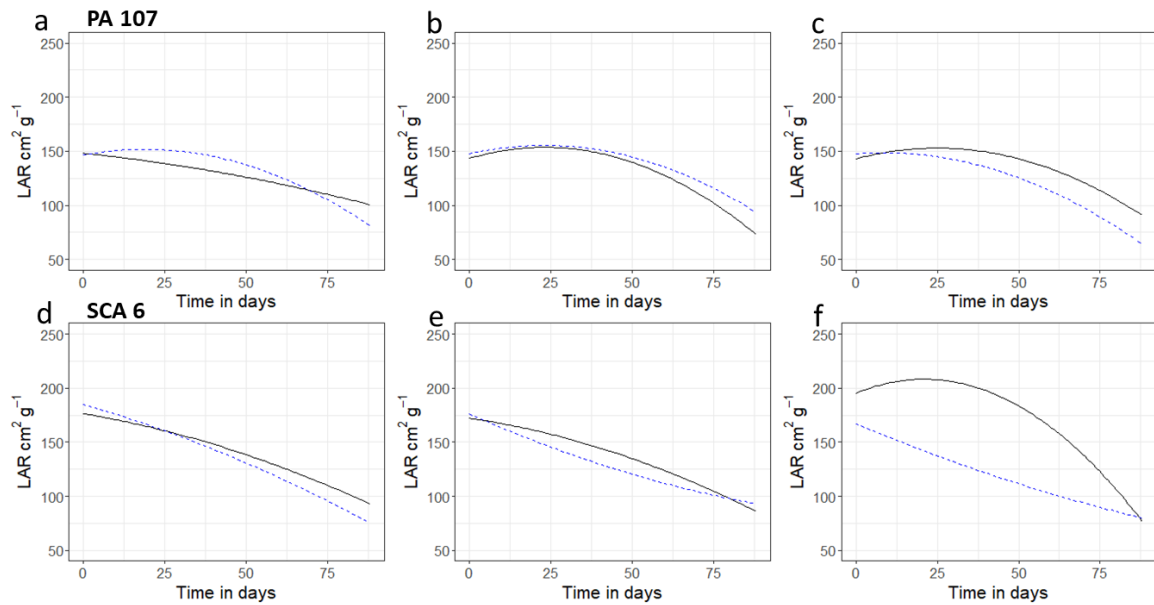
Figure 3.22 Progress curves of leaf area ratio (LAR) for PA 107 and SCA 6 juvenile cacao plants grown under two [CO_2] and three temperature regimes. Lines are calculated according to equation 3.5. Temperature treatments are 31/22°C (a),(d), 33.5/24.5°C (b),(e) and 36/27°C (c),(f). [CO_2] treatments are ambient (solid line) and elevated (dashed line).

Net assimilation rate (NAR). The effect of temperatures and [CO_2] on NAR over time differed between the genotypes (Figure 3.23). For PA 107, NAR followed a positive curvilinear increase over time at ambient [CO_2] with the highest rates at 33.5/24.5°C. In contrast, NAR was higher at elevated [CO_2] up to certain point in time, which varied with temperature, and then decreased towards the end of the experimental period. For SCA 6, a negative effect of the temperature on the net assimilation rate was evident at ambient [CO_2] over the experimental period. However, at elevated [CO_2] NAR remained stable despite the increase in temperature.
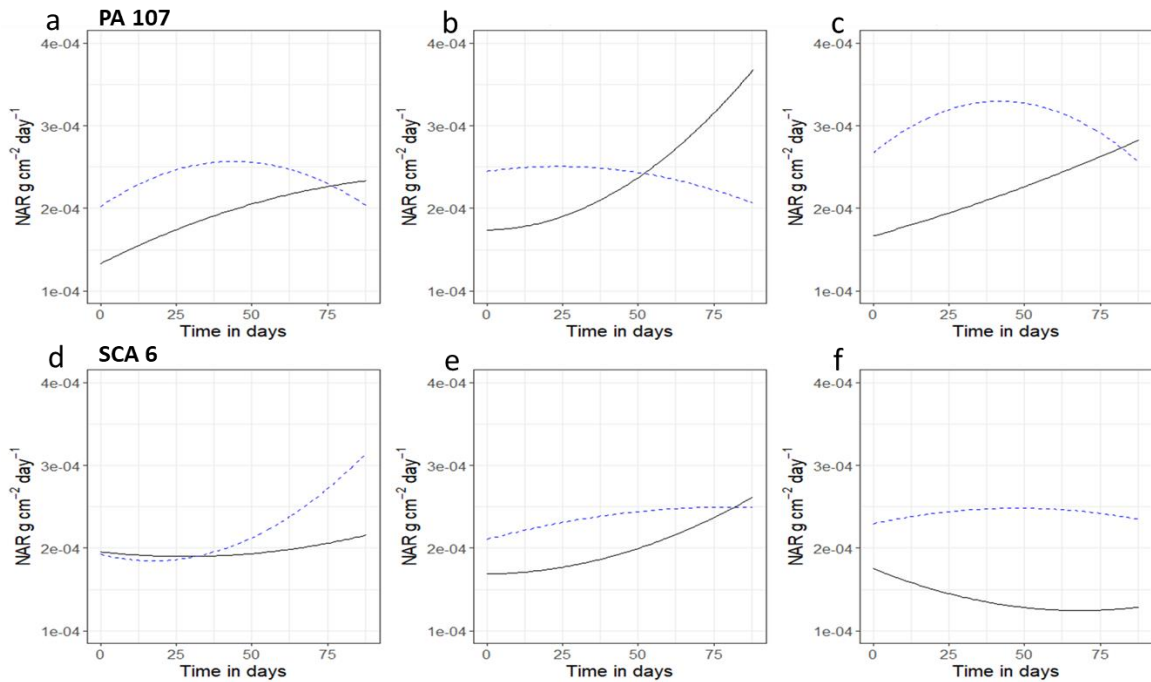
Figure 3.23 Progress curves of net assimilation rate from equations 3.4 and 3.5 for PA 107 and SCA 6 juvenile cacao plants grown under two [CO_2] and three temperature regimes. Temperature treatments are 31/22°C (a),(d), 33.5/24.5°C (b),(e) and 36/27°C (c),(f). [CO_2] treatments are ambient (solid line) and elevated (dashed line).

3.3.5   Leaf carbon and nitrogen concentration

Leaf carbon concentration. Effects of the treatment combinations on leaf carbon concentration for the genotypes PA 107 and SCA 6 are shown in Figure 3.24. There was a significant interaction between time and genotype ($P < 0.001$) for leaf carbon concentration, which was slightly higher for PA 107 than SCA 6 (46.81 ± 0.16 and 46.18 ± 0.17 % respectively) at 34 days, whereas SCA 6 had higher leaf carbon concentration (47.26 ± 0.07 %) than PA 107 (46.16 ± 0.14 %) at 88 days. Leaf carbon concentration did not differ statistically between genotypes at 50 and 65 days. Analysing the final point at 88 days, there was also a genotype x temperature interaction ($P < 0.01$) for leaf carbon concentration. Leaf carbon concentration decreased with an increase in temperature in PA 107 being significantly higher at 31/22°C (46.62 (±0.31) %) than at 36/27° (45.76 (±0.19) %). Leaf carbon did not differ statistically at 33.5/24.5°C (46.6 (±0.21 %) compared to 31/22°C and 36/27°C. Conversely, leaf carbon did no show significant differences across the temperature regimes for SCA 6 at final harvest. There was no effects of [CO_2] on leaf carbon concentration at final harvest.
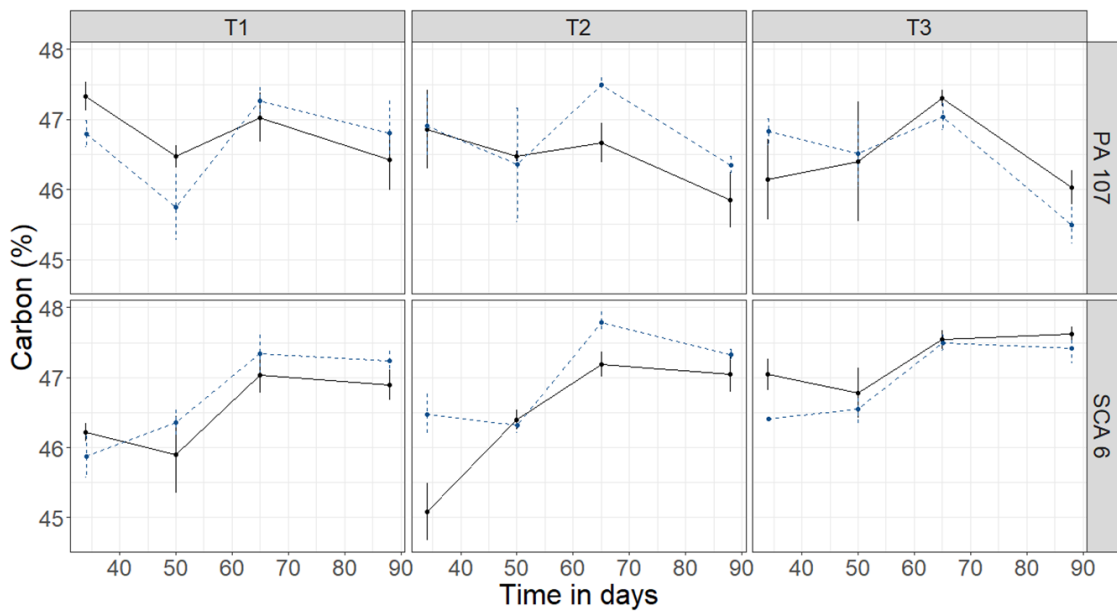
Figure 3.24 Effect of [CO_2] and temperature on leaf carbon concentration of two juvenile cacao genotypes at 34, 50, 65 and 88 days. Error bars show the standard error of the mean (n=3 at 0, 34 and 50 days; n=6 at 65 and 88 days). [CO_2] treatments are ambient (solid line) and elevated (dashed line). Temperature treatments are 31/22°C (T1), 36/27°C (T2) and 38.5/29.5°C (T3).

Leaf nitrogen concentration. Figure 3.25 shows the effects of the treatment combinations on leaf nitrogen concentration for the genotypes PA 107 and SCA 6. A significant interaction between time and genotype was observed ($P<0.001$). PA 107 had a higher leaf nitrogen concentration compared to SCA 6 at 50 and 88 days (2.77 (±0.07) and 2.53 (±0.04) % at 50 days; 2.57 (±0.04) and 2.27 (±0.05) % at 88 days) while there were no statistical differences at 34 and 65 days. Examining the final point at 88 days, there was a significant interaction among genotype, temperature and [CO_2] on leaf nitrogen concentration ($P<0.05$). For PA 107, leaf nitrogen concentration was significantly higher at 31/22°C (2.67 (±0.04) %) compared to 33.5/24.5°C (2.44 (±0.07) %) whereas there were no significant differences at 36/27°C compared to 31/22°C and 33.5/24.5°C. In contrast, for SCA 6 differences in leaf nitrogen concentration across temperature depended on the [CO_2] such that at 400 ppm there was a significant decrease from 31/22°C (2.74 (±0.01) %) to 33.5/24.5°C (2.08 (±0.07) %) from which it increased at 36/27°C (2.42 (±0.04) %). At 700 ppm, there were no differences in leaf nitrogen concentration of SCA 6 at 31/22°C and 36/27°C (2.18 (±0.06) and 2.17 (±0.09) % respectively) whereas a significant decrease was observed at 33.5/24.5°C (2.02 (±0.03) %).
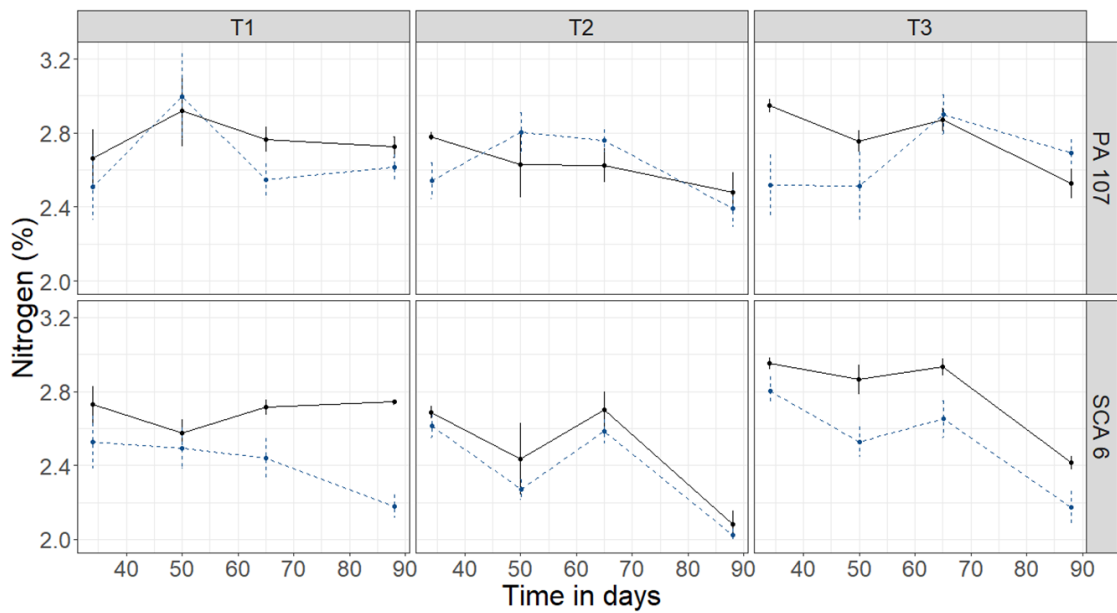
Figure 3.25 Effect of [CO_2] and temperature on leaf nitrogen concentration of two juvenile cacao genotypes at 34, 50, 65 and 88 days. Error bars show the standard error of the mean (n=3 at 0, 34 and 50 days; n=6 at 65 and 88 days). [CO_2] treatments are ambient (solid line) and elevated (dashed line). Temperature treatments are 31/22°C (T1), 36/27°C (T2) and 38.5/29.5°C (T3).

3.4   Discussion

This study aimed to determine how elevated temperature and [CO_2] affects the growth and physiology of two juvenile cacao genotypes grown under controlled environment conditions. The main findings were that: (i)   differences between the two genotypes in response to temperature and [CO_2] were observed; (ii) increasing temperature within the range of 31/22°C to 36/27°C improved photosynthesis parameters in both genotypes but the impact on growth varied between genotypes; (iii) photosynthesis and growth was increased by [CO_2] elevation; and (iv) elevated [CO_2] enhanced the positive effect of temperature increase in PA 107 and helped to ameliorate the negative effects of warming in SCA 6.

A general increase in plant height due to increases in temperature and elevated [CO_2] was observed in both genotypes. The positive effect of an increase in [CO_2] on height of juvenile cacao plants has also been reported in short-term experiments (Baligar *et al.*, 2005, 2021a, 2021b). Sena Gomes and Kozlowski (1987) also observed increases in plant height when temperatures increased up to 30.5°C in 55 days old cacao seedlings in growth chambers. However, Hebbar *et al.* (2020) observed no significant changes in the height of young cacao plants grown in open-top chambers at an average temperature of 3°C above the control day/night temperature of ~32.3/23.9°C. The present study also showed that the positive effect of increasing temperature up to 36/27°C was more evident in plants grown at elevated [CO_2] (700 ppm). A similar observation has been noted in other tree species. The greatest increase in

growth was also observed when *Cedrela odorata* and *Gliricidia sepium* seedlings were exposed to a combination of increased temperature and elevated [CO_2] (Esmail and Oelbermann, 2011). In contrast, increases in temperature caused an increase in stem diameter for PA 107 but this declined for SCA 6. At the early stages of growth in cacao, vigour estimated as a function of the stem growth has been identified as a trait for tolerant genotypes to water deficit and no-shade conditions (Ofori *et al.*, 2014). Here, the increases in temperature resulted in tall plants with a bigger diameter for PA 107 compared to SCA 6 which suggests that some genotypes might establish better under conditions of warming scenarios. A positive effect of an increase in temperature on stem growth was also reported by Sena Gomes and Kozlowski (1987) in cacao seedlings grown from 18.7 to 33.3°C for 60 days in controlled environment growth cabinets. Elevated [CO_2] increased the average stem diameter in young cacao plants by 9% and compensated for a negative effect of increased temperature for SCA 6. Although genotypic variation in stem growth of cacao plants in response to temperature has been observed in previous studies (Daymond and Hadley, 2004), here the elevated [CO_2] appeared to alleviate the effect of the more susceptible genotype to warmer temperatures.

Reductions in final leaf length were observed as temperatures increased in SCA 6, but this response was less evident in PA 107, whereas an inconsistent effect of elevated [CO_2] was observed in both genotypes. It has been suggested that plants at elevated temperature tend to produce small leaves in order to offset the leaf water loss due to the transpiration (Qaderi *et al.*, 2006) or as a thermoregulatory adaptive trait under elevated temperatures (Tserej and Feeley, 2021). This study showed that for cacao there appears to be genetic variation in the magnitude of high temperature induced leaf length reduction, as leaf length declined in SCA 6 but remained relatively stable in PA 107. Despite higher temperature leading to the production of smaller leaves, leaf growth rate was higher with leaves reaching their final length more quickly under warmer conditions.

Overall, large flushes were also observed in this study, which resulted in large number of leaves per flush. Elevated [CO_2] did not affect flushing interval nor the number of leaves per flush in both genotypes. Similarly, Lahive *et al.* (2018) did not observe changes in flushing in four-month-old Amelonado cacao seedlings grown under glasshouse conditions at elevated [CO_2], however, a greater number of leaves per flush was reported. An effect of temperature was observed on leaf production. Flushing interval decreased by 15 and 20% with temperature increases of 2.5 and 5.0°C, respectively compared with the control, while number of leaves per flush increased on average by 22% and 20% across the temperature regimes studied. Previous reports have shown a reduced interval of vegetative flushing with elevated temperature for some tropical fruits (Menzel and Simpson, 1988; Utsunomiya, 1992) and cacao grown in controlled environment growth chambers (Sale, 1968) and under field conditions (De Almeida and Valle, 2007). Although Sale (1968) reported that the highest leaf number per

flush occurred in plants grown at the lowest temperature of 23.3°C (compared to 26.7°C, or 30.0°C), a positive effect of an increase in temperature on leaf initiation was observed in cacao *Comum* seedlings during the first 30 days in plants grown at 33.3°C compared to those grown at 18.7°C (Sena Gomes and Kozlowski, 1987).

Stomatal density varied between the two genotypes and increased from the control temperature to control + 2.5°C from which it decreased at control + 5.0°C. Increases in stomatal density have been explained as an adaptive mechanism to face the evaporative demand under warm environments (Jumrani *et al.*, 2017), while declines have resulted from morphological adjustments in order to prevent water loss at the highest temperatures (Caine *et al.*, 2019). Such a decline in stomatal density might also lead to decreases in leaf stomatal conductance and photosynthesis rates (Xu and Zhou, 2008). However, in this study declining stomatal density at elevated temperatures did correlate to the general increase in gas exchange parameters. Here, it is suggested that the humidity control across the temperature may have impacted this enhancement in the gas exchange. On the other hand, stomatal density decreased by an average of 9% in leaves grown at elevated [CO_2] irrespective of genotype, which is consistent with a survey conducted by Woodward and Kelly (1995) who showed that in many species there was a reduction of stomatal density under elevated [CO_2]. However, stomatal density responses to elevated [CO_2] in cacao have not shown a conclusive trend. Increases of about 9 % in stomata density was shown in leaves of young Amelonado cacao plants grown at elevated [CO_2] under glasshouse conditions while there were no overall changes observed in six mature cacao clones grown under similar conditions (Lahive *et al.*, 2018, 2021).

Net photosynthetic rate varied between the two genotypes, increased with temperature and with elevated [CO_2]. Daymond *et al.* (2011) reported that cacao exhibits significant genotypic variation in several photosynthetic traits, in particular light-saturated photosynthetic rate. In this study, photosynthesis continued to increase up to growth temperatures of 36/27°C, which suggests that supra-optimal temperatures for cacao were not experienced in this study. Earlier studies have reported an optimum temperature for cocoa ranging from 31-35°C, above which photosynthesis declines (Balasimha *et al.*, 1991). However, Hebbar *et al.* (2020) observed a significant decline in photosynthesis of cocoa trees grown in open-top chambers at an average temperature of 36°C (measured at noon). This decline in photosynthesis at elevated temperature has been explained as a protective mechanism of stomata closure to prevent water loss in response to rising vapour pressure deficit (VPD) which normally increases with temperature. Previously, studies in cocoa have shown that photosynthesis declined as VPD increased with temperature (Raja Harun and Hardwick, 1988; Hernandez *et al.*, 1989; Baligar *et al.*, 2008). In this study, VPD was kept constant across each temperature treatment (~0.9 kPa) in order to explore the direct effect of temperature. These results suggest that the previously reported optimum

temperature range for photosynthesis in cocoa are likely to have been underestimated most likely due to the compounding effect of VPD. More studies are required to understand the impact VPD has on photosynthetic functioning in cocoa. Irrespective of temperature, photosynthesis considerably improved in plants grown at elevated $[CO_2]$ compared to those grown at ambient (68% increase on average). Increases in photosynthesis of 10 to 56% in response to elevated $[CO_2]$ were also reported in young cacao seedlings grown under different controlled-environment conditions (Lahive *et al.*, 2018; Hebbar *et al.*, 2020; Baligar *et al.*, 2021a). Increase in photosynthesis rate is expected in C_3 plants when exposed to elevated $[CO_2]$ (Drake *et al.*, 1997; Ainsworth and Long, 2005). This is explained due to increases in ribulose-1, 5-bisphosphate (RuBP) carboxylase/oxygenase (Rubisco) activity. This enzyme catalyses the carboxylation of RuBP for CO_2 fixation, but also may use O_2 substrate for photorespiration (Makino and Mae, 1999). The carboxylation process of RuBP is not saturated under current $[CO_2]$, consequently, increases in CO_2 may lead to increases in rate of carboxylation (Drake *et al.*, 1997).

Stomatal conductance and leaf transpiration increased linearly with temperature which was consistent with the increases in photosynthesis rates observed. Similarly, previous studies have shown that leaf transpiration increased with temperature increases up to 30°C (Raja Harun and Hardwick, 1988) and 33.3°C (Sena Gomes and Kozlowski, 1987) in young cocoa plants grown under controlled environment conditions. However, studies have shown decreases in cacao stomatal conductance with temperatures above 23.5°C. These studies have reported the stomatal responses to temperature elevation as a VPD function which may explain why stomatal conductance decreased with increases of both temperature and VPD in young cacao plants grown under controlled conditions (Sena Gomes and Kozlowski, 1987; Baligar *et al.*, 2008), in the field (Hernandez *et al.*, 1989), and in glasshouses (Raja Harun and Hardwick, 1988). Here, when VPD was kept constant and non-limiting no negative impact of temperature increases up to 36°C was observed. On the other hand, no effect of elevated $[CO_2]$ on stomatal conductance and transpiration were observed in this study. Differential responses in stomatal conductance to elevated $[CO_2]$ have previously been reported ranging from no changes in cacao seedlings growing under glasshouse conditions (Lahive *et al.*, 2018) to significant increases in young cacao plants grown in controlled environment growth chambers (Baligar *et al.*, 2008), glasshouses (Baligar *et al.*, 2021a) and open-top chambers (Hebbar *et al.*, 2020). Regarding transpiration, previous studies have shown different responses to the elevated $[CO_2]$ with significant decreases at elevated $[CO_2]$ (Baligar *et al.*, 2008, 2021a) or no changes (Lahive *et al.*, 2018; Hebbar *et al.*, 2020). Here, it was shown that under constant VPD, stomatal conductance and consequently photosynthesis rate and transpiration continued to increase with temperature increases up to 36°C.

In this study, there were differences between the two genotypes in their intrinsic water use efficiency (iWUE) defined as the ratio between leaf photosynthesis rate and stomatal conductance. In contrast,

working with a different set of cacao accessions, Balasimha *et al.* (1991) did not report genotypic differences in WUE (based on the ratio of photosynthesis to leaf transpiration). The same authors observed a reduction in WUE during the dry months when temperatures and VPD were higher. Later, Hebbar *et al.* (2020) observed a decrease of 30% in WUE (based on the whole plant) in trees grown at an average temperature of 3°C above the control day/night temperature of ~32.3/23.9°C in a single cacao genotype grown in open-top chambers. Here, an overall decline in iWUE of 14 and 21% was observed when temperatures increased by 2.5°C and 5.0°C, respectively, compared with the control. Although both photosynthesis rate and stomatal conductance increased across the temperatures, the stomatal conductance increase by a greater magnitude compared with photosynthesis rate, resulting in a decline in iWUE reflecting the potential water loss under warming conditions. Furthermore, iWUE increased by 86% in plants grown at elevated [CO_2]. Previous observations also reported a significant increase in the WUE of young cacao plants grown at elevated [CO_2] (Lahive *et al.*, 2018; Hebbar *et al.*, 2020; Baligar *et al.*, 2021a). Increases in iWUE at elevated [CO_2] in this study could be attributed to the increases of photosynthesis since there were no significant changes in stomatal conductance. In previous studies, the improvement of WUE at elevated [CO_2] in young cacao plants has been explained as increases in photosynthesis combined with either a decrease (Hebbar *et al.*, 2020; Baligar *et al.*, 2021a) or no change (Lahive *et al.*, 2018) in stomatal conductance and transpiration.

Chlorophyll fluorescence parameters (F_v/F_m and PI) and chlorophyll content differed between genotypes whereas no significant effects of elevated temperature and [CO_2] were observed. F_v/F_m, PI and chlorophyll content were higher in PA 107 than SCA 6. Genetic differences in chlorophyll content agreed with previously observations in cacao (Daymond and Hadley, 2004). On the other hand, a recent study in open-top chambers noted no change in F_v/F_m and decreases of 69% in chlorophyll content in a single genotype of cacao seedlings subjected to an average temperature of 36°C (Hebbar *et al.*, 2020). Irrespective of the temperature, no significant effects in F_v/F_m parameters (Hebbar *et al.*, 2020) and chlorophyll content (Baligar *et al.*, 2021a) have been reported in young cacao plants grown at elevated [CO_2]. In this study, the use of chlorophyll fluorescence parameters to monitor physiological status (Baker and Rosenqvist, 2004; Kalaji *et al.*, 2016) did not indicate stress either to temperature increase or elevated [CO_2], suggesting no changes in the photochemistry efficiency had occurred which was evident through the enhancement of photosynthesis in response to the imposed treatments. Additionally, the higher chlorophyll content observed in PA 107 may have been associated with the higher photosynthesis rates compared to SCA 6.

Independent effects of temperature and [CO_2] on dry weight and leaf area have been reported in young cacao plants, with significant reductions when temperature increases (Sale, 1968; Sena Gomes and Kozlowski, 1987; Hebbar *et al.*, 2020), and significant enhancements with increasing [CO_2] (Baligar *et al.*,

2005, 2021a, 2021b; Lahive *et al.*, 2018; Hebbar *et al.*, 2020). Under non-limiting water and nutrient conditions, elevated [CO_2] and temperature may stimulate plant growth and carbon assimilation compared to those grown at ambient [CO_2] (Ghannoum *et al.*, 2010; Wertin *et al.*, 2012b). In this study, under conditions of no limitation in water and nutrients, changes in dry weight and leaf area largely reflected the changes in leaf photosynthesis rates. However, the combined effect of elevated [CO_2] and temperature on dry weight and leaf area appeared to be genotype dependent. While biomass and leaf area increased with temperature and at elevated [CO_2] in PA 107, in SCA 6 the negative effect of the highest temperature at ambient [CO_2] was compensated for at elevated [CO_2]. A similar compensatory effect of [CO_2] to elevated temperature was also noted by Hebbar *et al.* (2020). These results suggest that elevated [CO_2] may ameliorate the negative impact of high temperatures in some genotypes, and stimulate growth in others.

RGR decreased slightly with temperature increases of 2.5°C and 5.0°C from the control with differential effects of [CO_2] between genotypes. Working with cocoa seedlings in controlled environment growth cabinets and testing a broad range of temperatures, Sena Gomes and Kozlowski (1987) observed increases of RGR up to 27.2°C from which RGR progressively decreased. Here it has been shown that, irrespective of temperature, RGR is higher at elevated [CO_2] although the extent varied between genotypes. RGR remained higher in SCA 6 across the experimental period while in PA 107 RGR started higher at the beginning and then fell rapidly and was lower than the ambient [CO_2] treatment by the end of the experimental period. A general increase in RGR in a range of genotypes of cacao plants grown at elevated [CO_2] has been reported in the literature (Baligar *et al.*, 2005, 2021a, 2021b). Relatively small effects of [CO_2] on RGR have been reported in other crops and the response is time dependent (Centritto *et al.*, 1999) taking place at the early stage of the plant growth with a particular stimulation where seedlings are raised at ambient levels of [CO_2] and the transferred to high [CO_2] (Poorter and Navas, 2003). Normally, RGR decreases with increases in plant size (Bush and Evans, 1988). However, irrespective of the temperature, there was a particular response of PA 107 to elevated [CO_2] at the beginning of the experiment with a rapid growth followed by the rapid decrease in relative growth rate (Figure 3.21) at the end of the experimental period. This may suggest a differential genotypic response to the short-term [CO_2] enrichment stimulus.

LAR decreased during the experimental period and slight differences between the [CO_2] treatments were observed at control and control+2.5°C temperature treatments. LAR reflects the efficiency of the production of leaf area per unit dry mass gained. Reductions in LAR at elevated [CO_2] have previously been reported in cacao plants (Baligar *et al.*, 2005, 2021a) grown at 28/25°C and 30/28°C, respectively. This reduction in LAR under [CO_2] enrichment was also noted in C_3 annual herbaceous plants (Bunce, 2001). In this study, differences in LAR between [CO_2] were more noticeable at control+5.0°C treatment

and in PA 107 rather than SCA 6. The LAR increase at ambient $[CO_2]$ may be explained by the negative effect of the temperature on plant biomass and leaf area which was more evident in SCA 6.

Increases in dry weight in response to elevated $[CO_2]$ cause a positive response of NAR in annual crops such as rice (Roy *et al.*, 2012), shrub species (Zheng *et al.*, 2010) and trees (Saxe *et al.*, 1998; Norby *et al.*, 1999). This positive effect was also reported in young cacao plants (Baligar *et al.*, 2005, 2021a, 2021b). Here, the response of NAR expressed as the RGR-LAR ratio to elevated $[CO_2]$ and temperature differed between the two genotypes. At elevated $[CO_2]$ and across the temperature treatments, NAR decreased in PA 107 but remained higher in SCA 6. In PA 107, although LAR decreased with biomass accumulation at elevated $[CO_2]$, the magnitude of RGR decline was greater from the beginning to the end of the experimental period. Conversely, the similar decrease in LAR and RGR in SCA 6 resulted in a more stable NAR suggesting a compensatory effect of the elevated $[CO_2]$ for genotypes that are more susceptible genotypes to warm environments. Similarly, in a study with 16 plant species including trees subjected to temperature increases from 18 to 28°C decreases at ambient $[CO_2]$ in growth parameters such as RGR and NAR (Loveys *et al.*, 2002) were observed.

Leaf carbon and nitrogen concentration differed between the two genotypes. Similarly, genotypic differences in leaf nitrogen content were also noted among a set of eight cacao clones grown under greenhouses conditions (Daymond *et al.*, 2011). Here, no evident changes in leaf carbon concentration along the temperature regimes and $[CO_2]$ were observed while irrespective of genotype the leaf nitrogen concentration decreased slightly in plants grown at elevated $[CO_2]$. Plants grown at elevated $[CO_2]$ generally show reduced tissue nitrogen concentrations compared to ambient $[CO_2]$ grown plants (Coleman *et al.*, 1993). This reduction has been explained due to the dilution effect of accumulated non-structural carbohydrates from stimulated photosynthesis rate at elevated $[CO_2]$ (Ainsworth and Long, 2005; Sun *et al.*, 2012). The results in this study are consistent with Lahive *et al.*, (2018) who also reported that leaf carbon content was not affected by elevated $[CO_2]$ while leaf nitrogen content decreased significantly in young cacao plants grown under glasshouses conditions. In combination both $[CO_2]$ and temperature caused a decline in the plant tissue nitrogen content being more sensitive in woody species than herbs (Jeong *et al.*, 2018). Here, at ambient $[CO_2]$ leaf nitrogen concentration varied between temperature regimes while in plants grown at elevated $[CO_2]$ no effect of temperature was observed.

To conclude, given that photosynthesis and consequently dry biomass did not decrease with an increase in temperature as much expected, suggests that under non-limiting VPD, the optimum temperature appears to be higher than previously reported in cocoa plants. This research has also provided evidence of how young cacao plants exhibit genotypic variation in their growth and physiological response to

warmer environments and elevated $[CO_2]$. Here it has been suggested, under non-limitation of water and nutrients that the positive effect of elevated $[CO_2]$ may increase in warm environments for high-temperature tolerant cacao genotypes while elevated $[CO_2]$ could compensate the negative effects of increases of 5°C above the average current temperatures in cacao on susceptible genotypes.

4   The impacts of a broader range of temperature, [CO$_2$] and water deficit on growth and physiology of juvenile cacao plants (*Theobroma cacao* L.)

4.1   Introduction

Major features of global climate change, such as rising atmospheric CO$_2$ concentration ([CO$_2$]), temperatures, as well as altered precipitation patterns have an impact on crops. Studies based on climate metrics have suggested that future scenarios might impact on the suitability for cacao production in the main production region of West Africa (Läderach *et al.*, 2013; Schroth *et al.*, 2016). However, new high-resolution climate model simulations based on the combination of plant physiological data and climate information could provide insights into cacao productivity in the future (Black *et al.*, 2021).

In Chapter 3, under elevated [CO$_2$], growth and photosynthesis of two juvenile cacao genotypes were stimulated over the short-term. The result was consistent with most C$_3$ species (Ainsworth *et al.*, 2002; Poorter and Navas, 2003) and cacao seedlings (Baligar *et al.*, 2005; Lahive *et al.*, 2018). Growth and development in cacao are also affected by temperature which is one of the main factors that limit its cultivation (Daymond and Hadley, 2004; De Almeida and Valle, 2007). Although cacao exhibits genotypic variation in several physiology traits (Daymond *et al.*, 2011), previous studies have shown overall increases in photosynthetic rates from sub-optimal temperatures up to an optimal temperature of between 31-33°C (Balasimha *et al.*, 1991). However, the decline in photosynthesis at supra-optimal temperatures has been attributed to the indirect effect to vapour pressure deficit (VPD) on stomatal conductance (Raja Harun and Hardwick, 1988; Balasimha *et al.*, 1991; Baligar *et al.*, 2008). In Chapter 3, when VPD was maintained at a non-limiting level, photosynthesis increased with increases in temperature up 36/27°C (max/min) although the impact on plant growth varied between genotypes. Based on this finding, there is a need to examine a broader range of temperatures to determine from which point the temperatures become supra-optimal independent of the confounding effect of VPD.

Among the most common environmental stresses that threaten crop production is drought, which negatively impacts photosynthetic processes, plant growth, hydraulic function and metabolism (Pflug *et al.*, 2018). It has been shown that plant physiology and development of cacao are affected by water deficit (De Almeida and Valle, 2007). Leaf water potential declined in response to drought (Balasimha *et al.*, 1991; Ávila-Lovera *et al.*, 2016) which reduces stomatal conductance and photosynthetic rate in

young cacao plants (Deng *et al.*, 1990; Mohd Razi *et al.*, 1992). As a result, growth and development are reduced, although the extent of this may vary among genotypes (Dos Santos *et al.*, 2018).

In the field, cacao plants are exposed to several climatic factors simultaneously. However, research examining such interactions is still scarce since such factorial studies are complex and expensive to establish under field conditions. To date, using controlled facilities, a few studies have addressed these interactive factors in cacao physiology and plant growth (Lahive *et al.*, 2018; Hebbar *et al.*, 2020). In other crops, studies have shown that water deficit may be alleviated by elevated [CO_2] by increasing water use efficiency through the maintenance of photosynthesis rates despite the reduction in stomatal conductance (Wullschleger *et al.*, 2002; Robredo *et al.*, 2007). Similarly, elevated CO_2 moderately mitigated the negative effects of water deficit on photosynthesis and plant growth of young cacao Amelonado plants grown under controlled glasshouses (Lahive *et al.*, 2018, 2021).

Effects of elevated [CO_2] on plant photosynthesis and growth can be counteracted by other climatic factors such as high temperature. Therefore, these factors must be considered together to create a better understanding of how future climate scenarios may impact plant physiology (Norby and Luo, 2004). For instance, in response to both elevated [CO_2] and temperature, grapevine showed increased photosynthesis which accelerated grape development (Kizildeniz *et al.*, 2021) and reductions of photosynthesis at supra-optimal temperatures were attenuated by CO_2 enrichment in coffee (Martins *et al.*, 2016; Rodrigues *et al.*, 2016). It has also been reported that the thermal optimum of photosynthesis is increased by high CO_2 concentrations (Sage and Kubien, 2007) and photosynthesis of C_3 plants is more tolerant to high temperatures when they are exposed to elevated [CO_2] (Taub *et al.*, 2000; Wang *et al.*, 2008). In cacao, Hebbar *et al.* (2020) working with seedlings, reported that the negative effect of warming conditions on photosynthesis rate, leaf water potential, and biomass accumulation, was attenuated in plants subjected to elevated [CO_2]. However, in Chapter 3 an additive effect of both elevated [CO_2] and increased temperature from 31/22°C to 36/27°C was observed on photosynthesis and plant growth, as well as genotypic variation in responses. Increases in temperature and water deficit are two of the most significant abiotic stresses in agricultural production that can occur in the field simultaneously (Mittler, 2006). Their negative interactive effect has been noticeable in annual crops (Cohen *et al.*, 2021), perennial grass (Xu and Zhou, 2006) and woody plants (Qaderi *et al.*, 2019). Under open top chambers (OTC), more severe effects of the combined elevated temperature and water deficit were observed on photosynthesis and biomass production of young cacao seedlings (Hebbar *et al.*, 2020). However, the magnitude of this exacerbation has not been examined across a wide range of temperatures and humidity.

Overall, elevated [CO_2] has usually been observed to mitigate water deficit stress on plant physiological traits, but high temperature has often been reported to exacerbate drought stress. Nevertheless, the interaction between elevated [CO_2] and temperature under water deficit may change based on the compensatory effect between [CO_2] and temperature, and how the three factors interact (Duan *et al.*, 2013). A recent study of cocoa net primary productivity (NPP) based on a land-surface model, predicted that high projected [CO_2] would ameliorate the impact of elevated temperature and rainfall variations in the West African region (Black *et al.*, 2021). However from a physiological perspective, such interactions in cocoa have not yet been sufficiently examined. Basic information on how different plants respond to climate change scenarios can be performed on seedlings under climate-controlled environments (Ghannoum *et al.*, 2010). Therefore, this study aimed to investigate whether elevated [CO_2] and a broader range of temperatures would modify the growth and physiology of juvenile cacao plants subjected to drought under controlled environment conditions (growth cabinets). More specifically, the hypotheses were: (i) elevated [CO_2] would ameliorate drought stress by increasing photosynthesis and growth; (ii) elevated temperature would exacerbate drought stress by increasing leaf respiration and decreasing growth; and (iii) elevated [CO_2] would ameliorate stress and high temperatures by increasing photosynthesis and growth and shifting optimal temperature in cacao; and.

4.2   Materials and Methods

4.2.1   Plant material and experimental setup

Mature pods of cacao genotype (T 63/971 x T 60/887) were received from the Seed Production Division (SPD) of the Cocoa Research Institute of Ghana (CRIG) on 28-11-18. This cross has been selected by CRIG on the basis of yield performance, for farmers in Ghana. The seeds were immediately sown into 5L pots containing a mixture of sand, gravel and vermiculite (1:2:2 vol:vol:vol) and plants were then maintained in a glasshouse specifically designed for climate change research on cacao at the Crops and Environment Laboratory (CEL) at The University of Reading, UK from 4-12-18 to 06-03-19. The glasshouse was set to provide a day/night temperature of 32/19°C and a minimum daytime light intensity of 148 µmol m^{-2} s^{-1} photosynthetically active radiation (PAR). Supplementary lighting was used to increase the day length to 12 hr (from 06:00 to 18:00) and a 50% shade screen closed when PAR was greater than 648 µmol m^{-2} s^{-1}. The seedlings were watered 6 times daily with a modified Long Ashton solution adjusted for cacao (End, 1990), which was supplied at a pH of 5.7 and an electrical conductivity (EC) of 2.0 mS.cm^{-1}.

On 07-03-19, eight cacao seedlings were transferred into each of twelve controlled environment growth cabinets (model HGC 1514; Weiss Gallenkamp, UK). The plants were irrigated with the nutrient solution as described above through an automatic drip irrigation system which was set to water four times per day (6:00, 11:00, 15:00 and 18:00 hr) for 5 min at each watering time. Within each cabinet, a 12-hr

photoperiod was maintained, with a light intensity at canopy level between 450 and 550 µmol m^{-2}s^{-1} (PAR). During the experimental period, plants were rotated at random within each growth cabinet twice per week to reduce potential position effects within the cabinets.

A specific combination of [CO_2] and temperature was maintained in each growth cabinet and a well-watered (WW), and water deficit (WS, hereafter referred to as 'water-stress') regime was randomly assigned to plants (n = 4 per temperature x [CO_2] treatment) (Figure 4.1). The [CO_2] treatments comprised ambient [CO_2] (a target of 400 ppm) and elevated [CO_2] (a target of 700 ppm). Six different temperature regimes were implemented, each following a daily sine wave profile with a maximum temperature at 14:00 h and minimum at 05:00 h. The maximum/ minimum values in each temperature treatment were set to: 28.5/19.5°C (T1), 31/22°C (T2), 33.5/24.5°C (T3), 36/27°C (T4), 38.5/29.5°C (T5) and 40/31°C (T6). T2 was the control regime which was designed to mimic the average temperature in the cacao-growing region of Ghana (data obtained from the Ghana Meteorological Service). The relative humidity within each cabinet was controlled and varied depending on the temperature treatment in order to maintain a constant VPD of 0.9 kPa, so as not to be limiting to photosynthesis (Balasimha *et al.*, 1991) and minimise the confounding effect of increasing VPD with temperature. The daily average temperature, [CO_2] (including day and night values) and relative humidity recorded within each treatment combination during the experimental period is summarised in Table 4.1

The pots were manually watered to field capacity (defined as the amount of water held in the substrate after gravitational water has drained away) the day before the start of the experiment and allowed to drain overnight. To reduce water loss from the substrate surface, all the pots were covered with black rubber matting. To monitor evaporative water loss, an additional pot containing the same substrate without a cacao seedling, and also covered with rubber matting was placed in each cabinet.

Cab	[CO$_2$]	Temperature (°C)
24	elevated	28.5/19.5
25	ambient	28.5/19.5
26	elevated	31/22
27	ambient	31/22
28	elevated	33.5/24.5
29	ambient	33.5/24.5
30	elevated	36/27
31	ambient	36/27
32	elevated	38.5/29.5
33	ambient	38.5/29.5
34	elevated	40/31
35	ambient	40/31

Cabinet arrangement:

28 WS / WW	29 WW / WS	36 WS / WW
27 WW / WS	30 WS / WW	35 WW / WS
25 WS / WW	31 WW / WS	34 WS / WW
24 WW / WS	32 WS / WW	33 WW / WS

WS / WW pot arrangement (green dots are cacao seedlings, grey dot is control pot)

Figure 4.1 Arrangement of climatic treatments ([CO$_2$] x Temperature) and water (WW – well watered; WS – water stress) across 12 controlled environment growth cabinets used for the experiment, located at Crops and Environment Laboratory, School of Agriculture, Policy and Development, University of Reading. Each box represents a growth cabinet. Green dots are cacao seedlings and grey dot, control pot.

Table 4.1 Climatic cabinet conditions during the experimental period (mean over 81 days). T1 a[CO_2]= 28.5/19.5°C and ambient [CO_2], T1 e[CO_2]= 28.5/19.5°C and elevated [CO_2], T2 a[CO_2]= 31/22°C and ambient [CO_2], T2 e[CO_2]= 31/22°C and elevated [CO_2], T3 a[CO_2]= 33.5/24.5°C and ambient [CO_2], T3 e[CO_2]= 33.5/24.5°C and elevated [CO_2], T4 a[CO_2]= 36/27°C and ambient [CO_2], T4 e[CO_2]= 36/27°C and elevated [CO_2], T5 a[CO_2]= 38.5/29.5°C and ambient [CO_2], T5 e[CO_2]= 38.5/29.5°C and elevated [CO_2], T6 a[CO_2]= 40/31°C and ambient [CO_2], T6 e[CO_2]= 40/31°C and elevated [CO_2] .

	Mean daily temperature (°C)	[CO_2] (ppm)	Relative Humidity (%)
T1 e[CO_2]	23.8	698.6	67.5
T1 a[CO_2]	23.7	451.4	65.7
T2 e[CO_2]	26.3	701.2	71.2
T2 a[CO_2]	26.3	470.6	72.2
T3 e[CO_2]	28.8	699.8	76.4
T3 a[CO_2]	28.8	453.8	76.2
T4 e[CO_2]	31.2	698.9	78.5
T4 a[CO_2]	31.1	433.1	77.0
T5 e[CO_2]	33.2	701.0	78.2
T5 a[CO_2]	33.8	448.7	79.2
T6 e[CO_2]	35.3	697.9	83.0
T6 a[CO_2]	35.3	459.4	83.1

Plants were grown under the experimental conditions for 81 days (from 25-03-19 to 14-06-19). On the morning of 26-03-19, each pot was weighed to determine the weight at field capacity (FC) and soil moisture within each pot was measured using a ML2 *ThetaProbe* sensor (Delta T Devices, Cambridge, UK). The average soil moisture at FC was 11 %. Theoretically, the FC in sandy soils is around 10 % and the wilting point is around 3 % (Brandt *et al.*, 2017). Every three days throughout the experimental period, each pot was weighed to determine plant water use over that period (ml). The soil moisture (%, vol) of each pot was also recorded at the same time. For the well-watered treatment and the control pot, the total water use from each pot over the three days was replenished to return the soil to FC. To create a similar rate of soil moisture decline across all water-stress * temperature * CO_2 treatments, each of the water-stress treatment pots was weighed every three days and the plant with the lowest water use was determined. The difference between this minimum water-use value and the water-use in every other pot was calculated to determine the volume of water each pot received at each measurement time. This procedure was applied until the soil moisture within the pot reached between 3-4%. From this point, the average water-use across all the water-stressed plants, as determined

through weighing, was added to each pot to keep the soil moisture close to the wilting point (3%). Figure 4.2 shows the average soil moisture before each watering event and the cumulative volume of water added over the experimental period.
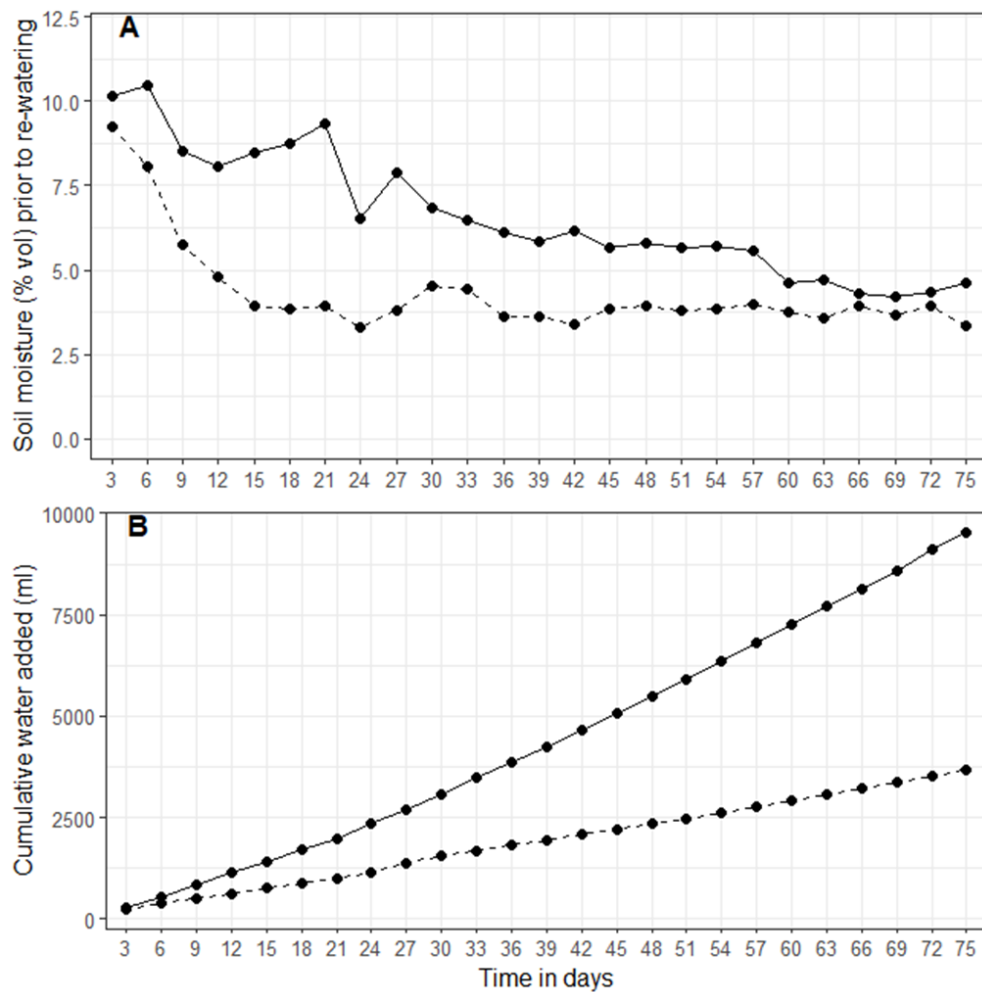
Figure 4.2 Average soil moisture, taken prior to re-watering (A) and cumulative water added per plant (B) across the experimental period. Well-watered (solid line) and water-stress (dashed line)

4.2.2 Photosynthetic measurements

Light-response curve parameters. Measurements of photosynthetic light-response curves were carried out on the youngest, fully expanded and hardened leaf from four plants per watering treatment in each cabinet using a portable infrared gas analyser (IRGA) fitted with a light attachment and an internal CO_2 source (*LC pro*-SD, *ADC BioScientific*, Great Amwell, Herts., UK). The measurements were conducted 72 days after imposing the treatments, between 21-05-19 and 30-05-19, between 09:00 and 14:00 hr each day. In plants grown in the ambient and elevated [CO_2] treatments at T2, T4 and T5 photosynthesis was measured at eight irradiances: 696, 435, 348, 261, 174, 87, 44 and 0 µmol m^{-2} s^{-1} PAR. The CO_2 concentration within the IRGA chamber was set to 400 or 700 ppm depending on the treatment of the

plants being measured. The temperature in the leaf chamber was set to the corresponding maximum temperature for each treatment. At the highest irradiance, photosynthetic rate was allowed to stabilise for 20 min before data was recorded. The irradiance level was then reduced using the IRGA automatic sequencing program and maintained at each irradiance for five minutes before data was recorded. Photosynthetic light-response curves were fitted to a non-rectangular hyperbola (Prioul and Chartier, 1977) in the form:

$$Pn= \{\emptyset\,Q + A_{max} - \sqrt{[(\emptyset\,Q + A_{max}) - 4\,\emptyset\,Q\,k\,A_{max}]/2\,k}\} - Rd \qquad\qquad 4.1$$

Where k is the convexity, $\emptyset$ apparent quantum yield, Q is irradiance, A_{max} is light-saturated gross photosynthetic rate (hereafter referred to as light-saturated photosynthesis rate) and Rd is apparent dark respiration. The photosynthetic parameters A_{max}, $\emptyset$, Rd, light compensation point, and light saturation point were estimated from the fitted curves. Fitting was carried out using the Microsoft Excel spreadsheet tool provided by Lobo *et al.* (2013).

Instantaneous parameters. On days 44 and 45 of the experiment instantaneous net photosynthesis measurements were made between 09:00 and 14:00 hr on the youngest fully expanded and hardened leaf from four plants per water treatment in each cabinet. The irradiance in the cuvette was set to 696 μmol m^{-2} s^{-1} PAR, CO_2 flux was set to the treatment [CO_2], i.e., 400 and 700 ppm for ambient and elevated CO_2 treatments respectively, and also the temperature to reflect the maximum temperature within each cabinet. The readings were recorded after 10 min of stabilization. Stomatal conductance (g_s), leaf transpiration (E) and leaf intercellular (Ci) to ambient (Ca) [CO_2] concentration ratio (Ci/Ca) were also recorded alongside the net photosynthetic rate (P_n). Intrinsic water use efficiency (iWUE) was calculated as P_n/g_s.

Fluorescence measurements were also carried out on the same leaves after they were dark-adapted for at least 30 min. Maximum quantum efficiency of photosystem II (measured as *Fv/Fm* ratio) and the performance index (*PI*) was measured following illumination at a wavelength of 650 nm at a maximum intensity of 3,500 μmol m^{-2} s^{-1} using a Handy PEA chlorophyll fluorimeter (*Hansatech Instruments Ltd*, Norfolk, UK).

Water relation parameters. After the instantaneous gas exchange measurements, the same leaves were dark adapted using aluminium foil. The next morning, stem water potential (Ψ_{stem}) was measured using a Scholander pressure chamber (SKPM 1405*, Skye Instruments Ltd*, Llandrindod Wells, UK) between 09:00 and 11:00 hr. Plant transpiration was measured gravimetrically from 26-03-19 to 09-06-19 as described in section 4.2.1. Cumulative plant transpiration was calculated as the difference between the total water lost per pot (ml) and the total water lost from control pot (ml). The plant transpiration

efficiency (TE) was calculated for each plant as the ratio of increase in dry biomass (final biomass – initial biomass) per cumulative unit of water transpired over the experimental period.

4.2.3   Plant growth measurements

Non-destructive observations. Plant height and stem diameter were measured on day 0 and 97 in four plants per water treatment in each growth cabinet and the increase calculated as (value D_0 –D_{97}). Height was measured from the surface of the substrate to the shoot apex using a measuring tape. Stem diameter was recorded at 5 cm above the substrate using a digital calliper.

Destructive observations. Six representative plants were destructively harvested at the beginning of the experiment (D_0) to determine initial biomass. Between 12-06-19 and 14-06-19 (Day_{97}), the remaining plants were harvested to determine final biomass. Seedlings were cut at the base of the stem and divided into stems, leaves and roots and fresh weight was recorded using a balance (KERN, model PCB 250-3, *KERN & SOHN*, Balingen, Germany). Number of leaves and leaf area (LA) of plants was measured. LA was measured using a WD3 WinDIAS leaf image analysis system (*Delta-T Devices Ltd,* Cambridge, UK). Stems, leaves and roots dry weights (DW) were recorded after samples were oven-dried at 70°C to a constant weight. Root-shoot ratio was calculated as the ratio of root dry weight (g) to aboveground dry weight (g). Complementary growth parameters were estimated as follows:

Leaf area ratio (LAR) = [LA/ TotalDW]   4.2

Where TotalDW, is the total dry biomass (stem + leaves + roots)

Relative growth rate (RGR) = (lnTotalDW$_0$-lnTotalDW$_{97}$)/ D_0 – D_{97} 4.3

Where D is time in days, subscripts 0 and 97 refer to initial and final destructive plant harvest

Net assimilation rate (NAR) = RGR/LAR   4.4

Specific leaf area (SLA) = [LA/ total leaf DW]   4.5

Leaf trait parameters. On 04-06-19, clear nail polish imprints were taken from the abaxial surface of the youngest fully expanded and hardened leaf from four plants per treatment combination. The imprints were observed using a Leitz Dialux 20 light microscope with a Leica DFC450 digital camera attached; and images obtained using *Leica Application suite* version 4.6.2 (*Leica Microsystems*, Wetzlar, Germany). Three random images from each imprint were taken and the number of stomata and epidermal cells in each image were counted using *ImageJ* version 2.2 analysis software (Rueden *et al.*, 2017) at 400 magnification (×40 objective and ×10 eyepiece). Stomatal density (SD) was calculated as the number of

stomata per unit area of each image. Stomatal index (SI) was calculated as: (stomatal number/ [stomata + epidermal cells]) *100 (Salisbury, 1927). On 20-04-19, chlorophyll content (ChlCont) was measured on the same leaves used for the gas exchange measurements using a CL-01 field-portable chlorophyll meter (*Hansatech Instruments Ltd.*, Norfolk, UK). The units were transformed to chlorophyll content (μg cm^{-2}) using the conversion provided by (Daymond *et al.*, 2011), ChlCont = (1.945 × chlorophyll meter reading) + 11.392.

4.2.4   Data analysis

A completely randomized split-plot experimental design with three factors was used, where the combination of [CO_2] and temperature (growth cabinets) were the main plots, and water treatments were the sub-plots. The main effects and their interactions were examined by analysis of variance (ANOVA), prior to checks of homogeneity of variances and normality of distributions for each variable under study, using the Levene and Shapiro–Wilk's tests respectively. When the ANOVA showed a significant treatment effect ($P < 0.05$), group means comparisons were performed using a Bonferroni *post hoc* test. All analyses and graphics were performed using R version 4.0.4 (R Core Team, 2021).

4.3   Results

4.3.1   Light- response curve parameters

Light-saturated photosynthetic rate. Light-saturated photosynthetic rate (A_{max}) was significantly lower under water deficit than under well-watered conditions by an average of 39% (P <0.001; Figure 4.3). The response to temperature varied at each [CO_2] (P <0.01 for temperature*[CO_2]). Under ambient [CO_2], there was a significant increase in A_{max} from 31/22°C to 36/27°C (2.36 (±0.21) and 3.87 (±0.15) μmol m^{-2} s^{-1} respectively), further increases in temperature to 38.5/29.5°C, resulted in a significant decline in A_{max} to 1.98 (±0.13) μmol m^{-2} s^{-1}. Although a similar trend was seen under elevated [CO_2], the differences were not significant. The response of A_{max} to [CO_2] also varied at each water regime (P <0.001 for [CO_2]*water treatment). In the well-watered treatment A_{max} was 59 % higher under elevated [CO_2] compared with ambient [CO_2], whereas under water-stress conditions, A_{max} was 15% higher at elevated [CO_2] compared to ambient [CO_2] treatment.
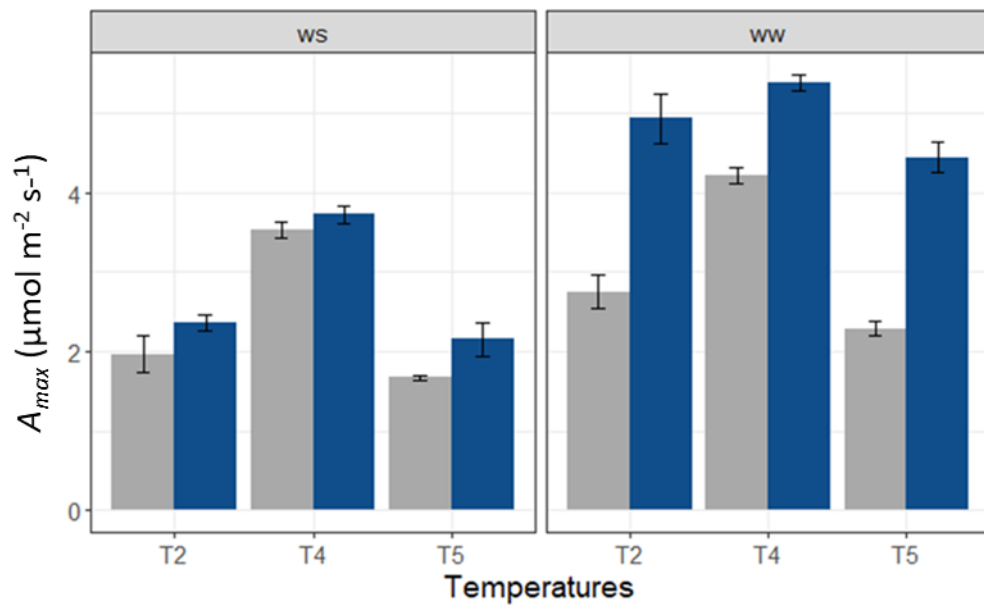
Figure 4.3 Light-saturated photosynthesis rate (A_{max}) of cocoa seedlings grown at ambient and elevated [CO_2], three temperatures and under well-watered (WW) and water-stress conditions (WS). Error bars show the standard error of the mean (n=4). [CO_2] treatments are ambient (grey bar) and elevated (blue bar). Temperature treatments are 31/22°C (T2), 36/27°C (T4) and 38.5/29.5°C (T5).

Quantum efficiency. Quantum efficiency (Ø) was 19 % lower under water deficit compared to the well-watered treatment (P <0.01; Figure 4.4). The response to temperature varied at each [CO_2] treatment (P <0.001 for temperature*[CO_2]). Under ambient [CO_2], there was a trend of a decrease in Ø with increasing temperature from 0.13 (±0.08) mol mol^{-1} at 31/22°C to 0.07 (±0.08) mol mol^{-1} at 38.5/29.5°C, whereas in plants grown under elevated [CO_2], Ø did not differ across the temperature regimes.
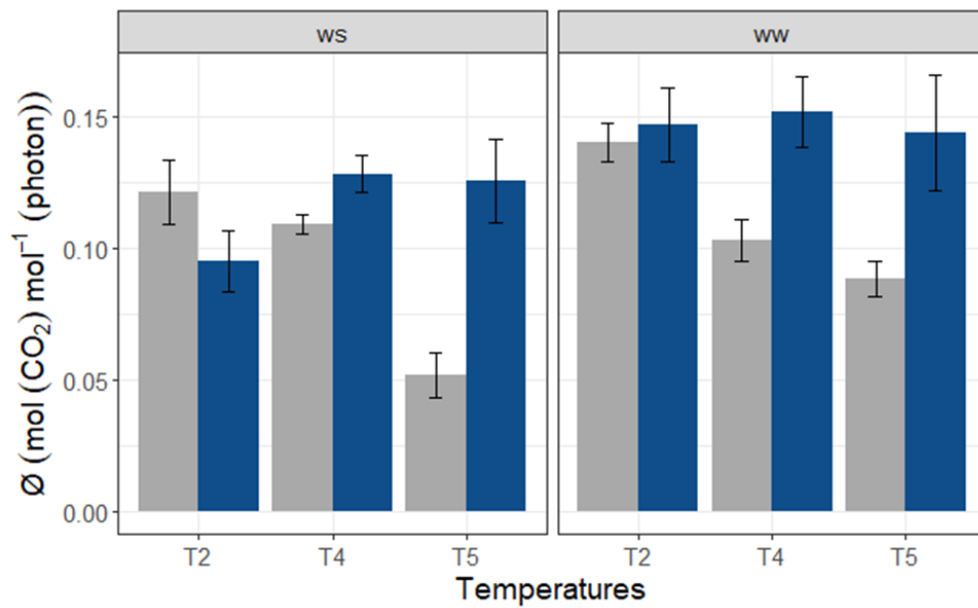
Figure 4.4 Quantum efficiency (Ø) of cocoa seedlings grown at ambient and elevated [CO_2], three temperatures and under well-watered (WW) and water-stress conditions (WS). Error bars show the standard error of the mean (n=4). [CO_2] treatments are ambient (grey bar) and elevated (blue bar). Temperature treatments are 31/22°C (T2), 36/27°C (T4) and 38.5/29.5°C (T5).

Dark respiration rate. Dark respiration rate (*Rd*) was significantly lower under water deficit by an average of 20.3% (P <0.001; Figure 4.5). The response to temperature differed for each [CO_2] treatment (P <0.01 for temperature*[CO_2]). Under ambient [CO_2], although not significant, *Rd* showed a trend increasing across the temperature regimes. In plants grown under elevated [CO_2], *Rd* increased significantly from 31/22°C (0.87 (±0.05) µmol mol^{-2} s^{-1}) to 36/27°C (1.27 (±0.04) µmol mol^{-2} s^{-1}); *Rd* did not increase significantly with a further increase in temperature to 38.5/29.5°C (1.34 (±0.06) µmol mol^{-2} s^{-1}).
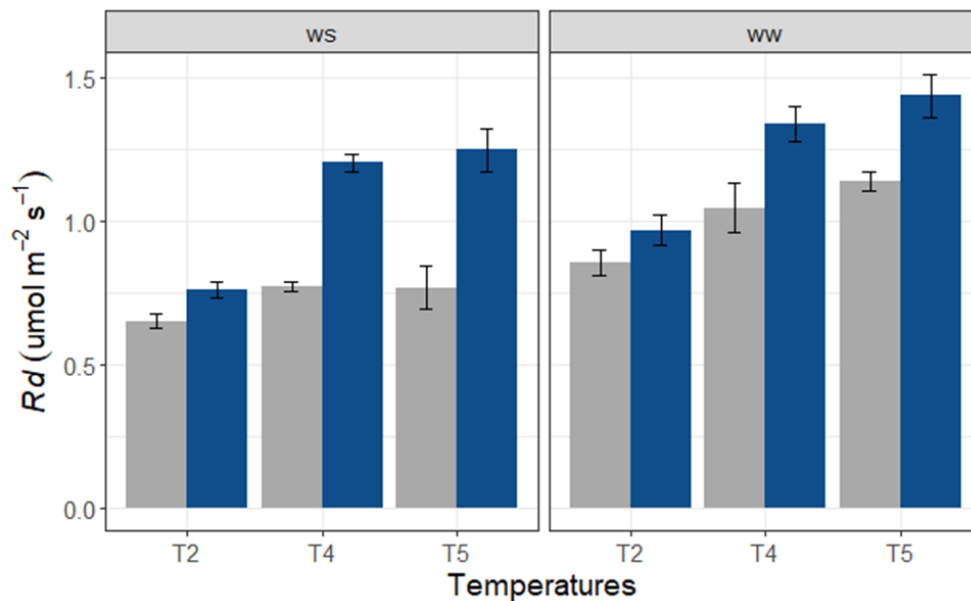
Figure 4.5 Respiration rate (*Rd*) of cocoa seedlings grown at ambient and elevated [CO_2], three temperatures and under well-watered (WW) and water-stress conditions (WS). Error bars show the standard error of the mean (n=4). [CO_2] treatments are ambient (grey bar) and elevated (blue bar). Temperature treatments are 31/22°C (T2), 36/27°C (T4) and 38.5/29.5°C (T5).

Light saturation point. Light saturation point was 29% lower under water deficit than in the WW treatment (*P* <0.001; Figure 4.6). The response to temperature varied at each [CO_2] (*P* <0.01 for temperature*[CO_2]). Under ambient [CO_2], light saturation point was significantly higher at 36/27°C (222.5 (±11.44) μmol m^{-2} s^{-1}) compared with 31/22°C and 38.5/29.5°C (124.6 (±11.01) μmol m^{-2} s^{-1} and 136.0 (±6.41) μmol m^{-2} s^{-1}, respectively). Although a similar trend was observed under elevated [CO_2], the differences were not significant. The response of light saturation point to [CO_2] also varied between the water regimes (*P* <0.001 for [CO_2]*water treatment). In well-watered conditions light saturation point increased by 38 % under elevated [CO_2] compared with ambient [CO_2], whereas under water-stress conditions, light saturation point did not differ significantly between [CO_2] treatments.

Figure 4.6 Light saturation point of cocoa seedlings grown at ambient and elevated [CO_2], three temperatures and under well-watered (WW) and water-stress conditions (WS). Error bars show the standard error of the mean (n=4). [CO_2] treatments are ambient (grey bar) and elevated (blue bar). Temperature treatments are 31/22°C (T2), 36/27°C (T4) and 38.5/29.5°C (T5).

Light compensation point. Light compensation point increased with temperature ($P< 0.001$). However, the increase from 31/22°C to 38.5/29.5°C was greater at ambient [CO_2] than elevated [CO_2] (135% and 62%, respectively) ($P <0.001$ for temperature*[CO_2]; Figure 4.7). The response to both temperature and [CO_2] also varied between water regimes ($P<0.05$ for temperature*water; $P<0.001$ for [CO_2]*water). For well-watered plants, temperature increases from 31/22°C to 38.5/29.5°C resulted in an increase in light compensation point by 117% and 65% at ambient and elevated [CO_2], respectively. Under water-stress conditions, the increase in temperature from 31/22°C to 38.5/29.5°C resulted in a 155% and 60% increase in the light compensation point at ambient and elevated [CO_2], respectively.
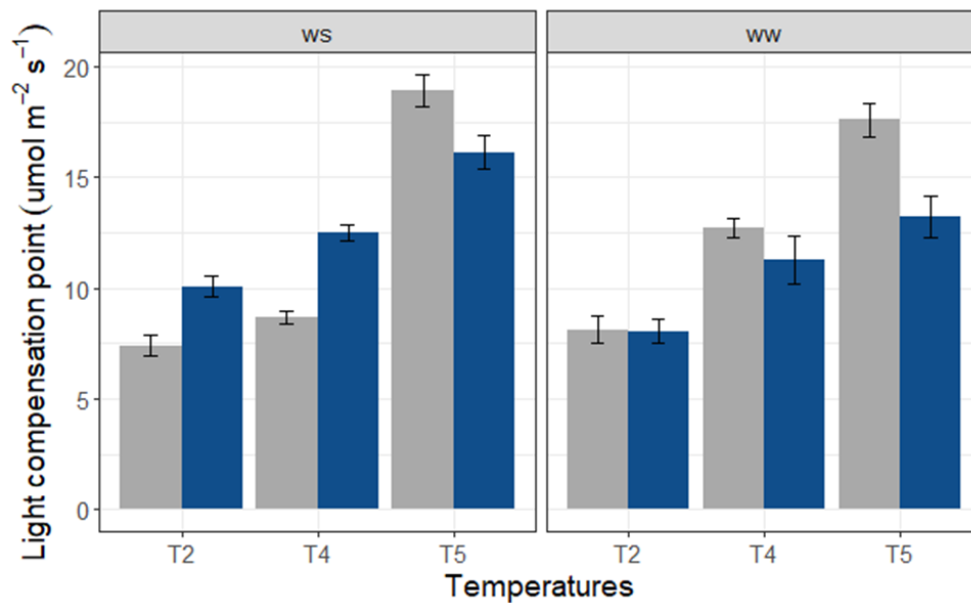
Figure 4.7 Light compensation point of cocoa seedlings grown at ambient and elevated [CO_2], three temperatures and under well-watered (WW) and water-stress conditions (WS). Error bars show the standard error of the mean (n=4). [CO_2] treatments are ambient (grey bar) and elevated (blue bar). Temperature treatments are 31/22°C (T2), 36/27°C (T4) and 38.5/29.5°C (T5).

4.3.2   Instantaneous gas exchange parameters

Net photosynthetic rate. Net photosynthetic rate (P_n) was significantly lower under water deficit by an average of 35% and higher at elevated [CO_2] (47%) (P <0.001; Figure 4.8). The response to temperature varied between treatment combinations (P <0.01 for [CO_2]* temperature*water regime). In the water stress treatment at ambient [CO_2], P_n did not differ significantly from 28.5/19.5°C (1.43 (±0.21) µmol m^{-2} s^{-1}) to 36/27°C (1.91(±0.27) µmol m^{-2} s^{-1}); above this temperature, P_n decreased significantly.   Under water stress and elevated [CO_2], P_n was similar in the 28.5/19.5°C and 31/22°C treatments, with significantly higher rates at 33.5/24.5°C and 36/27°C, while further increases in temperature caused in a significant decline in P_n. Under the well-watered *ambient [CO_2], P_n did not differ between 28.5/19.5°C and 31/22°C whereas temperature increases up to 36/27°C resulted in a significant increase in P_n which declined again with increases in temperature beyond 38.5/29.5°C. In the well-watered* elevated [CO_2] treatment, although P_n increased by 20% from 28.5/19.5°C to 38.5/29.5°C the difference was not significant. However, at 40/31°C there was a significant reduction in P_n, although this remained higher than that of plants grown at the same temperature under ambient [CO_2].

Figure 4.8 Net photosynthesis rate of cocoa seedlings grown at ambient and elevated $[CO_2]$, six temperatures and under well-watered (WW) and water-stress conditions (WS). Error bars show the standard error of the mean (n=4). $[CO_2]$ treatments are ambient (blue solid line) and elevated (red dashed line). Temperature treatments are 28.5/19.5°C (T1), 31/22°C (T2), 33.5/24.5°C (T3), 36/27°C (T4), 38.5/29.5°C (T5) and 40/31°C (T6).

Stomatal conductance. Stomatal conductance (g_s) was significantly lower (23%) under water deficit compared to the WW treatment. (P <0.001; Figure 4.9). However, the reduction was proportionally greater at ambient $[CO_2]$ than elevated $[CO_2]$ (24% and 15%, respectively) (P <0.001 for $[CO_2]$*water treatment). The response to temperature and $[CO_2]$ also varied at each water regime (P <0.05 for temperature*water; P <0.001 for $[CO_2]$*water). For plants grown under the water stress*elevated $[CO_2]$ treatment combination, g_s did not differ from 28.5/19.5°C (0.020 (±0.001) mol m^{-2} s^{-1}) to 31/22°C (0.021 (±0.001) mol m^{-2} s^{-1}); from here gs increase significantly for temperatures up to 38.5/29.5°C (0.031 (±0.003) mol m^{-2} s^{-1}), but further temperature increase beyond this resulted in a decline in g_s to 0.026 (±0.003) mol m^{-2} s^{-1}. In plants grown under the water stress *ambient $[CO_2]$ treatment combination increases in temperature from 31/22°C up to 40/31°C, caused a significant increase in stomatal conductance from 0.025 (±0.003) mol m^{-2} s^{-1} at 31/22°C to 0.045 (±0.003) mol m^{-2} s^{-1} at 40/31°C. For plants grown in the well-watered *elevated $[CO_2]$ treatment combination, g_s did not differ between 28.5/19.5°C and 31/22°C, it followed by a slight decline at 33.5/24.5°C. Increases in temperature from 36/27°C resulted in a significant increase to 0.028 (±0.003) mol m^{-2} s^{-1}, g_s did not change significantly with further temperature increases. In the well-watered*ambient $[CO_2]$ treatment combination, g_s was highest at 28.5/19.5°C (0.056 (±0.002) mol m^{-2} s^{-1}) and declined in the 31/22°C regime (0.045(±0.003) mol m^{-2} s^{-1}), further increases in temperature (36/27°C) resulted in a significant increase to 0.055

(±0.005) mol m^{-2} s^{-1}. Temperatures above 36/27°C resulted in a decline g_s to 0.045 (±0.003) mol m^{-2} s^{-1} at 40/31°C.
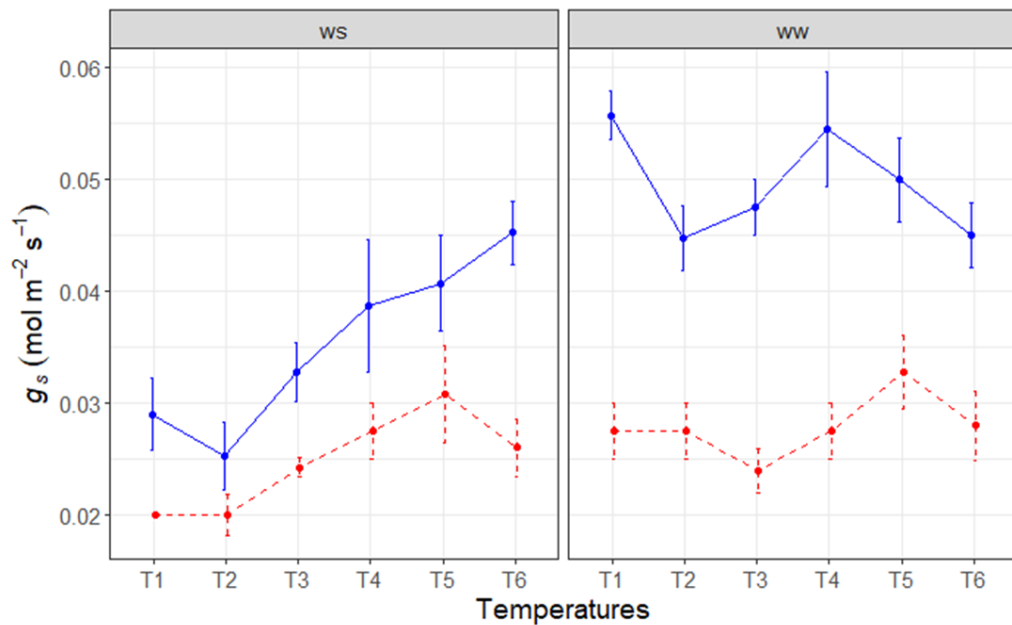
Figure 4.9 Stomatal conductance (g_s) of cocoa seedlings grown at ambient and elevated [CO_2], six temperatures and under well-watered (WW) and water-stress conditions (WS). Error bars show the standard error of the mean (n=4). [CO_2] treatments are ambient (blue solid line) and elevated (red dashed line). Temperature treatments are 28.5/19.5°C (T1), 31/22°C (T2), 33.5/24.5°C (T3), 36/27°C (T4), 38.5/29.5°C (T5) and 40/31°C (T6).

Leaf transpiration rate. Leaf transpiration rate (*E*) decreased under the water deficit treatment by an average of 21% (*P* <0.001; Figure 4.10). However, the reduction was proportionally greater at ambient [CO_2] than elevated [CO_2] (29 and 11%, respectively) (*P* <0.001 for [CO_2]*water treatment). The response to temperature and [CO_2] also varied under each water regime (*P* <0.05 for temperature*water treatment; *P* <0.001 for [CO_2]*water treatment). In the water stress treatment at ambient [CO_2], *E* did not differ between 28.5/19.5°C (0.46 (±0.16) mmol m^{-2} s^{-1}) and 33.5/24.5°C (0.59 (±0.03) mmol m^{-2} s^{-1}), above this temperature, there was a significant increase in *E*, rising to 1.31 (±0.07) mmol m^{-2} s^{-1} at 40/31°C. A similar response was seen in plants grown under water stress and elevated [CO_2], although was lower than in the ambient [CO_2] treatment. *E* was similar in the 28.5/19.5°C and 33.5/24.5°C regimes (0.32 (±0.02) mmol m^{-2} s^{-1}) and increased between 36/27°C and 40/31°C to a maximum of 0.93 (±0.13) mmol m^{-2} s^{-1}. For well-watered plants at ambient [CO_2], *E* declined slightly between 28.5/19.5°C and 31/22°C, whilst for temperatures above this *E* increased significantly to a maximum of 1.52 (±0.04) mmol m^{-2} s^{-1} at 40/31°C. In plants grown at elevated [CO_2] in the well-watered treatment, *E* remained stable between 28.5/19.5°C and 33.5/24.5°C. However, subsequent increases in temperature resulted in a significant increase *E*, rising to a maximum of 1.17 (±0.03) mmol m^{-2} s^{-1} at 40/31°C.

Figure 4.10 Leaf transpiration rate (*E*) of cocoa seedlings grown at ambient and elevated [CO_2], six temperatures and under well-watered (WW) and water-stress conditions (WS). Error bars show the standard error of the mean (n=4). [CO_2] treatments are ambient (blue solid line) and elevated (red dashed line). Temperature treatments are 28.5/19.5°C (T1), 31/22°C (T2), 33.5/24.5°C (T3), 36/27°C (T4), 38.5/29.5°C (T5) and 40/31°C (T6).

Ratio of intercellular (Ci) to atmospheric (Ca). Ci/Ca ratio was on average 21% lower under water deficit compared to the WW control (P <0.001; Figure 4.11). In contrast, there was an overall significant increase of 10.1% in the Ci/Ca of plants grown at elevated [CO_2] (0.61 (±0.01)) compared to those grown at ambient [CO_2] (0.54 (±0.01)) (P <0.001). Ci/Ca varied across the temperature regimes (P <0.001). Ci/Ca remained stable from 28.5/19.5°C to 36/27°C and increased significantly at 38.5/29.5°C and 40/31°C. There were no significant interactions between treatments.
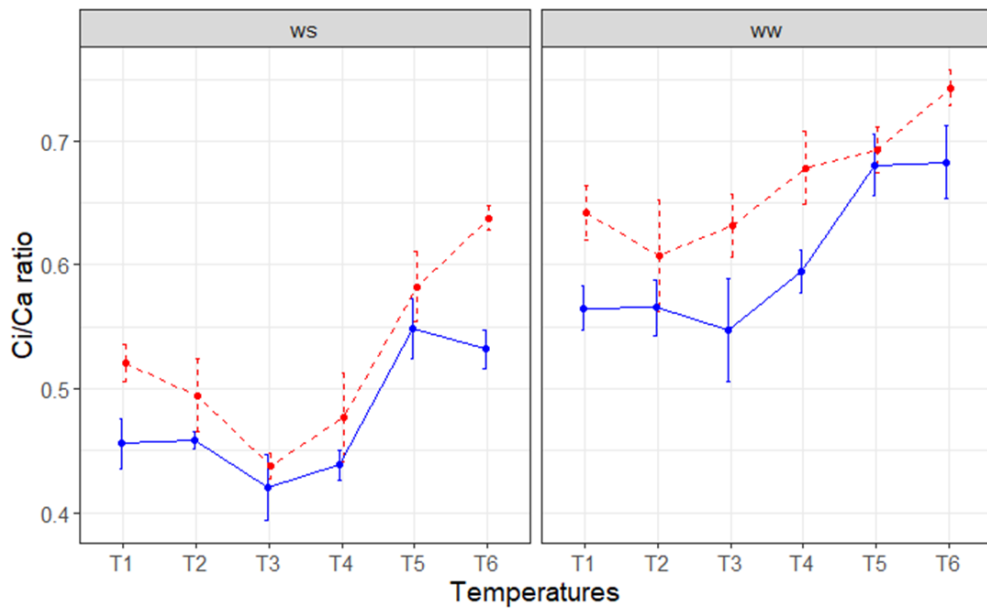
Figure 4.11 Ratio of intercellular [CO_2] (Ci) to atmospheric [CO_2] (Ca) **(Ci/Ca)** of cocoa seedlings grown at ambient and elevated [CO_2], six temperatures and under well-watered (WW) and water-stress conditions (WS). Error bars show the standard error of the mean (n=4). [CO_2] treatments are ambient (blue solid line) and elevated (red dashed line). Temperature treatments are 28.5/19.5°C (T1), 31/22°C (T2), 33.5/24.5°C (T3), 36/27°C (T4), 38.5/29.5°C (T5) and 40/31°C (T6).

Maximum quantum efficiency of PS II. Maximum quantum efficiency (*Fv/Fm*) was 7.1% lower under water deficit compared to the well-watered treatment (*P* <0.001; Figure 4.12). The response to temperature varied for each water regime (*P* <0.001 for temperature*water treatment). Under well-watered conditions, *Fv/*Fm was not affected by temperature within the range of 28.5/19.5°C (0.75 (±0.01)) to 38.5/29.5°C (0.76 (±0.01)); however, there was a significant decline at 40/31°C (0.71 (±0.01)). In the water-stress treatment, *Fv/Fm* was not affected by temperature regime. No clear effect of elevated [CO_2] on *Fv/Fm* was observed.
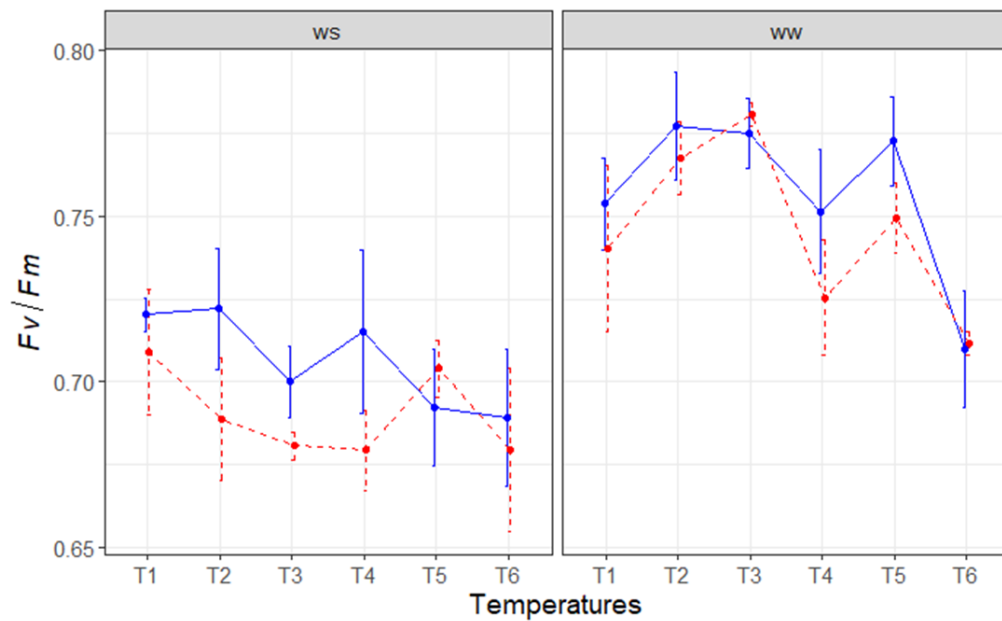
Figure 4.12 Maximum quantum efficiency (*Fv/Fm*) of cocoa seedlings grown at ambient and elevated [CO_2], six temperatures and under well-watered (WW) and water-stress conditions (WS). Error bars show the standard error of the mean (n=4). [CO_2] treatments are ambient (blue solid line) and elevated (red dashed line). Temperature treatments are 28.5/19.5°C (T1), 31/22°C (T2), 33.5/24.5°C (T3), 36/27°C (T4), 38.5/29.5°C (T5) and 40/31°C (T6).

Performance index. Performance index (*PI*) was significantly lower under water deficit by an average of 51.7% (*P* <0.001; Figure 4.13). The response to temperature varied under each water regime (*P* <0.001 for temperature*water treatment). In plants grown under well-watered conditions, there was an increase in *PI* from 28.5/19.5°C (1.01 (±0.23)) to 31/22°C (1.66 (±0.09)). At temperatures above 33.5/24.5°C *PI* declined reaching a minimum of 0.28 (±0.05) at 40/31°C, which was similar to the values measured under water stressed conditions. Overall, *PI* was significantly lower under water deficit and no effects of temperature were observed. No significant effect of elevated [CO_2] on *PI* was observed.

Figure 4.13 Performance index (*PI*) of cocoa seedlings grown at ambient and elevated [CO_2], six temperatures and under well-watered (WW) and water-stress conditions (WS). Error bars show the standard error of the mean (n=4). [CO_2] treatments are ambient (blue solid line) and elevated (red dashed line). Temperature treatments are 28.5/19.5°C (T1), 31/22°C (T2), 33.5/24.5°C (T3), 36/27°C (T4), 38.5/29.5°C (T5) and 40/31°C (T6).

4.3.3   Water relation parameters

Stem water potential. Stem water potential (Ψ_{stem}) was significantly lower in plants grown under water deficit by an average of 44.4% (*P* <0.001), and declined with an increase in temperature (*P* <0.001; Figure 4.14). The response of Ψ_{stem} to [CO_2] also varied at each water regime (P<0.01 for [CO_2]*water treatment). For well-watered plants, Ψ_{stem} declined from 28.5/19.5°C (-0.63 (±0.07) MPa) to 40/31°C (-1.56 (±0.11) MPa), but no impact of [CO_2] was observed. However, in the water-stress treatment, Ψ_{stem} was higher at elevated [CO_2] between28.5/19.5°C and 36/27°C.
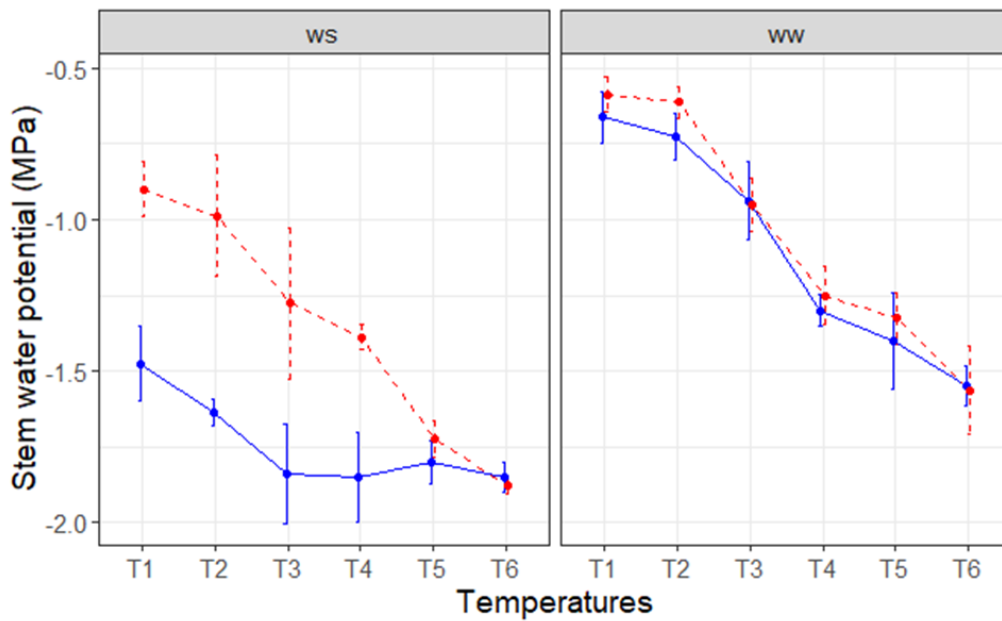
Figure 4.14 Stem water potential (Ψ_{stem}) of cocoa seedlings grown at ambient and elevated [CO_2], six temperatures and under well-watered (WW) and water-stress conditions (WS). Error bars show the standard error of the mean (n=4). [CO_2] treatments are ambient (blue solid line) and elevated (red dashed line). Temperature treatments are 28.5/19.5°C (T1), 31/22°C (T2), 33.5/24.5°C (T3), 36/27°C (T4), 38.5/29.5°C (T5) and 40/31°C (T6).

Total plant transpiration. Total plant transpiration (measured over 81 days) (E_{plant}) was significantly lower under water deficit compared to well-watered plants by an average of 69% (*P* <0.001; Figure 4.15) and the response to temperature was dependent on water regime (*P* <0.001 for temperature*water treatment). For well-watered plants, E_{plant} doubled between the 28.5/19.5°C (5204 (±534) ml) and 33.5/24.5°C (9640 (±885) ml) temperature regimes. However, a significant decline was observed at 40/31°C (6370 (±685) ml). In plants in the water-stress treatment, there was a significant increase in E_{plant} from 28.5/19.5°C to 31/22°C (1784 (±107.7) and 2974 (±209.4) ml respectively). E_{plant} did not change with increases in temperature up to 38.5/29.5°C but a decrease was observed at 40/31°C (1618 (±112) ml). No significant effect of elevated [CO2] on E_{plant} was observed.
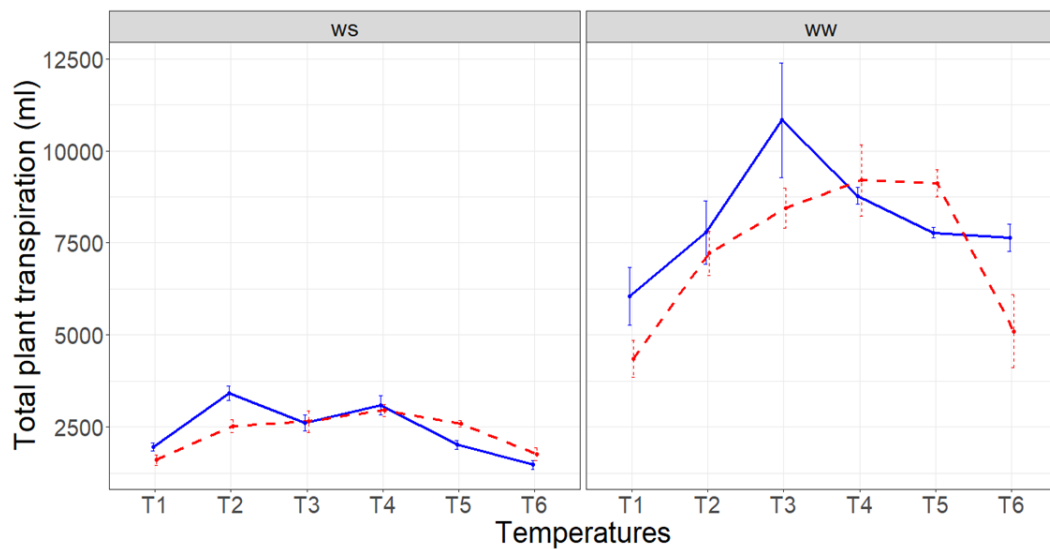
Figure 4.15 Total plant transpiration (E_{plant}) of cocoa seedlings grown at ambient and elevated [CO_2], six temperatures and under well-watered (WW) and water-stress conditions (WS). Error bars show the standard error of the mean (n=4). [CO_2] treatments are ambient (blue solid line) and elevated (red dashed line). Temperature treatments are 28.5/19.5°C (T1), 31/22°C (T2), 33.5/24.5°C (T3), 36/27°C (T4), 38.5/29.5°C (T5) and 40/31°C (T6).

Plant transpiration efficiency. Plant transpiration efficiency (*TE*) was 70% higher in the water deficit compared to the well-watered treatment (*P* <0.001) and 66% higher under elevated [CO_2] than ambient [CO_2] (*P*<0.001; Figure 4.16). The magnitude of the response to temperature varied under each water regime (*P* <0.05 for temperature*water treatment). Under well-watered conditions, an increase in temperature from 28.5/19.5°C to 40/31°C resulted in a decline in *TE* from 0.011 (±0.001) to 0.003 (±0.001) g ml^{-1} at ambient [CO_2], and 0.018 (±0.001) to 0.006 (±0.001) g ml^{-1} elevated [CO_2]. In water-stressed plants, *TE* decreased from 0.019 (±0.001) to 0.009 (±0.001) g ml^{-1}, and 0.032 (±0.002) to 0.014 (±0.001) g ml^{-1} at ambient [CO_2] and elevated [CO_2] with an increase in temperature from 28.5/19.5°C and 40/31°C.
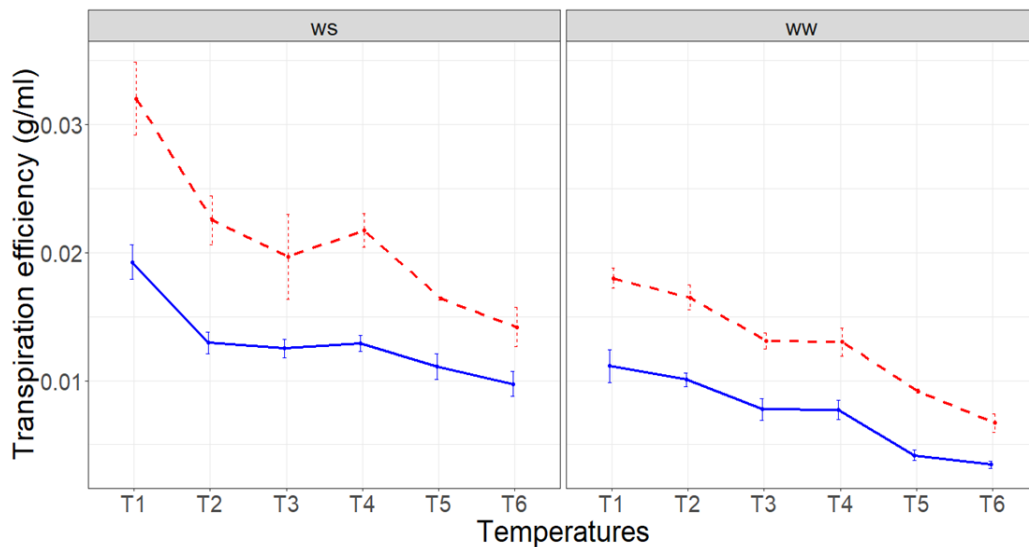
Figure 4.16 Plant transpiration efficiency (*TE*) of cocoa seedlings grown at ambient and elevated [CO_2], six temperatures and under well-watered (WW) and water-stress conditions (WS). Error bars show the standard error of the mean (n=4). [CO_2] treatments are ambient (blue solid line) and elevated (red dashed line). Temperature treatments are 28.5/19.5°C (T1), 31/22°C (T2), 33.5/24.5°C (T3), 36/27°C (T4), 38.5/29.5°C (T5) and 40/31°C (T6).

Intrinsic water use efficiency. Intrinsic water use efficiency (iWUE) was significantly lower in the water deficit compared to the well-watered treatment, by an average of 18% (*P* <0.001) and was higher under elevated [CO_2] than ambient [CO_2], by an average of 137% (*P* <0.001; Figure 4.17). For both CO_2 and water treatments, there was a general trend of an increase in iWUE with temperature up to 36/27°C followed by a decline with further temperature increases (*P* <0.001 for [CO_2]*water treatment; *P* <0.05 for [CO_2]*temperature). There was also a greater decline in intrinsic water use efficiency at 40/31°C (74.16 (±10.83) μmol mol^{-1}) under elevated [CO_2] compared to ambient [CO_2].
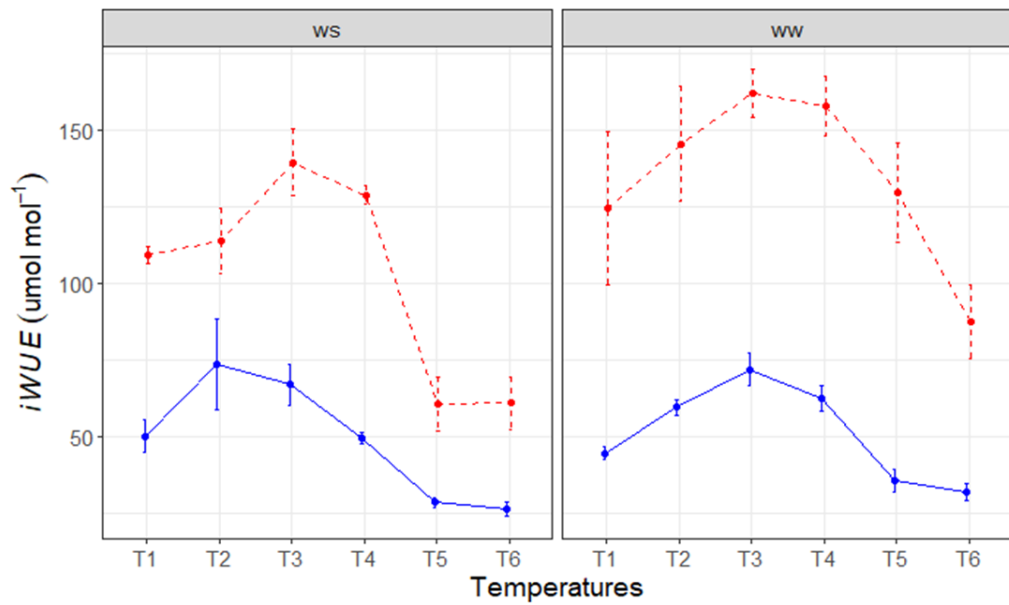
Figure 4.17 Intrinsic water use efficiency (iWUE) of cocoa seedlings grown at ambient and elevated [CO_2], six temperatures and under well-watered (WW) and water-stress conditions (WS). Error bars show the standard error of the mean (n=4). [CO_2] treatments are ambient (blue solid line) and elevated (red dashed line). Temperature treatments are 28.5/19.5°C (T1), 31/22°C (T2), 33.5/24.5°C (T3), 36/27°C (T4), 38.5/29.5°C (T5) and 40/31°C (T6).

4.3.4 Plant growth parameters

Height increase. Plants were 37% shorter in the water deficit treatment than the well-watered treatment (*P* <0.001) and 12% taller at elevated [CO_2] compared to ambient [CO_2] (*P* <0.01; Figure 4.18). For both [CO_2] levels and water treatments, plant height increased with temperature up to 36/27°C followed by a decline with further temperature increases. However, the magnitude of the response to temperature was higher under well-watered conditions (*P* <0.01 for temperature*water treatment). Maximum plant height was achieved at 36/27°C in the water-stress treatment under both ambient and elevated [CO_2] (60.62 (±2.09) and 64.25 (±2.14) cm respectively), and between 33.5/25.4°C and 36/27°C in the well-watered treatment under both ambient and elevated [CO_2] (33.5/25.4°C: 90.28 (±3.75) and 93.35 (±4.29) cm; 36/27°C: 86.72 (±5.27) and 98.58 (±3.72) cm, respectively).
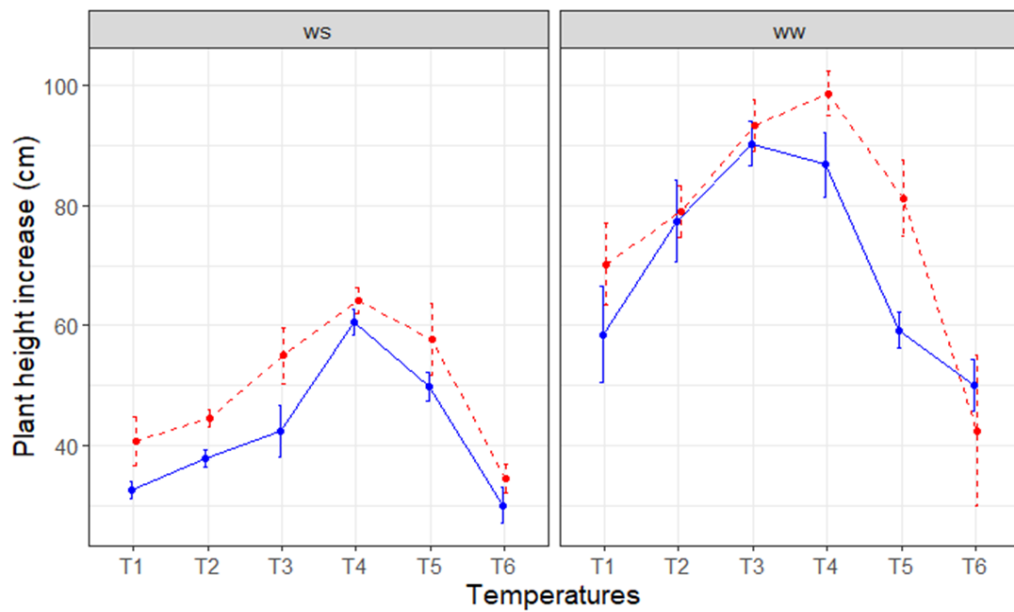
Figure 4.18 Plant height increase of cocoa seedlings grown at ambient and elevated [CO_2], six temperatures and under well-watered (WW) and water-stress conditions (WS). Error bars show the standard error of the mean (n=4). [CO_2] treatments are ambient (blue solid line) and elevated (red dashed line). Temperature treatments are 28.5/19.5°C (T1), 31/22°C (T2), 33.5/24.5°C (T3), 36/27°C (T4), 38.5/29.5°C (T5) and 40/31°C (T6).

Stem diameter increase. The increase in stem diameter over the course of the experiment was 31% lower under water deficit compared to the well-watered treatment (P <0.001; Figure 4.19). The increment in stem diameter in response to the temperature varied at each [CO_2] (P <0.01 for [CO_2]*temperature) and water treatment (P <0.01 for water*temperature). In the water stress*ambient [CO_2] treatment combination, stem diameter increase did not differ significantly from 28.5/19.5°C to 36/27°C, above this temperature there was a significant decrease in stem growth. For the water stress*elevated [CO_2] treatment combination, stem diameter increase did not change significantly between 28.5/19.5°C and 31/22°C, it increased up to 36/27°C, but further increases in temperatures resulted in a significant decline. In the well-watered*ambient [CO_2] treatment combination, stem diameter increment increased with temperature between 28.5/19.5°C and 33.5/24.5°C, further increases up to 40/31°C resulted in a significant decline in stem diameter increment. In the well-watered*elevated [CO_2] treatment combination, stem diameter increase increased across a wider range of temperatures, from 28.5/19.5°C to 38.5/29.5°C, with a decrease at 40/31°C.
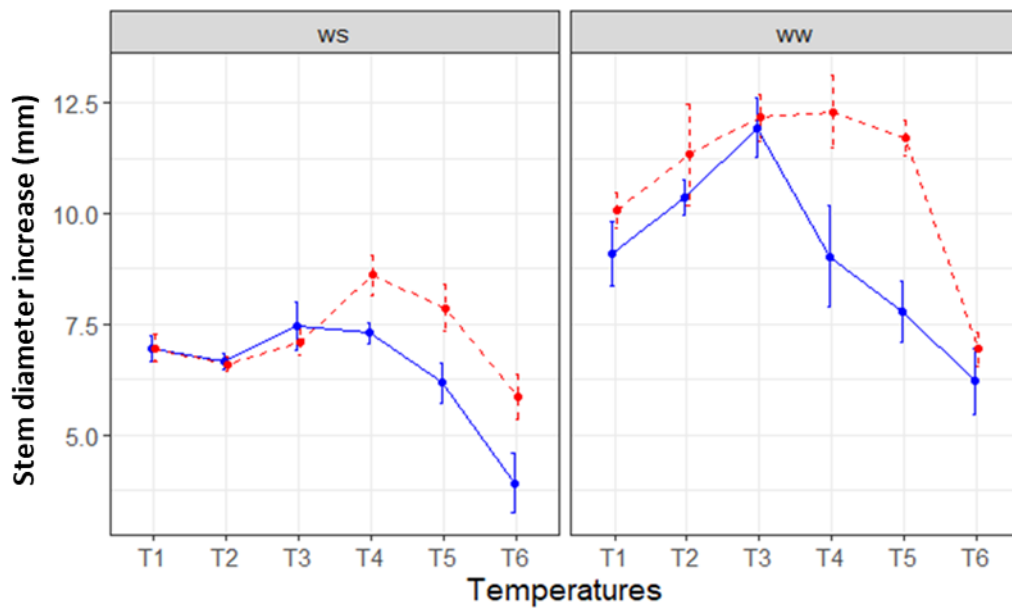
Figure 4.19 Stem diameter increase of cocoa seedlings grown at ambient and elevated [CO_2], six temperatures and under well-watered (WW) and water-stress conditions (WS). Error bars show the standard error of the mean (n=4). [CO_2] treatments are ambient (blue solid line) and elevated (red dashed line). Temperature treatments are 28.5/19.5°C (T1), 31/22°C (T2), 33.5/24.5°C (T3), 36/27°C (T4), 38.5/29.5°C (T5) and 40/31°C (T6).

Leaf number. Leaf number per plant was significantly lower for plants grown under water deficit compared plants grown under the well-watered treatment, by an average of 27% (P <0.001; Figure 4.20). The effect of temperature on leaf number differed between [CO_2], (P <0.001 for [CO_2] * temperature), and water treatments (P < 0.05 for water*temperature). In the water stress*ambient [CO_2] treatment, leaf number did not differ significantly between 28.5/19.5°C (42 (±3)) and 31/22°C (44 (±3)), however number of leaves increased in the 33.5/24.5°C and 36/27°C treatments (71 (±4) and 103 (±8) respectively). Further increases in temperature caused a significant reduction in leaf number to 71 (±7) and 73 (±13), respectively. For water stress*elevated [CO_2] treatment, leaf number did not differ from 28.5/19.5°C (38 (±5)) to 33.5/24.5°C (58 (±11)); temperature increases of 36/27°C, 38.5/29.5°C, and 40/31°C caused a significant increase in leaf number of 139 (±29), 165 (±24), and 147 (±16), respectively. In the well-watered*ambient [CO_2] treatment, there was a significant increase in leaf number across the temperature regimes, ranging from 57 (±7)) at 28.5/19.5°C to 188 (±15) at 40/31°C. In the well-watered*elevated [CO_2] treatment, leaf number increased significantly from 28.5/19.5°C (51 (±7)) to 36/27°C (211 (±25)), above which a significant reduction was observed.
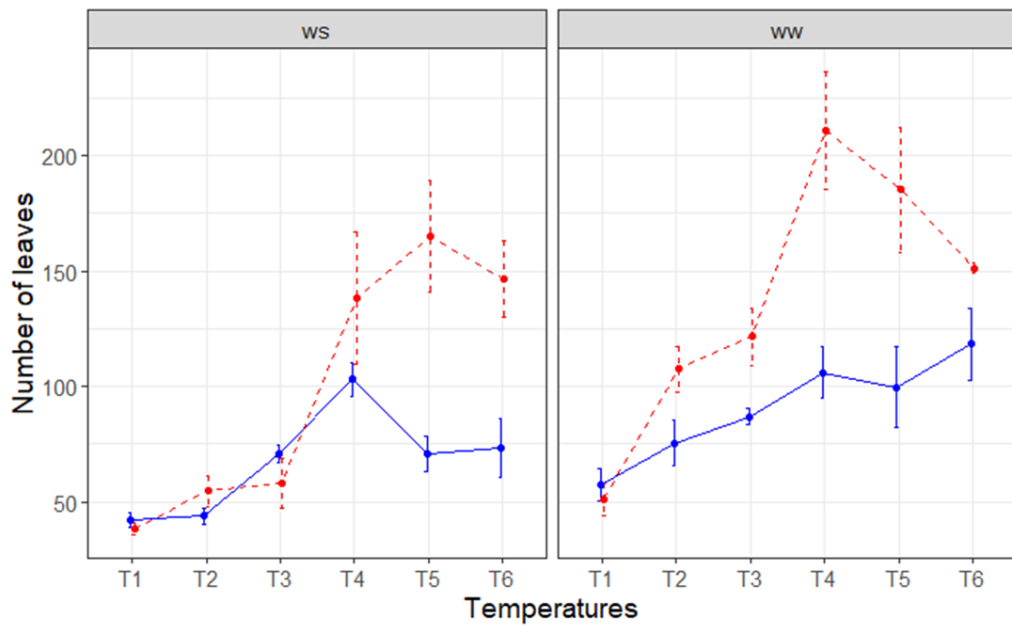
Figure 4.20 Number of leaves of cocoa seedlings grown at ambient and elevated [CO_2], six temperatures and under well-watered (WW) and water-stress conditions (WS). Error bars show the standard error of the mean (n=4). [CO_2] treatments are ambient (blue solid line) and elevated (red dashed line). Temperature treatments are 28.5/19.5°C (T1), 31/22°C (T2), 33.5/24.5°C (T3), 36/27°C (T4), 38.5/29.5°C (T5) and 40/31°C (T6).

Total leaf area. Total leaf area (LA) per plant was significantly lower in plants grown under water deficit compared to well-watered plants, by an average of 58% (*P* <0.001; Figure 4.21) but significantly higher (36.3%) under elevated [CO_2] compared to ambient [CO_2] (*P* <0.001). The response to temperature varied between treatments (*P* <0.01 for [CO_2]*temperature*water regime). In the water stress*ambient [CO_2] treatment, LA did not differ significantly in plants grown from 28.5/19.5°C to 36/27°C, increases in temperature above this caused a significant reduction at 40/31°C. Similarly, in the water stress*elevated [CO_2] treatment, LA followed the same response to temperature although the overall values were greater at elevated [CO_2]. In the well-watered*ambient [CO_2] treatment, LA increased significantly from 6365 (±326) cm^2 to 8284 (±373) cm^2 between 28.5/19.5°C and 31/22°C, LA decreased significantly at temperatures above this to a value of 3029 (±123) cm^2 at 40/31°C. The same response pattern was seen in the well-watered*elevated [CO_2] treatment, LA increased significantly from 6500 (±936) to 10913 (±1034) cm^2 between 28.5/19.5°C and 31/22°C, further increases in temperature resulted in a decline in LA to 7766 (±145) and 3115(±123) cm^2 at 38.5/29.5°C and 40/31°C°, respectively.
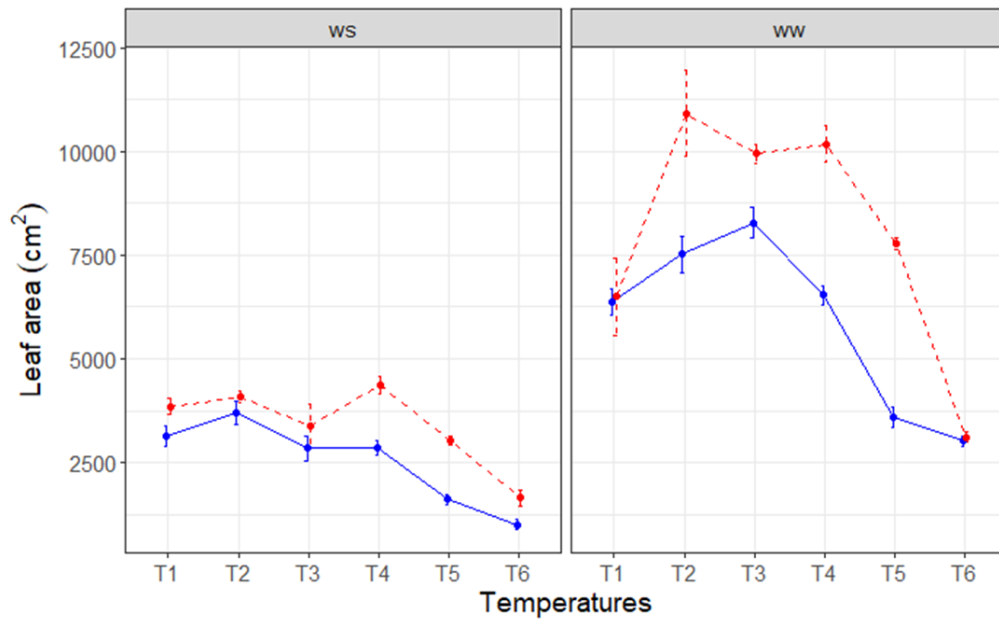
Figure 4.21 Total leaf area (LA) of cocoa seedlings grown at ambient and elevated $[CO_2]$, six temperatures and under well-watered (WW) and water-stress conditions (WS). Error bars show the standard error of the mean (n=4). $[CO_2]$ treatments are ambient (blue solid line) and elevated (red dashed line). Temperature treatments are 28.5/19.5°C (T1), 31/22°C (T2), 33.5/24.5°C (T3), 36/27°C (T4), 38.5/29.5°C (T5) and 40/31°C (T6).

Shoot dry weight. Shoot dry weight was significantly lower under water deficit by an average of 63% (P <0.001; Figure 4.22). The response to $[CO_2]$ and temperature varied across the water treatments ($P <$ 0.01 for $[CO_2]$*water treatment; $P < 0.001$ for temperature*water treatment). In the water stress *ambient $[CO_2]$ treatment, shoot dry weight did not differ from 28.5/19.5°C (14.26 (±1.13) g) to 36/27°C (16.67 (± 0.94) g); above this temperature, shoot dry weight decreased at 38.5/29.5°C and 40/31°C (9.82 (±0.68) and 5.68 (±0.41) g, respectively). For the water stress*elevated $[CO_2]$ treatment, shoot dry weight increased from 28.5/19.5°C (18.87 (±2.77) g) to 36/27°C (31.12 (±0.81) g). Further increases in temperature to 38.5/29.5°C and 40/31°C, caused a significant decline in shoot dry weight (20.16 (±0.42) and (12.01 (±1.46) g, respectively). For the well-watered*ambient $[CO_2]$ treatment, there was a slight increase in shoot dry weight from 28.5/19.5°C (24.81 (±2.22 g) to 36/27°C (29.84 (±1.83) g), from which increases in temperature up to 40/31°C resulted in a decrease in shoot dry weight to 12.77 (±1.59) g. In the well-watered*elevated $[CO_2]$ treatment, shoot dry weight increased significantly from 28.5/19.5°C (29.81 (±4.15) g) to 36/27°C (55.31 (±2.59) g). However, increases in temperature to 38.5/29.5°C and 40/31°C, resulted in a significant decline to 39.97 (±1.72) and 13.96 (±3.68) g, respectively.
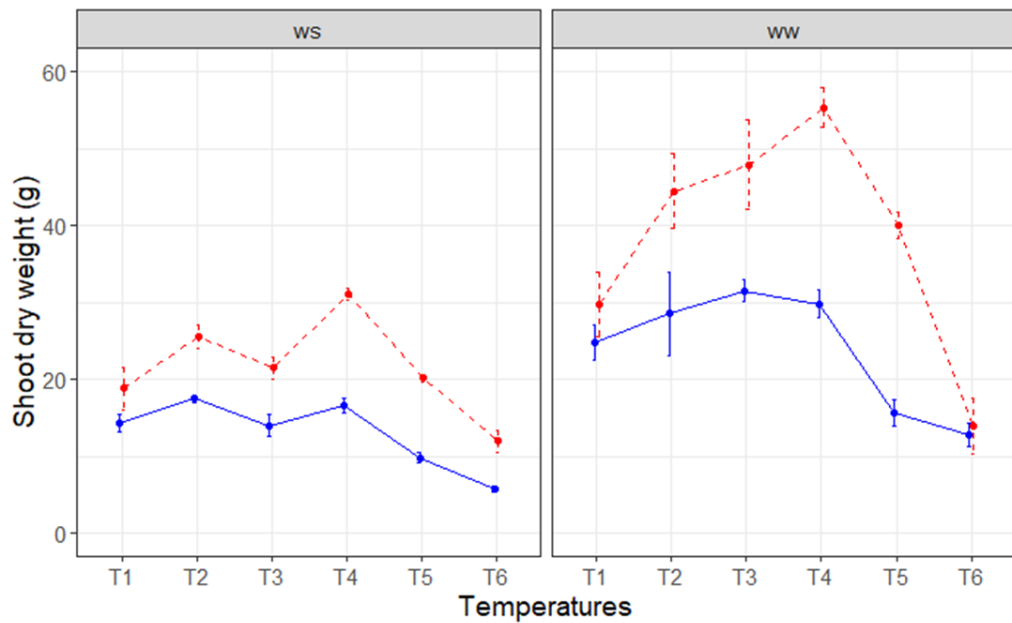
Figure 4.22 Shoot dry weight of cocoa seedlings grown at ambient and elevated [CO_2], six temperatures and under well-watered (WW) and water-stress conditions (WS). Error bars show the standard error of the mean (n=4). [CO_2] treatments are ambient (blue solid line) and elevated (red dashed line). Temperature treatments are 28.5/19.5°C (T1), 31/22°C (T2), 33.5/24.5°C (T3), 36/27°C (T4), 38.5/29.5°C (T5) and 40/31°C (T6).

Total leaf dry weight. Total leaf dry weight was significantly lower under the water deficit treatment compared to the well-watered treatment, by an average of 48% (P <0.001; Figure 4.23) and increased by 39% in the elevated [CO_2] treatment compared to the ambient [CO_2] treatment (P <0.001). The response to temperature varied between treatments (P <0.01 for [CO_2]* temperature*water regime). In the water stress *ambient [CO_2] treatment, little difference in total leaf dry weight was observed from 28.5/19.5°C to 36/27°C; above this temperature, there was a significant decrease in total leaf dry weight at 38.5/29.5°C and 40/31°C (11.92 (±0.73) and 9.78 (±0.71) g, respectively). For the water stress *elevated [CO_2] treatment, total leaf dry weight did not change significantly from 28.5/19.5°C to 38.5/29.5°C (21.81 (±1.12) and 19.41 (±1.01) g, respectively). However, there was a significant decline at 40/31°C (11.94 ± (1.18) g). In the well-watered * ambient [CO_2] treatment, the total leaf dry weight did not differ significantly between 28.5/19.5°C and 36/27°C, further increases in temperature up 40/31°C caused a significant reduction to 12.6 (±1.03) g. In the well-watered*elevated [CO_2] treatment, total leaf dry weight increased significantly from 36.16 (±3.26) g to 54.81 (±4.49) g between 28.5/19.5°C and 31/22°C. Increases in temperature to 38.5/29.5°C and 40/31°C°, resulted in a decline in leaf dry weight to 36.12 (±1.41) g and 17.12(±0.44) g, respectively.
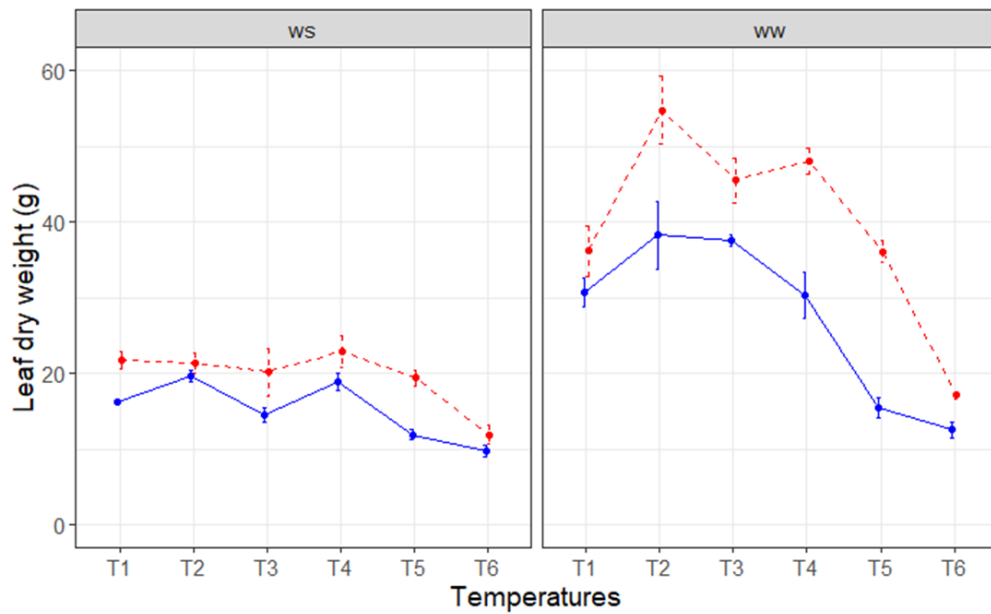
Figure 4.23 Leaf dry weight of cocoa seedlings grown at ambient and elevated [CO_2], six temperatures and under well-watered (WW) and water-stress conditions (WS). Error bars show the standard error of the mean (n=4). [CO_2] treatments are ambient (blue solid line) and elevated (red dashed line). Temperature treatments are 28.5/19.5°C (T1), 31/22°C (T2), 33.5/24.5°C (T3), 36/27°C (T4), 38.5/29.5°C (T5) and 40/31°C (T6).

Root dry weight. Root dry weight was 33% lower under water deficit compared to the well-watered treatment (P <0.001; Figure 4.24) and increased by 46% between ambient and elevated [CO_2] (P <0.001). The response of root dry weight to temperature varied across the water treatments (P < 0.001 for temperature*water treatment) and differed in magnitude between [CO_2] treatments. In the water stress treatment at both ambient and elevated [CO_2], root dry weight declined significantly from 28.5/19.5°C to 40/31°C, although root dry weight was higher in the elevated [CO_2] treatment. In the well-watered * ambient [CO_2] treatment, root dry weight increased between 28.5/19.5°C and 33.5/24.5°C (12.9 (±1.73) and 14.57 (±1.09) g) and decreased significantly at temperatures above this, declining to 4.15 (±0.91) g at 40/31°C. In the well-watered *elevated [CO_2] treatment, root dry weight increased between 28.5/19.5°C and 31/22°C (15.38 (±2.12) and 22.4 (±2.01) g, respectively), and declined at temperatures higher than this.
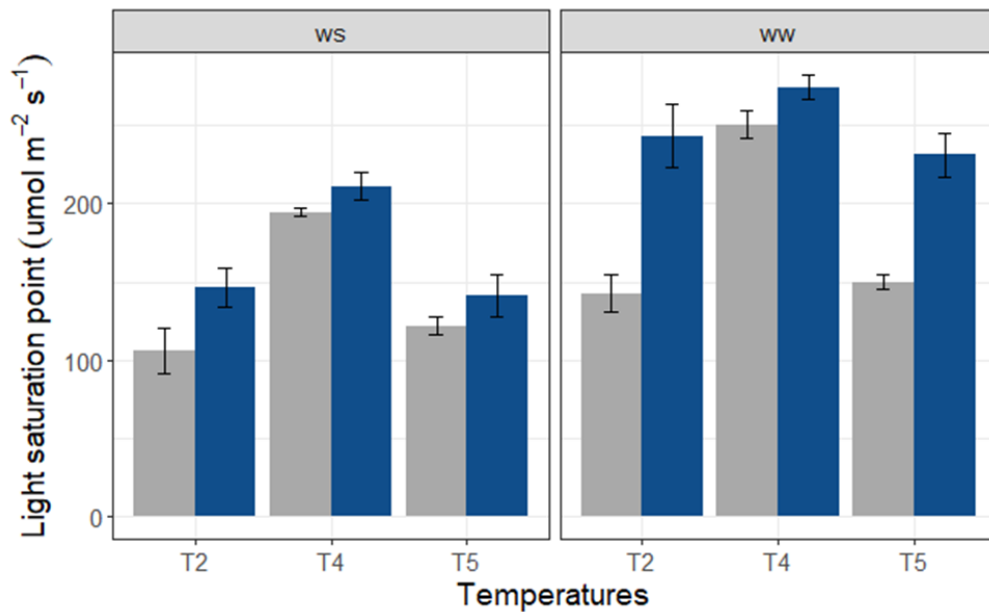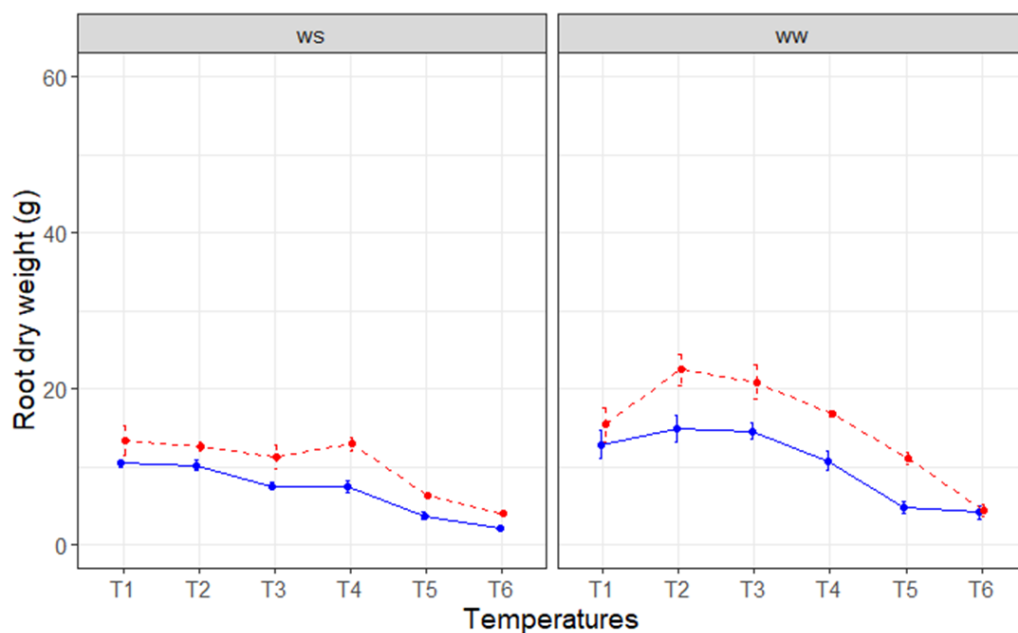
Figure 4.24 Root dry weight of cocoa seedlings grown at ambient and elevated [CO_2], six temperatures and under well-watered (WW) and water-stress conditions (WS). Error bars show the standard error of the mean (n=4). [CO_2] treatments are ambient (blue solid line) and elevated (red dashed line). Temperature treatments are 28.5/19.5°C (T1), 31/22°C (T2), 33.5/24.5°C (T3), 36/27°C (T4), 38.5/29.5°C (T5) and 40/31°C (T6).

Total plant dry weight. Total plant dry weight was 44% lower in the water deficit treatment compared to the well-watered treatment (P <0.001; Figure 4.25) and was 49% greater under elevated [CO_2] compared to ambient [CO_2] (P <0.001). The response to temperature varied between treatments (P <0.01 for [CO_2]* temperature*water regime). For the water stress*ambient [CO_2] treatment, there was little difference in the total plant dry weight from 28.5/19.5°C to 36/27°C (40.91 (±1.41) g and 43.12 (±2.67) g); above this temperature, there was a significant decrease in total dry weight at 38.5/29.5°C and 40/31°C (25.5 (±1.48) g and 17.55(±1.07) g, respectively). For the water stress*elevated [CO_2] treatment, total plant dry weight did not change significantly from 28.5/19.5°C to 36/27°C (54.05 (±4.87) g and 66.96 (±1.90), g respectively); however, there was a significant decline at higher temperatures. In the well-watered*ambient [CO_2] treatments, the total plant dry weight did not differ significantly in plants grown between 28.5/19.5°C and 36/27°C, temperatures above this caused a significant reduction in plant dry weight. In the well-watered *elevated [CO_2] treatment, the total plant dry weight increased considerably from 81.34 (±7.95) g to 121.72 (±9.25) g between 28.5/19.5°C and 31/22°C, with little change at temperature increases up to 36/27°C, further increases in temperature up to 40/31°C° resulted in a decline in total plant dry weight 35.52(±3.94) g.
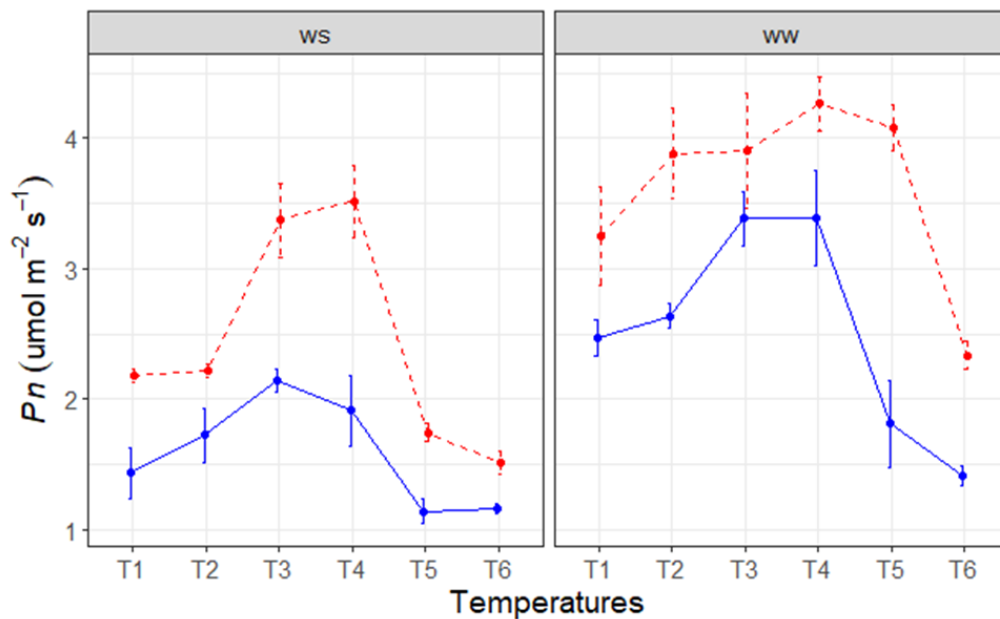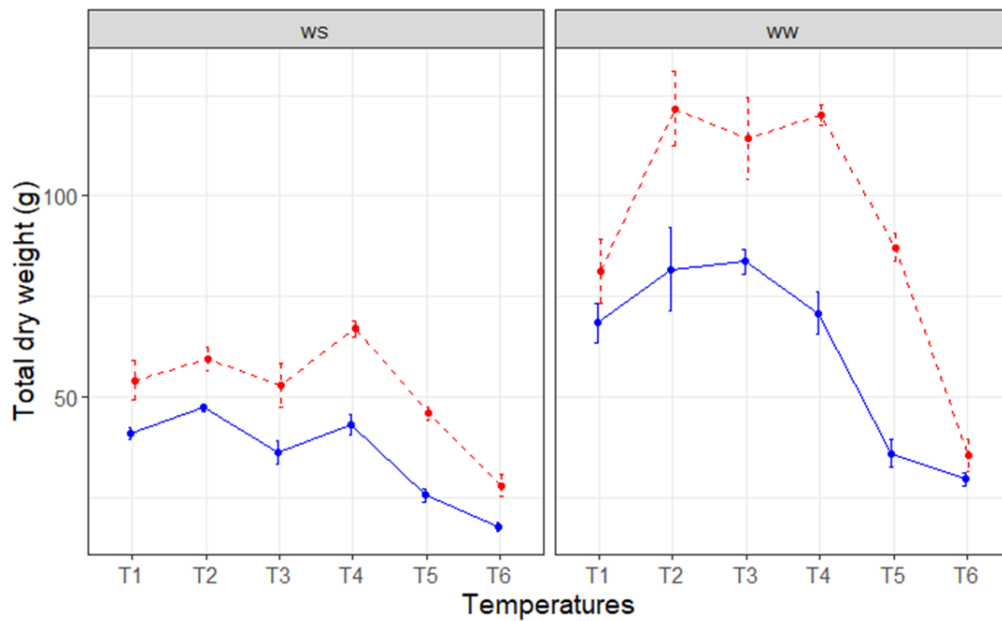
Figure 4.25 Total plant dry weight of cocoa seedlings grown at ambient and elevated [CO_2], six temperatures and under well-watered (WW) and water-stress conditions (WS). Error bars show the standard error of the mean (n=4). [CO_2] treatments are ambient (blue solid line) and elevated (red dashed line). Temperature treatments are 28.5/19.5°C (T1), 31/22°C (T2), 33.5/24.5°C (T3), 36/27°C (T4), 38.5/29.5°C (T5) and 40/31°C (T6).

Root-shoot ratio. Root-shoot ratio (R-S) was 24% higher in the water deficit treatment compared to the well-watered treatment (*P* <0.001; Figure 4.26) whereas root-shoot ratio (R-S) did not differ significantly between [CO_2] treatments. The response to temperature varied for each water regime (*P* <0.01 for temperature*water treatment). At 28.5/19.5°C R-S was higher under the water stressed treatment than in the well-watered treatment. Under well-watered conditions, R-S did not change from 28.5/19.5°C to 33.5/24.5°C, however, R-S declined significantly with further increases in temperature. In the water-stress treatment, R-S decreased linearly with increases in temperature ranging from 0.34 (±0.02) at 28.5/19.5°C to 0.15 (±0.01) at 40/31°C.
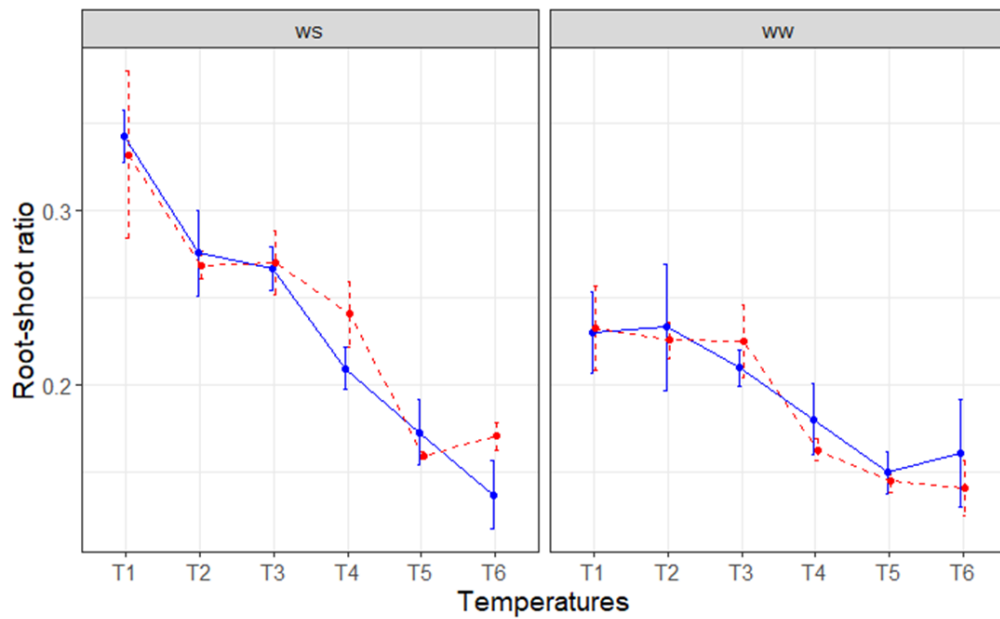
Figure 4.26 Root-shoot ratio (R-S) of cocoa seedlings grown at ambient and elevated [CO_2], six temperatures and under well-watered (WW) and water-stress conditions (WS). Error bars show the standard error of the mean (n=4). [CO_2] treatments are ambient (blue solid line) and elevated (red dashed line). Temperature treatments are 28.5/19.5°C (T1), 31/22°C (T2), 33.5/24.5°C (T3), 36/27°C (T4), 38.5/29.5°C (T5) and 40/31°C (T6).

Leaf area ratio. Leaf area ratio (LAR) was significantly lower under water deficit compared to the well-watered treatments, by an average of 26% (P <0.001; Figure 4.27) and was 9% lower under elevated [CO_2] compared to ambient [CO_2] (P <0.01). The response to temperature varied at each water regime (P < 0.01 for temperature*water treatment). In well-watered plants, although not significant, LAR increased slightly across the temperature regimes ranging from 86.72 (±5.67) cm^2 g^{-1} at 28.5/19.5°C to 96.37 (±4.12) cm^2 g^{-1} at 40/31°C. In the water-stress treatment, LAR remained stable between 28.5/19.5°C and 33.5/24.5°C (75.04 (±4.66) and 71.31 (±5.41) cm^2 g^{-1}, respectively), followed by a significant decrease in LAR at 36/27°C and above, declining to 58.71 (±4.16) cm^2 g^{-1} at 40/31°C.
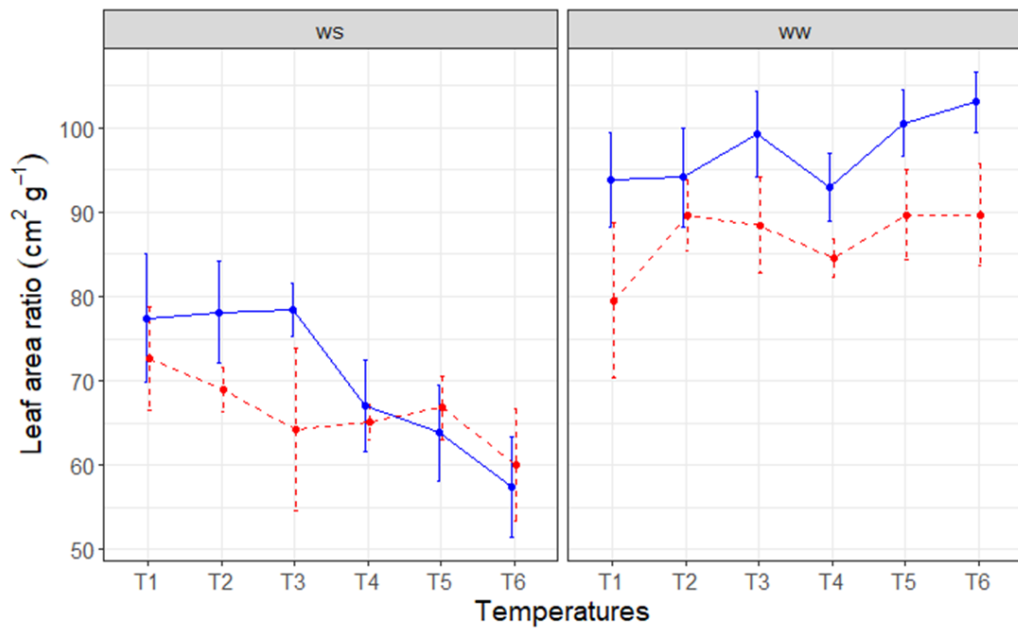
Figure 4.27 Leaf area ratio (LAR) of cocoa seedlings grown at ambient and elevated [CO_2], six temperatures and under well-watered (WW) and water-stress conditions (WS). Error bars show the standard error of the mean (n=4). [CO_2] treatments are ambient (blue solid line) and elevated (red dashed line). Temperature treatments are 28.5/19.5°C (T1), 31/22°C (T2), 33.5/24.5°C (T3), 36/27°C (T4), 38.5/29.5°C (T5) and 40/31°C (T6).

Relative growth rate. Relative growth rate (RGR) was significantly lower in the water deficit compared to the well-watered treatments, by an average of 18% (*P* <0.001; Figure 4.28). The response of RGR to temperature varied with [CO_2] (*P* <0.001 for [CO_2]*temperature) and water treatment (*P* <0.001 for temperature*water treatment). For all treatments, there was a general trend of little change in RGR from 28.5/19.5°C (0.029 (±0.001) g g^{-1} d^{-1}) to 36/27°C (0.031 (±0.001) g g^{-1} d^{-1}), above this temperature, there was a significant decrease in RGR at 38.5/29.5°C and 40/31°C (0.026 (±0.001) and 0.019 (±0.001) g g^{-1} d^{-1}, respectively) (*P* <0.001). Overall, RGR was higher under elevated [CO_2] than at ambient [CO_2] but this effect was not evident at 40/31°C in the well-watered treatment.
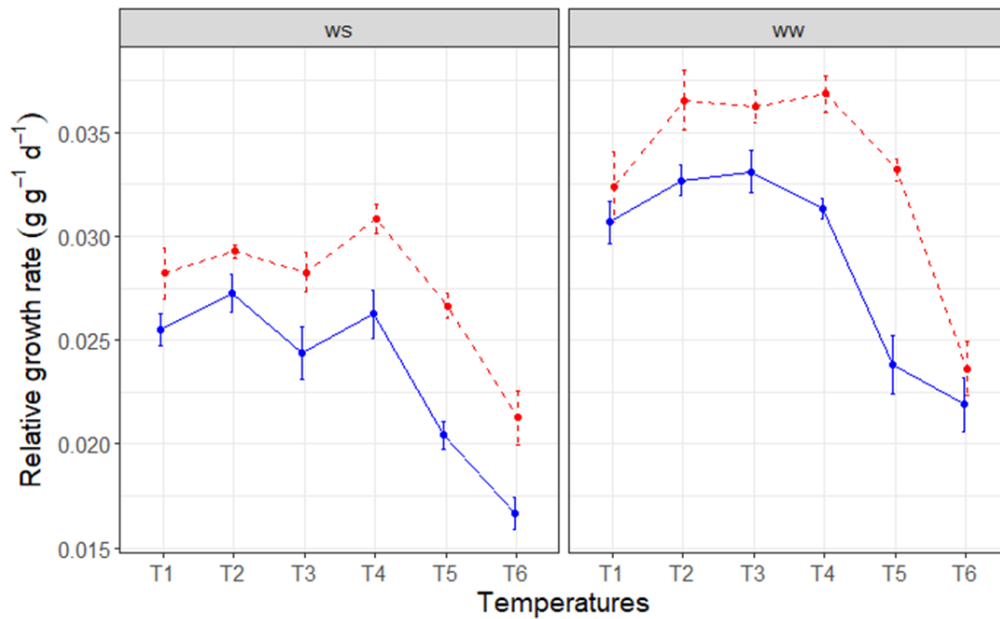
Figure 4.28 Relative growth rate (RGR) of cocoa seedlings grown at ambient and elevated [CO_2], six temperatures and under well-watered (WW) and water-stress conditions (WS). Error bars show the standard error of the mean (n=4). [CO_2] treatments are ambient (blue solid line) and elevated (red dashed line). Temperature treatments are 28.5/19.5°C (T1), 31/22°C (T2), 33.5/24.5°C (T3), 36/27°C (T4), 38.5/29.5°C (T5) and 40/31°C (T6).

Net assimilation ratio. Net assimilation rate (NAR) was significantly higher at elevated [CO_2] compared to at ambient [CO_2], by an average of 23% (P <0.01). The response to temperature was dependent on water regime (P <0.05 for temperature*water treatment). In well-watered plants, NAR did not differ from 28.5/19.5°C to 36/27°C, however, there was a significant reduction at 38.5/29.5°C and 40/31°C, respectively. In plants grown under the water-stress treatment, the NAR did not differ across the temperature regimes (Figure 4.29).
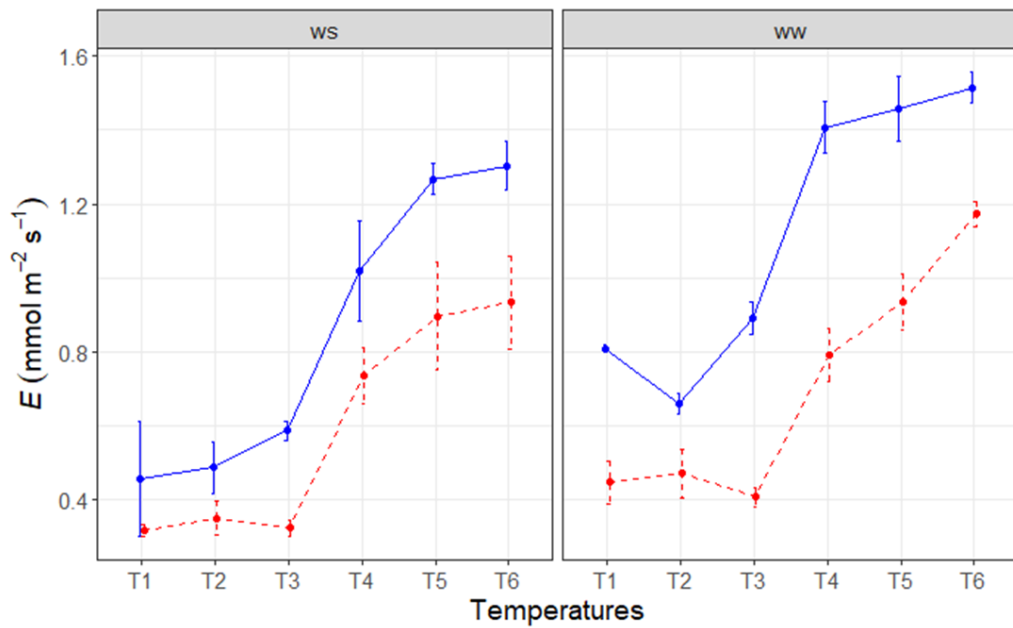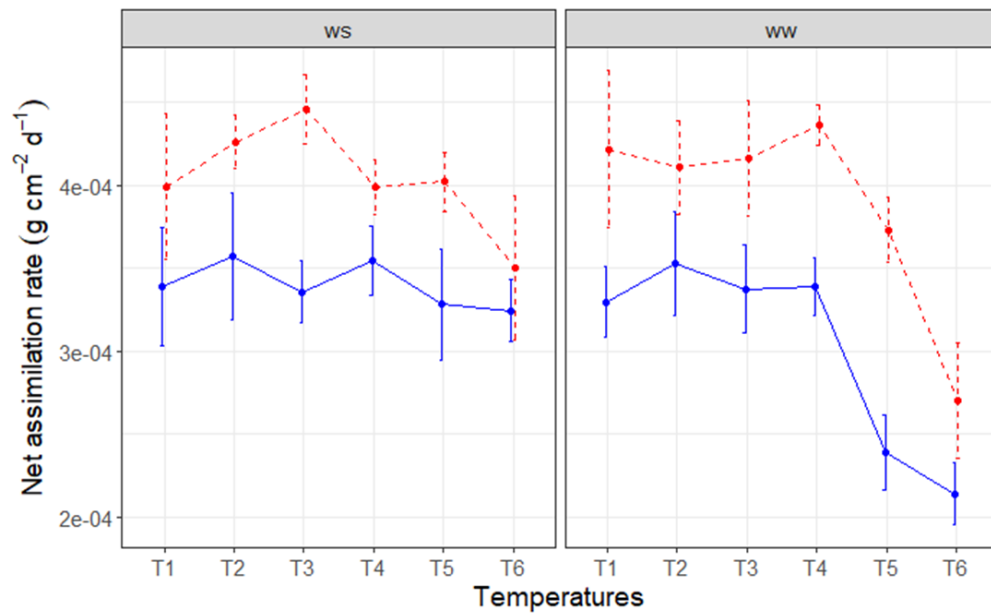
Figure 4.29 Net assimilation rate (NAR) of cocoa seedlings grown at ambient and elevated [CO_2], six temperatures and under well-watered (WW) and water-stress conditions (WS). Error bars show the standard error of the mean (n=4). [CO_2] treatments are ambient (blue solid line) and elevated (red dashed line). Temperature treatments are 28.5/19.5°C (T1), 31/22°C (T2), 33.5/24.5°C (T3), 36/27°C (T4), 38.5/29.5°C (T5) and 40/31°C (T6).

4.3.5   Leaf trait parameters

Stomatal density. Whilst there were no main effects of the water deficit and [CO_2] treatments on stomatal density (SD), the response to temperature varied between treatments (P <0.01 for [CO_2]* temperature*water treatment; Figure 4.30). In the water stress*ambient [CO_2] treatment, SD did not differ significantly from 28.5/19.5°C to 36/27°C (1162 (±47) and 1239 (±46) stomata mm^{-2}); above this temperature, there was a significant increase at 38.5/29.5°C (1350 (±46) stomata mm^{-2}) and a subsequent decrease at 40/31°C (1125 (±63) stomata mm^{-2}). For water stress*elevated [CO_2] treatment, there was a general trend of a decline with temperature from 28.5/19.5°C to 40/31°C, with the exception of 36/27°C at which SD was significantly higher (1362 (±87) stomata mm^{-1}). In well-watered*ambient [CO_2] treatment, SD decreased significantly from 28.5/19.5°C to 31/22°C (1312 (±32) and 1100 (±35) stomata mm^{-2}), between 33.5/24.5°C and 36/27°C there was a significant increase in SD (1325(±72) and 1388 (±77) stomata mm^{-2} respectively). Further temperature increases, resulted in a decline to 1112 (±66) stomata mm^{-2} at 38.5/29.5°C and 1112 (±32) stomata mm^{-2} at 40/31°C. In the well-watered*elevated [CO_2] treatment, SD did not differ between 28.5/19.5°C and 31/22°C (1088 (±24) and 1050 (±29) stomata mm^{-2}). It increased significantly at 33.5/24.5°C (1482 (±52) stomata mm^{-2}); remaining stable until it decline at 40/31°C to 1250 (±29) stomata mm^{-2}.
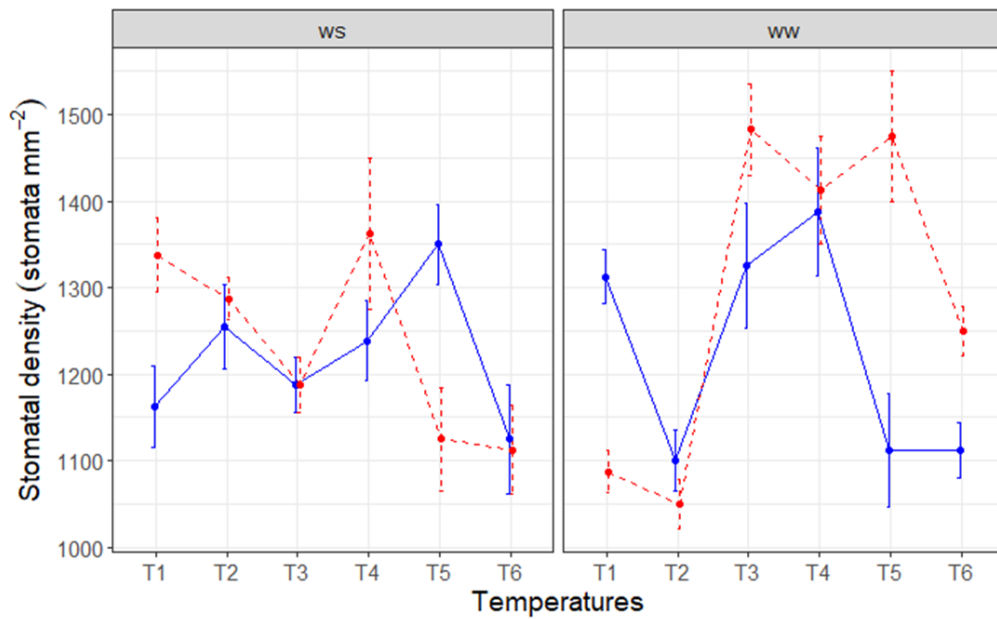
Figure 4.30 Stomatal density (SD) of cocoa seedlings grown at ambient and elevated [CO_2], six temperatures and under well-watered (WW) and water-stress conditions (WS). Error bars show the standard error of the mean (n=4). [CO_2] treatments are ambient (blue solid line) and elevated (red dashed line). Temperature treatments are 28.5/19.5°C (T1), 31/22°C (T2), 33.5/24.5°C (T3), 36/27°C (T4), 38.5/29.5°C (T5) and 40/31°C (T6).

Stomatal index. Whilst there were no main effects of water deficit and [CO_2] on stomatal index (SI), the response to temperature varied between treatments ($P <0.01$ for [CO_2]* temperature*water treatment; Figure 4.31). In the water stress treatment SI did not differ significantly across the temperature regimes at either ambient or elevated [CO_2]. In the well-watered*ambient [CO_2] treatment, there were no significant differences in stomata index with changes in temperature. In the well-watered*elevated [CO_2] treatment, SI did not differ significantly between 28.5/19.5°C and 31/22°C (18.24 (±0.59) and 16.86 (±0.28) %) but increased at 33.5/24.5°C (21.33 (±0.89) %). However, further temperature increases caused a subsequent decline to 19.44 (±0.84) % at 36/27°C, above which there were no significant differences.
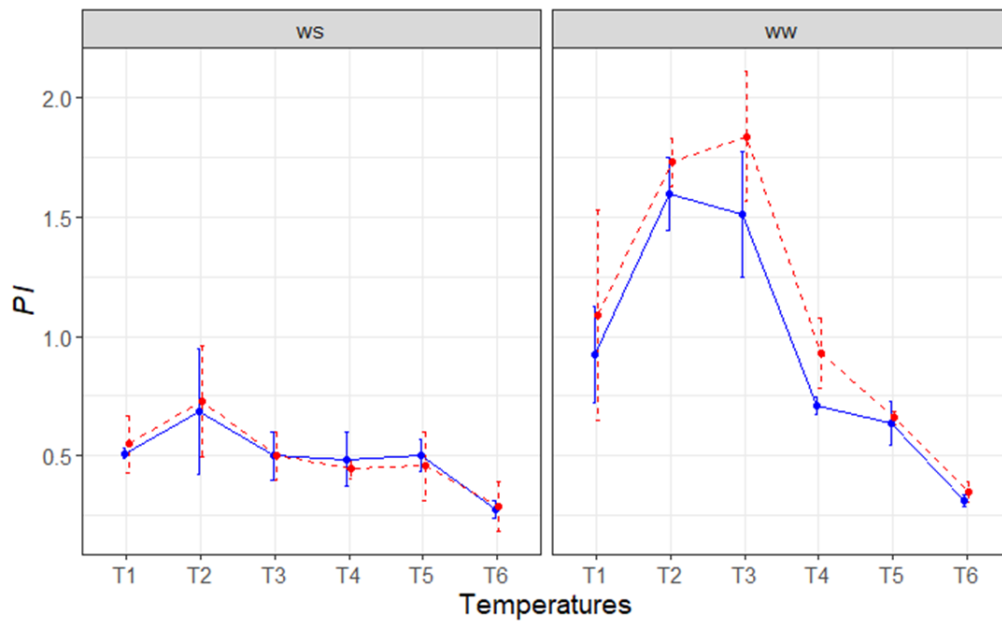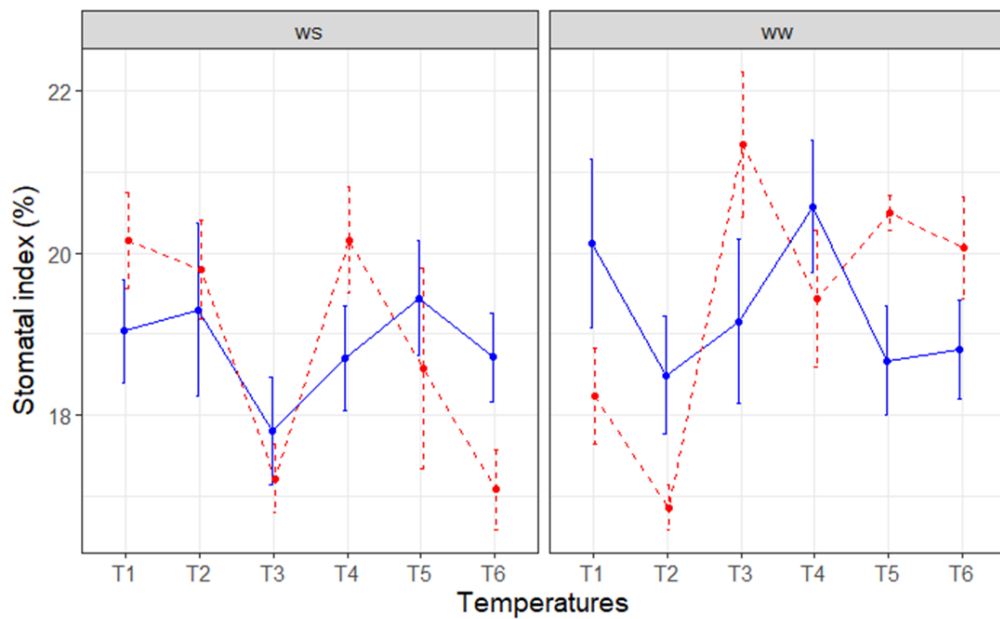
Figure 4.31 Stomatal index of cocoa seedlings grown at ambient and elevated [CO_2], six temperatures and under well-watered (WW) and water-stress conditions (WS). Error bars show the standard error of the mean (n=4). [CO_2] treatments are ambient (blue solid line) and elevated (red dashed line). Temperature treatments are 28.5/19.5°C (T1), 31/22°C (T2), 33.5/24.5°C (T3), 36/27°C (T4), 38.5/29.5°C (T5) and 40/31°C (T6).

Specific leaf area. The response of specific leaf area (SLA) to temperature varied between treatments (*P* <0.01 for [CO_2]* temperature*water regime; Figure 4.31). In the water stress*ambient [CO_2] treatment, SLA did not differ significantly from 28.5/19.5°C to 33.5/25.5°C (193.7 (±14.6) and 194.8 (±11.1) cm^2 g^{-1}), there was a significant decrease above this temperature, falling to 103.3 (±11.1) cm^2 g^{-1} at 40/31°C. For water stress*elevated [CO_2] treatment, SLA did not change significantly from 28.5/19.5°C to 38.5/29.5°C (177.1 (±9.1) and 158.6 (±11.1) cm^2 g^{-1} respectively); however, there was a significant decline in SLA at 40/31°C (139.3 (±12.0) cm^2 g^{-1}). In well-watered *ambient [CO_2] treatment, SLA increased across the temperature range from 200.6 (±4.1) cm^2 g^{-1} at 28.5/19.5°C to 246.7 (±27.7) cm^2 g^{-1} at 40/31°C. In the well-watered*elevated [CO_2] treatment SLA increased from 28.5/19.5°C (176.8 (±13.3) cm^2 g^{-1}) to 33.5/24.5°C (220.3 (±10.0) cm^2 g^{-1}). Further temperature increase resulted in a decline in SLA, falling to 182.8 (±11.2) cm^2 g^{-1} at 40/31°C.
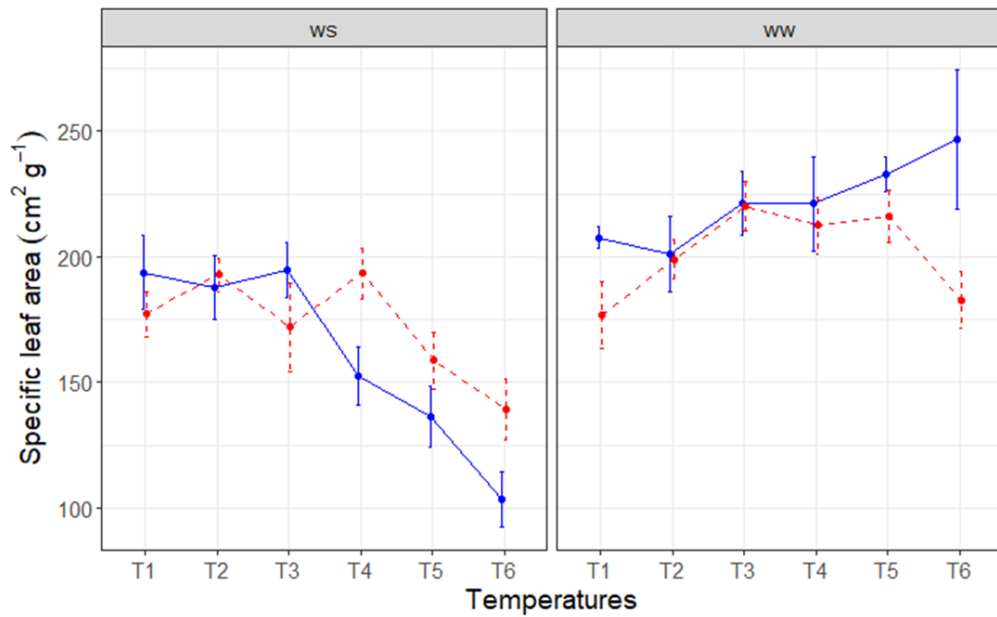
Figure 4.32 Specific leaf area (SLA) of cocoa seedlings grown at ambient and elevated $[CO_2]$, six temperatures and under well-watered (WW) and water-stress conditions (WS). Error bars show the standard error of the mean (n=4). $[CO_2]$ treatments are ambient (blue solid line) and elevated (red dashed line). Temperature treatments are 28.5/19.5°C (T1), 31/22°C (T2), 33.5/24.5°C (T3), 36/27°C (T4), 38.5/29.5°C (T5) and 40/31°C (T6).

Chlorophyll content. Chlorophyll content was significantly lower in the water deficit compared to the well-watered treatment, by an average of 6% (P <0.001; Figure 4.33). The response to temperature varied at each $[CO_2]$ treatment (P <0.05 for temperature*$[CO_2]$). In both $[CO_2]$ treatments, ambient and elevated $[CO_2]$ chlorophyll content increased between 28.5/19.5°C and 36/27°C (28.5/19.5°C: 22.70 (±1.03) µg cm^{-2} and 27.11 (±1.03) µg cm^{-2}; 36/27°C: 23.41 (±0.62) µg cm^{-2}) and 31.38 (±0.85) µg cm^{-2}, respectively). Further temperature increases caused a subsequent decline in chlorophyll content. At 40/31°C, chlorophyll content was similar at both ambient and elevated $[CO_2]$ specifically in the well-watered treatment.
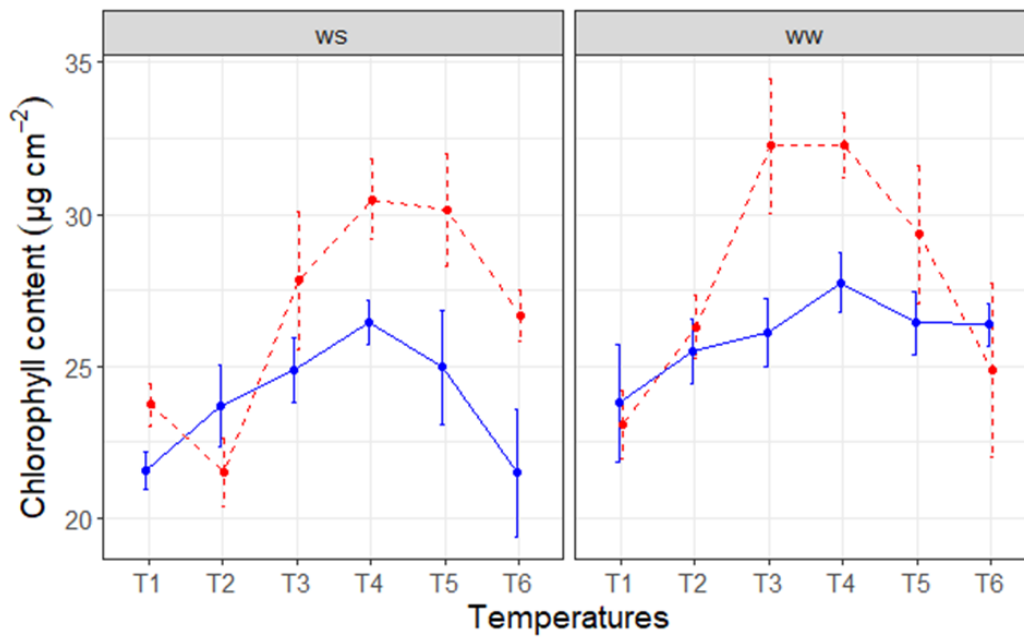
Figure 4.33 Chlorophyll content of cocoa seedlings grown at ambient and elevated [CO_2], six temperatures and under well-watered (WW) and water-stress conditions (WS). Error bars show the standard error of the mean (n=4). [CO_2] treatments are ambient (blue solid line) and elevated (red dashed line). Temperature treatments are 28.5/19.5°C (T1), 31/22°C (T2), 33.5/24.5°C (T3), 36/27°C (T4), 38.5/29.5°C (T5) and 40/31°C (T6).

4.4   Discussion

Climate change has the potential to alter cacao growth and physiology. In this experiment, cacao seedlings were grown at two CO_2 concentrations, six growth temperatures and two water regimes in growth cabinets for 81 days, in order to elucidate how these climate variables interact to alter the growth and physiology of these plants. Here, elevated [CO_2] enhanced photosynthesis and consequently growth, whereas temperatures above 36/27°C and water deficit conditions had negative effects. The unique aspect of this experiment was to examine the interaction between these variables. Elevated [CO_2] could ameliorate the negative effects of water deficit but this was dependent on growth temperature, whilst- supra-optimal growth temperatures exacerbated the negative impacts of water deficit.

Elevated [CO_2] had a positive effect on photosynthetic parameters. Here, the improvement in photosynthesis was approximately 40% and 47% based on measurements from A_{max} and P_n, respectively. A similar response has been previously reported in a wide variety of plant species (Long *et al.*, 2004; Ainsworth and Rogers, 2007) as well as cocoa seedlings (Baligar *et al.*, 2008, 2021a; Lahive *et al.*, 2018). Ø defined as the molar ratio of carbon fixed or oxygen evolved during photosynthesis per photon absorbed represents the photochemical efficiency of light utilization. Similar enhancement of Ø was reported in four-month-old Amelonado cacao seedlings grown under glasshouse conditions and

subjected to [CO_2] elevation (Lahive *et al.*, 2018). Elevated [CO_2] enables plants to use available light more efficiently, resulting in a higher light saturation point as observed here, suggesting that shade-tolerant species might perform better under both low and elevated light intensities and improve their carbon balance under climate change future scenarios (Kubiske and Pregitzer, 1996).

As expected, a negative effect of water deficit was observed for photosynthesis rate, Ø and light saturation point with overall reductions of 35%, 19% and 29%, respectively. Previous studies have shown a strong negative effect of water deficit on photosynthesis of cocoa plants grown under glasshouses conditions (Joly and Hahn, 1989b; Deng *et al.*, 1990; Mohd Razi *et al.*, 1992; De Almeida *et al.*, 2016; Lahive *et al.*, 2018; Osorio Zambrano *et al.*, 2021), open top chambers (Hebbar *et al.*, 2020) and in the field (Balasimha *et al.*, 1991; Rada *et al.*, 2005; Araque *et al.*, 2012; Ávila-Lovera *et al.*, 2016; De Almeida *et al.*, 2016). When water is scarce, plants can use stomatal closure as one of their primary mechanisms for reducing transpiration and maintaining turgor. Here, under water deficit, the reduction in photosynthesis was coupled with an average reduction of 23% in g_s, suggesting a stomatal limitation to photosynthesis. However, a non-stomatal effect on photosynthesis was also indicated by a reduction in *Fv/Fm* and *PI* under water deficit conditions. Furthermore, the observed decline in Ø and light saturation point, suggested that the light-use efficiency was reduced under water deficit. A similar response was also observed when 4-month-old cacao plants were subjected to water deficit (Lahive *et al.*, 2018). The same authors, reported a significant increase in light compensation point. However, light compensation point was unaffected by water deficit in the present study.

The fact that light compensation point and light saturation point did not change with elevated [CO_2] under water deficit conditions suggests that the positive effect of elevated [CO_2] on these parameters is more evident under well-watered conditions. However, elevated [CO_2] enhanced Ø compared to ambient [CO_2], but the enhancement was greater in the well-watered treatment compared to water deficit. It has been suggested that elevated [CO_2] may not have the same beneficial effects on cacao growing in regions that experience severe dry periods (Lahive *et al.*, 2018). The results presented here, suggest that the water-stress treatment implemented could be considered mild. Therefore, to a certain extent, increased atmospheric [CO_2] could help alleviate the negative effects of drought stress in cacao as has been seen in other species (Li *et al.*, 2008; Jiang *et al.*, 2016; Catarino *et al.*, 2021). Similar to the results reported by (Lahive *et al.*, 2018), the enhancement of net photosynthesis under elevated [CO_2] in the water deficit conditions resulted in rates of photosynthesis that were similar to well-watered plants at ambient [CO_2], across the range of temperatures studied. Similarly, working with 6-month-old cacao seedlings grown under water deficit in open-top chambers, (Hebbar *et al.*, 2020) observed an improvement in photosynthesis in plants at [CO_2] elevation of 550 and 700 ppm and an ambient temperature regime of 32.3/23.9°C. This positive effect was also sustained in plants grown at 550 ppm

and 3°C above ambient. It has been suggested that plants grown at elevated [CO_2] are less susceptible to moderate drought than those grown at ambient [CO_2] (Hamim, 2005; Wang *et al.*, 2017). In the present study, it has been shown that when day temperatures reach 40°C and water deficit is applied, the improvement of photosynthesis due to elevated [CO_2] remained apparent.

When considering the response of photosynthetic parameters to temperature only, overall the highest values for photosynthesis, $\emptyset$ and light saturation point were observed at 36/27°C. However, the light compensation point increased across the full range of temperatures suggesting that light utilisation under shaded conditions may become less efficient with increasing temperature. As discussed in Chapter 3, the higher optimal temperature observed here compared to other cacao studies (Balasimha *et al.*, 1991; Yapp, 1992; Hebbar *et al.*, 2020) is likely to be due to the fact that VPD was maintained at a constant non-limiting value. When temperature rises, the concurrent increase in VPD causes plants to close their stomata to minimize water loss through the leaf surface impacting indirectly on photosynthesis (Raja Harun and Hardwick, 1988; Balasimha *et al.*, 1991; Baligar *et al.*, 2008). This suggests that appropriate cropping systems that improve the microclimate (i.e. raise humidity) at the leaf and canopy level may help mitigate the negative effects of warmer temperatures on photosynthesis. The reductions in $\emptyset$ and light saturation point above 36/27°C suggest that light capture and utilization are less efficient above optimal temperatures for cacao. After a certain optimal point, high temperatures affect photosynthesis through the inhibition of photochemical activity due to membrane injury and damage to the components of electron transport (Berry and Bjorkman, 1980). This also coincides with a decline in chlorophyll content observed with temperature regimes above 36/27°C (discussed paragraphs below).

Elevated [CO_2] mitigated the negative effect of high-temperature stress on photosynthesis, $\emptyset$ and light saturation point up to 38.5/29.5°C. In the present study, under elevated [CO_2] a sustained photosynthesis rate was achieved up to 38.5/29.5°C in comparison to plants grown at ambient [CO_2] where the highest photosynthesis rate was recorded at 36/27°C. Similarly, it has been found that elevated [CO_2] mitigates the negative effect of heat stress on photosynthesis in pea, wheat, soybean, sunflower, tomato, white goosefoot (Hamilton *et al.*, 2008; Wang *et al.*, 2008), eucalypt (Ghannoum *et al.*, 2010), and coffee (Rodrigues *et al.*, 2016). Depending on the growth environment, C_3 photosynthesis is known to vary in temperature dependence. Additionally, because Rubisco is not substrate-saturated and oxygenation is inhibited by higher [CO_2] concentrations in C_3 plants, a rise in the [CO_2] above current atmospheric levels increases photosynthesis (Long *et al.*, 2004). Plants might be affected by changes in both air temperature and [CO_2], as biochemical models predict that the assimilation of CO_2 by C_3 plants will be enhanced with temperature increases (Long, 1991; Sage and Kubien, 2007), which may also cause the raise in the optimal thermal point as was seen in this study.

E and g_s decreased by 34% and 38% respectively in plants grown at elevated [CO_2]. In similar, short-term experiments, high [CO_2] generally reduced stomatal aperture with variability among different functional groups of plants (Ainsworth and Rogers, 2007). Earlier studies in cacao, have also reported decreases in g_s and *E* at elevated [CO_2] in young plants in growth cabinets (Baligar *et al.*, 2008), glasshouses (Baligar *et al.*, 2021a) and open top chambers (Hebbar *et al.*, 2020). However, an increase in g_s in cacao plants under elevated [CO_2] was observed by Lahive *et al.* (2018) in 4-month-old cacao seedlings grown in glasshouses conditions which was associated with a higher SI. Increasing [CO_2] may limit photosynthesis if Ci/Ca decreases compared to that in current atmospheric [CO_2] due to a large reduction in g_s (Xiao *et al.*, 2021). In this study, although g_s declined at elevated [CO_2], there was a positive response in Ci/Ca in young cacao plants that might explain the enhancement in photosynthesis rate. The 137% increase in iWUE in plants grown at elevated [CO_2] compared to ambient [CO_2] observed here, was due to a combination of a reduction in g_s and an improvement in P_n. Similar responses in iWUE to elevated [CO_2] have been reported in young cacao plants (Baligar *et al.*, 2008, 2021a; Lahive *et al.*, 2018; Hebbar *et al.*, 2020). Extending from leaf level (iWUE) to more integrative plant level (Transpiration efficiency – *TE*), the water use efficiency involves a combination of physiological and morphological characteristics (Hatfield and Dold, 2019). Here, *TE* based on total dry biomass per unit of water consumed also increased on average by 66% in plants grown at elevated [CO_2] compared to ambient [CO_2]. Hebbar *et al.* (2020) also reported a sustained increase in *TE* of 6-month-old cacao seedlings subjected to [CO_2] of 550 and 700 ppm. The same authors reported no changes in leaf water potential (LWP) with [CO_2] elevation compared to ambient [CO_2] in well-watered conditions. However, here a small overall improvement in Ψ_{stem} (+16%) was observed with [CO_2] elevation. Although it has been noted that reducing the g_s and *E* does not always result in changes in LWP at high CO_2 concentrations (Bunce and Ziska, 1998), research has revealed that plants growing at elevated [CO_2] can lead to conditions in which LWP is higher (Wullschleger *et al.*, 2002).

An overall decline in g_s and *E* was observed under water deficit conditions. Stomatal closure is the most common response to drought stress preventing water loss through transpiration (Pirasteh-Anosheh *et al.*, 2016). Previous reports have shown similar responses in young cacao seedlings grown under water deficit conditions (Joly and Hahn, 1989a; Mohd Razi *et al.*, 1992; Araque *et al.*, 2012; Osorio Zambrano *et al.*, 2021). Stomatal closure contributed to drought-induced decreases in photosynthesis. The decline in both P_n and g_s led to a decrease in iWUE by 19%. Studies on the effects of water deficit in cacao have shown variable responses for young plants with increases and decreases in iWUE depending on the genotype (Araque *et al.*, 2012; Ávila-Lovera *et al.*, 2016; De Almeida *et al.*, 2016; Osorio Zambrano *et al.*, 2021) and it has been suggested as an indicator when comparing cultivars for drought tolerance (Daymond *et al.*, 2011; Alban *et al.*, 2016). However, when considering *TE* at the plant level, the present

results indicate an increase of 70% under water deficit conditions. In this study, although water deficit resulted in a decline in both dry plant biomass and water consumption, the reduction in total water transpired was much greater which would have been explained by reductions in LA. The 44% decrease in Ψ_{stem} under water deficit follows a similar response reported by previous authors (Joly and Hahn, 1989b; Deng *et al.*, 1990; Mohd Razi *et al.*, 1992; Araque *et al.*, 2012; Hebbar *et al.*, 2020; Osorio Zambrano *et al.*, 2021).

Although not significant, there was overall slight increase in g_s with an increase in temperature under water deficit conditions, whereas a sustained increase in E was observed irrespective of [CO_2] and watering treatments. This increase in E with increasing temperature is in contrast to previous studies, (Sena Gomes and Kozlowski, 1987; Raja Harun and Hardwick, 1988) and is, likely to be due to the removal of the confounding effect of VPD. This was coupled with an increase in Ci/Ca. It has been suggested that with a rise in temperature Ci/Ca should increase, due to lower water viscosity and higher photorespiration (Prentice *et al.*, 2014). In this study, the sustained increase of Ci/Ca with temperature increases was not reflected in photosynthesis, which declined above 36/27°C, which might be related to photochemical inhibition as observed through the chlorophyll fluorescence measurements.

iWUE increased with temperature up to 36/27°C followed by a decline was observed with further increases in temperature. These mirrored the response of photosynthesis which responded to temperature to a greater extent than g_s. However, when analysing whole-plant water use, there was a decrease in *TE* which is consistent with reductions in leaf area (discussed later) across the temperature treatments. Similarly, Hebbar *et al.* (2020) reported a decline in gravimetric water use efficiency in 6-month-old cacao seedlings grown in open top chambers at an average temperature of 3°C above ambient (~35.27/26.93°C). In the present study, the reduction in *TE* is explained by the impact of elevated temperature in the final biomass achieved and the high transpiration rate observed. Despite the slight increase in g_s under water stress, rising temperatures caused a significant decrease (more negative) in LWP as well as in R-S ratio under all the treatments. According to Sena Gomes and Kozlowski (1987), a decrease in the root-shoot ratio at elevated temperatures may lead to a decrease in LWP because roots are not able to absorb enough water to match the transpiration losses. Furthermore, leaf transpiration lowers plant water potential due to intermolecular forces between water molecules causing water to be under tension during transpiration (Vesala *et al.*, 2017).

At elevated [CO_2], g_s and E followed the temperature trend described above but at a lower magnitude. Using 6-months-old cacao seedlings grown under open-top chambers in India, Hebbar *et al.* (2020) did not observe significant changes in either g_s nor E from control temperature and ambient [CO_2] when plants were subjected to temperature elevation of 3°C and 550 ppm. As noted before, temperature

sensitivity is likely to be influenced by several variables, including VPD, genotype, whether plants were grown in the laboratory or in the field, and the temperature regimes applied (Slot *et al.*, 2016). Although Ci/Ca increased at temperatures above 36/27°C, it remained higher at elevated [CO_2] compared to ambient [CO_2]. This result suggests that the reduction in g_s, associated with high Ci, responds to the Ca even at elevated temperatures. Amelioration of the negative effect of increasing temperatures by elevated [CO_2] on LWP was more evident under water deficit conditions from 28.5/19.5°C to 36/27°C. However, above 36/27°C, elevated [CO_2] did not compensate the high temperature effect. Under controlled conditions, a similar mitigation effect of elevated [CO_2] to high temperatures (from 25/20°C to 42/34°C) on physiological parameters was observed in coffee plants up to an optimal temperature (37/30°C), above which the effect declined (Rodrigues *et al.*, 2016).

Extreme heat stress and drought often occur simultaneously and each of these stresses can aggravate the severity of the other (Duan *et al.*, 2014). As shown in this study, g_s and *E* declined under water deficit in order to avoid water loss through transpiration (Pirasteh-Anosheh *et al.*, 2016). However, stomatal control seems to be decoupled from transpiration under a warming scenario (above 33.5/24.5°C), which has been explained as a mechanism for leaf cooling to prevent overheating of the photosynthetic apparatus (Schulze *et al.*, 1973). Although stomatal opening facilitated [CO_2] diffusion from ambient air to the leaf, which led to increases in Ci/Ca ratio across the temperatures, the magnitude was much lower under water deficit conditions. However, the increases in Ci/Ca across the range of temperatures were not reflected in photosynthesis rate. Similarly, severe reductions in photosynthesis have been reported under both elevated temperatures and water deficit in several crops (Shah and Paulsen, 2003; Xu and Zhou, 2006; Sehgal *et al.*, 2017) including cacao seedlings (Hebbar *et al.*, 2020). In combination with water deficit, elevated temperatures also negatively impacted iWUE and LWP. Previously, Hebbar *et al.*(2020) conducted a study using 6-months-old cacao seedlings grown under open top chambers in India and reported significant decreases (more negative) in LWP in combination of water deficit and elevated temperatures 3°C above ambient. Typically, g_s has a strong relationship with leaf water status (Klein, 2014). However, the present study has shown that stomata control was also decoupled from Ψ_{stem}. The increase in *E* across the temperature treatments under water deficit conditions could be explained by the increase in g_s.

Chlorophyll fluorescence measurements are an easy and non-destructive method of looking at the relationship between metabolism and energy processes, which can be affected by environmental factors (Paknejad *et al.*, 2007). Plants that are exposed to environmental stress show a declining slope of maximum quantum yield in PSII (*Fv/Fm*) which is a useful criterion for measuring photo-inhibition (Angelopoulos *et al.*, 1996), while in terms of a plant's general state and vitality, the performance index (*PI*) provides important quantitative information regarding the function of both photosystems I and II

(Kalaji *et al.*, 2016). Here, *Fv/Fm* and *PI* were unaffected by elevated [CO_2], which is consistent with observations in Chapter 3. However, both parameters were affected by water deficit and elevated temperatures alone or in combination. Previously, *Fv/Fm* has been used as an indicator of water deficit stress in young cacao plants which varied from no effects (Ávila-Lovera *et al.*, 2016), significant decreases (Hebbar *et al.*, 2020) and decreases with differential genotypic responses (Araque *et al.*, 2012; Osorio Zambrano *et al.*, 2021). Furthermore *Fv/Fm* and *PI* declined (by 4.5% and 60.1% respectively) between 28.5/19°C and 40/31°C. *Fv/Fm*, has previously been used for assessing temperature stress in cacao with genotypic differences (Daymond and Hadley, 2004). However, working with 6-month-old cacao seedlings grown under open top chambers at an average temperature of 3°C above chamber control (~35.27/26.93°C), Hebbar *et al.* (2020) did not observe differences in *Fv/Fm*. In the present study, *Fv/Fm* declined above 38.5/29.5°C suggesting a certain temperature tolerance in cacao when VPD is low. In contrast *PI* declined above 31/22°C implying a major sensitivity to temperature. Similar observations were reported by Kalaji *et al.* (2012) exploring fluorescence parameters as early indicators for stress in Barley. The decline of both parameters with temperature elevation is much greater under water deficit condition suggesting an exacerbation of water stress with temperature. However, once 40/31°C is reached, no differences were apparent between well-watered and water deficit treatments.

Chlorophyll content was more responsive to temperature changes with increases up to 36/27°C, above which a decline was observed, whereas elevated [CO_2] enhanced chlorophyll content by 10% and water deficit caused a slight reduction of 6%. This contrasts with the experiment presented in Chapter 3 where neither temperature nor elevated [CO_2] had an effect on chlorophyll content. One explanation might be the wider range of temperatures tested here, and the responsiveness of different genotypes. Previous studies on the effect of environmental factors on chlorophyll content, have shown no differences at elevated [CO_2] (Hebbar *et al.*, 2020; Baligar *et al.*, 2021a), decreases under water deficit (Hebbar *et al.*, 2020) and varied responses to temperature (Sale, 1968; Daymond and Hadley, 2004; Hebbar *et al.*, 2020). It has been noted that photosynthesis depends largely on chlorophyll pigments, which are essential for photosynthetic processes and thus, plant growth (Y. Li *et al.*, 2018). Here, as expected, a relationship between chlorophyll content and photosynthesis was evidenced.

Stomatal density (SD) was affected by higher temperature, with an overall increase from 31/22°C to 36/27°C, above which there was a decline with further temperature increases. Similar temperature responses, were observed in the experiment presented in Chapter 3, working with two different genotypes. It has been reported that plants may acclimate to warmer environments by adjusting stomatal characteristics (Drake *et al.*, 2018). However, when the temperature was analysed in combination with elevated [CO_2] and water deficit, SD was variable. Additionally, no significant effects

of elevated [CO_2] and water treatment were observed in stomata index (SI), whilst increases in both SD and SI of 4-months-old Amelonado cacao seedlings grown in glasshouses conditions were observed under elevated [CO_2] and water deficit (Lahive *et al.*, 2018). Previous work has highlighted variability in stomatal responses to environmental factors for several crops (Wu *et al.*, 2018). The lack of conclusive observations about the impacts of environment on SD and SI may be attributed to short experimental periods. Stomatal pore openings and closures are generally adjusted when plants respond to short-term environmental changes (Zhou *et al.*, 2010) as was observed in this study (g_s response). However, long-term changes in the environment may affect the size of stomatal apertures, density, and distribution of stomatal cells in leaves (Yan *et al.*, 2017).

The stimulation of growth parameters such as height, stem diameter, number of leaves and total dry weight in response to elevated [CO_2] is consistent with a number of previous studies on juvenile cacao plants grown under controlled environment conditions (Baligar *et al.*, 2005, 2021a, 2021b; Lahive *et al.*, 2018; Hebbar *et al.*, 2020). Overall, the increase in total dry biomass was comparable to that observed for photosynthesis (47.3%). No differences in root-shoot ratio were observed between ambient and elevated [CO_2]. This shows that under well-watered conditions, elevated [CO_2] affected above and belowground components similarly during the experimental period. A review by Rogers *et al.* (1996) has noted substantial variations in root-shoot ratio to elevated [CO_2]: overall increases (59.6%), no changes (3.0%), and decreases (37.5%) explained by crop type, resources supply, and other experimental factors such as pot size. However, they also highlight that experimental duration had a strong influence on the lack of correlation between root-shoot ratio and pot size among the studies analysed. Here, under the short-term, there were no roots emerging from the bottom of the pots. Thus, it was assumed that root restriction did not occur and therefore did not exert sufficient influence to affect carbon partitioning under elevated [CO_2]. On the other hand, there was a notable decline in growth when plants were grown under water deficit conditions. The negative impact of water deficit on growth of young cacao plant has been noted in several studies (Deng *et al.*, 1990; Mohd Razi *et al.*, 1992; Dos Santos *et al.*, 2014; Lahive *et al.*, 2018; Hebbar *et al.*, 2020). When plants experience water deficit, their growth is reduced, and their biomass ratio changes, because plants tend to allocate more biomass to the roots instead of the shoots (Chaves *et al.*, 2002). Here, the decline in aboveground components was higher (47%) than the belowground (33%) reflecting a 24% increase in root-shoot ratio under water deficit.

In combination with water deficit, the enhancement of growth by elevated [CO_2] was generally maintained. The positive effect of elevated [CO_2] under water deficit conditions has also been noted by Lahive *et al.* (2018) who reported that young cocoa plants grown at ~32/20°C under glasshouse conditions showed a slight enhancement in leaf number, leaf dry weight and stem diameter. Furthermore, increases in plant height and total dry biomass was observed by Hebbar *et al.* (2020) when

6-months-old cacao seedlings grown in open top chambers at an average temperature of ~32/24°C were subjected to 700 ppm and water deficit. A meta-analysis of the combined effects of elevated [CO_2] and water supply on different crops, revealed that the stimulating effect of [CO_2] on biomass under drought conditions was closely related to increases in photosynthesis (van der Kooi *et al.*, 2016). The same correlation has been observed in the present study, where changes in photosynthesis and iWUE of plants under both elevated [CO_2] and water deficit, were reflected in the growth parameters. The lack of impact of elevated [CO_2] on total dry weight at the highest temperature (40/31°C) was consistent with the suppression of net photosynthesis at this temperature.

Previously, most studies in cacao have looked at the effect of sub-optimal temperatures on plant growth. Growth enhanced at daytime temperature of 30°C (Sale, 1969b), whilst increases in leaf number, stem diameter and plant height have been reported between 23.3 and 30°C (Sale, 1968; Sena Gomes and Kozlowski, 1987). Dry matter biomass has also been reported to vary with temperature, with increases of plant dry weight up to 26.7°C (Sale, 1968). However, other studies have reported a decline in plant dry weight at supra-optimal temperatures of 33.3°C (Sena Gomes and Kozlowski, 1987), and 3°C above the control temperature (~35.27/26.93°C) in Open Top Chambers (Hebbar *et al.*, 2020). These contrasting responses to increased temperature reported in cacao may be associated with differences in experimental conditions and genotypes. Here, looking at a wide range of temperatures with no confounding of VPD, there was a positive response across the range of temperatures between 31/22°C to 36/27°C for height and stem diameter, leaf area, shoot and root dry weight and total plant dry weight. However, there was a consistent increase in the number of leaves produced whereas root-shoot ratio declined across the temperature treatments. The sustained decline of root-shoot ratio observed here, suggests that increasing temperatures result in a greater retention of dry matter by aerial parts and less of it being translocated to roots. Similarly, Sena Gomes and Kozlowski (1987) reported a decrease in root-shoot ratio when cacao seedlings were subjected to growth temperatures above 22.2°C. Therefore for young plants, roots might be not able to absorb sufficient water to meet transpiration demands under high evaporative conditions

Despite the fact that plant height was consistently increased by elevated [CO_2] across the temperature ranges, the positive effect on stem diameter and number of leaves was mostly above 33.5/24.5°C. The fact that elevated [CO_2] did not significantly affect the root-shoot ratio suggests that extra assimilates were partitioned by the same proportion among plant tissues across the temperature regimes. The decline in total leaf area and increases in leaf number resulted in smaller individual leaves being produced under warmer temperatures, which was more noticeable under elevated [CO_2]. Esmail and Oelbermann (2011) reported increases in plant height, number of leaves, shoot and root biomass of *Cedrela odorata* L. and *Gliricidia sepium* (Jacp.) Walp seedlings with increases of temperature and in

combination with elevated [CO_2]. Similarly, in open top chambers, growth (plant height and dry matter biomass) increased in young cacao plants subjected to both elevated temperature (3°C above chamber control, ~35.27/26.93°C) and 550 ppm CO_2 Hebbar *et al.* (2020). However, although increases in [CO_2] have an important impact on plant carbon metabolism, increasing temperatures can counteract this (Norby and Luo, 2004). This study has shown that at the highest temperature of 40/31°C, the positive effect of elevated [CO_2] on the growth traits was overridden.

Drought and high temperature together have greater effects on plant growth and productivity compared to their individual effects (De Boeck *et al.*, 2016; Sehgal *et al.*, 2017). Under water deficit, increased growth reduction occurred at 40/31°C in terms of plant height, stem diameter and leaf dry weight, and above of 38.5/29.5°C in terms of shoot dry weight, root dry weight and total plant dry weight. Similarly, Hebbar *et al.* (2020) also noted the negative impact of high temperatures on biomass of young cacao plants grown under water stress conditions. Elevated temperatures can exacerbate the effect of severe water deficit on plant photosynthetic activity (Xu and Zhou, 2006; Yu *et al.*, 2012). This study has shown that photosynthesis was supressed under the combined effects of drought and high temperature which was reflected in the growth parameters.

This study has shown that plants grown at elevated [CO_2] have increased leaf area (LA), relative growth rate (RGR), net assimilation rate (NAR), decreased leaf area ratio (LAR) whilst specific leaf area (SLA) was unaffected whereas plants grown under water deficit exhibited in a significant decline in all these parameters. Increases in LA, RGR and NAR and a decline in SLA have been previously observed in young cacao plants grown at elevated [CO_2] (Baligar *et al.*, 2005, 2021a, 2021b; Lahive *et al.*, 2018; Hebbar *et al.*, 2020). However, a decline in LA, SLA, RGR and NAR with no changes in LAR were reported when cacao seedling were subjected to water deficit (Mohd Razi *et al.*, 1992; Dos Santos *et al.*, 2014; Lahive *et al.*, 2018; Hebbar *et al.*, 2020). Under water deficit conditions, CO_2 enrichment increased LA and SLA above 36/27, and RGR and NAR across the temperature range which suggests that elevated [CO_2] had a compensating effect on growth parameters. A few reports on how elevated [CO_2] and water deficit act on these parameters in cocoa have been published with varied outcome reporting slight improvements or no changes in LA, and no changes in SLA in plants grown under water deficit and subjected to elevated [CO_2] (Lahive *et al.*, 2018; Hebbar *et al.*, 2020).

Increased temperature did not change LAR but a decline in LA, SLA, RGR and NAR above 36/27°C was observed. Here, the reduction in LA at high temperatures suggests an adaptive mechanism in order to reduce water loss from leaves by controlling transpiration as observed earlier. The LA, SLA, RGR and NAR trend, correlates with photosynthesis which showed a strong decline above 36/27°C under well-watered conditions. A lack of an effect on LAR indicates that both LA and total plant dry weight decline

similarly across the temperature range. Previous research has shown progressive increases in LA and RGR from 18.7°C to 33.3°C in cocoa seedlings growth under controlled environment conditions (Sena Gomes and Kozlowski, 1987). The same authors, suggested that decreases in plant growth at high temperatures might have be associated with increases in respiration. It has been noted that photorespiration increases faster than photosynthetic rates when leaf temperatures rise (Long, 1991). Here, the decline in photosynthesis above 36/27°C and the sustained increase in dark respiration with temperature elevation, may explain the observed reduction in growth parameters.

Light interception is determined by leaf area, which is a crucial component of plant productivity (Gifford *et al.*, 1984). Although LA and RGR declined above 36/27°C, they were higher at elevated [CO_2] compared to ambient [CO_2], and the difference was more evident in well-watered plants compared to water stressed plants. However, at 40/31°C the positive effect of elevated [CO_2] on LA and RGR was not significant suggesting a strong negative effect on these parameters of the highest temperature. As discussed previously, elevated [CO_2] stimulates crop biomass accumulation and potentially increases yield in C_3 plants. However, the effectiveness of [CO_2] enrichment depends on growth temperature (Kimball *et al.*, 1995; Morison and Lawlor, 1999). A positive effect of elevated [CO_2] in NAR across the temperatures was also noted, although a strong decline above 36/27°C was evident under well-watered conditions but not in the water deficit treatment. The reduction in NAR under well-watered conditions, can be explained by the decline in RGR while LAR remained unchanged across the temperature range. This study indicates how elevated [CO_2] may improve photosynthetic thermo-tolerance in young cacao plants under these experimental conditions. Consequently, the combined effects of elevated [CO_2] and temperature should be considered when predicting future changes in productivity and suitable areas for production.

To conclude, the results of this experiment showed that elevated [CO_2] could mitigate the effects of water deficit in young cocoa plants under warmer conditions by increasing photosynthesis and growth. However, the extent of this amelioration depends on the intensity of temperature stress and water deficit and whether they occur as a single factor or combined. Additionally, under non-limiting aerial and soil water conditions, elevated [CO_2] shifted the optimum temperature for net photosynthesis rate from 36/27°C to 38.5/29.5. However, at 40/31°C the positive effect of elevated [CO_2] was no longer evident. Furthermore, the compensatory effect of elevated [CO_2] evident on growth and biomass parameters was related with changes in photosynthesis rate. The information provided here is based only on a relatively short-term experiment, using young cacao plants and a single genotype. Therefore, whether this response is reflected in mature plants and on a range of genotypes is explored in the next chapter.

5   Combined effect of elevated [CO_2] and temperature on plant growth and physiology of six contrasting mature cacao genotypes (*Theobroma cacao* L.)

5.1   Introduction

In the short-term, elevated [CO_2] may promote growth, biomass and yield by improving the net photosynthesis rate (Norby *et al.*, 1999; Ainsworth and Long, 2005; van der Kooi *et al.*, 2016) as seen in the previous two chapters. Although positive effects of elevated [CO_2] on photosynthesis and vegetative growth has been seen in cacao, much of the available data are based on juvenile seedlings (Baligar *et al.*, 2005, 2008, 2021a, 2021b; Lahive *et al.*, 2018). However, studies in cacao mature trees are still scarce. Recently, Lahive *et al.* (2021) examining the effects of elevated [CO_2] on growth, reported that the enhancement in growth appeared to be lower in magnitude in older trees compared to young trees (Lahive *et al.*, 2021). The authors suggested that respiratory maintenance and reproductive sinks in the mature trees may have resulted in the decline of the vegetative components. It has been noted that the long-term impact of elevated [CO_2] will depend on how photosynthesis adjusts to future conditions (Ghildiyal and Sharma-Natu, 2000). When leaves are exposed to elevated [CO_2] for a long period their photosynthetic capacity might decline (acclimation or downregulation). This decline can occur due to lower nitrogen concentration and Rubisco activity, a change in the source-sink balance due to leaf carbohydrate accumulation and a decline in stomatal conductance (Drake *et al.*, 1997; Cotrufo *et al.*, 1998; Makino and Mae, 1999; Jifon and Wolfe, 2002; Long *et al.*, 2004; Ainsworth and Long, 2005; Kanemoto *et al.*, 2009). However, a sustained enhancement of dry biomass and leaf and canopy-level photosynthesis were observed on six mature cacao genotypes grown for 23 months under elevated [CO_2] in controlled conditions (Lahive *et al.*, 2021). The authors also noted that the positive effect of elevated [CO_2] was maintained even under water-limiting conditions, and that increase in photosynthesis was greater than the stimulation in growth. However, whilst elevated [CO_2] might be expected to stimulate photosynthesis, the extent to which this takes place depends on other environmental factors (Leakey *et al.*, 2009; Dusenge *et al.*, 2019). To date, there is limited information on the long-term effects on plant growth and physiology of adult cacao trees exposed to elevated [CO_2] and how mature trees will respond in combination with a warming scenario.

Among the most important controls on the distribution of species around the world is temperature, and most biological processes are temperature-sensitive (Dusenge *et al.*, 2019). Under ambient [CO_2] conditions, temperatures above optimal result in a decline in photosynthesis and an increase in respiration rate, affecting the carbohydrate availability for plant growth (Salvucci and Crafts-Brandner,

2004; Liu and Huang, 2008). Although limited, studies have shown that temperature variation is an important factor for cacao physiology, growth, and development (Daymond and Hadley, 2004; De Almeida and Valle, 2007). The optimum temperature for photosynthesis in cacao has been reported as being between 31-33°C (Balasimha *et al.*, 1991) and 33-35°C (Yapp, 1992). However, as was shown in Chapters 3 and 4, when vapour pressure deficit (VPD) was not a limiting factor, the optimum temperature for growth and photosynthesis appeared to be higher. Changes in temperature may also impact stomatal conductance (Sena Gomes and Kozlowski, 1987; Raja Harun and Hardwick, 1988), plant growth and development traits (Sale, 1968, 1969b; Sena Gomes and Kozlowski, 1987; Cazorla *et al.*, 1989).

Considering the challenge of climate change, it is crucial to evaluate the potential impact of future environmental conditions, particularly those associated with air temperature and increases in [CO_2]. Way *et al.* (2015) noted that these two environmental factors may either exacerbate or counteract their independent effects while other reports have highlighted the alleviation of high temperature stress by enhanced [CO_2], by increasing tree growth and productivity (Boisvenue and Running, 2006). Whilst efforts have been taken to examine the independent effects of elevated [CO_2] and temperature on plant growth and physiology of cacao, studies addressing the combined impact of [CO_2] and temperature are still scarce. In a recent study, using 6-month-old cacao seedlings of VTLCC1 variety grown for 7 months under open-top chambers (OTC) and subjected to elevated [CO_2] (550 ppm) and high temperature (average maximum temperature of 36.5°C), Hebbar *et al.* (2020), observed that increasing [CO_2] improved photosynthesis and biomass accumulation while the impact of high temperatures resulted in a severe reduction of photosynthetic parameters and plant growth. The authors also suggested that, in combination, elevated [CO_2] would mitigate the negative effect of elevated temperatures. It has been hypothesized that the CO_2 enrichment mitigation of the adverse effects on high temperatures could be associated with the maintenance of a positive carbon balance through stimulation of photosynthesis and reductions in leaf respiration rates (Song *et al.*, 2014). Using cacao seedlings in short-term (3 months) experiment in this study (Chapters 3 and 4), a compensatory effect of elevated [CO_2] was noted under non-limiting water conditions being more evident in some genotypes than in others. However, it is unclear as to whether this compensation will remain stable over a prolonged period of time in older trees and if it is similar in different cacao genotypes.

Important genotypic variation in physiology and plant growth responses to both elevated [CO_2] (Lahive, 2015; Handley, 2016; Baligar *et al.*, 2021a; Lahive *et al.*, 2021) and temperature (Daymond and Hadley, 2004) have been reported for cacao. Understanding how genotypic variation affects physiological characteristics in response to environmental changes might facilitate the identification of germplasm with better performance under future climate scenarios, and could also be implemented in the breeding

of new materials better adapted to elevated [CO_2] and/or high temperatures. Little is known about the long-term interactive effect of elevated [CO_2] and temperatures on mature cacao trees. Therefore, the aim of this study was to examine the plant growth and photosynthesis responses of six mature cacao genotypes to the combined effects of elevated [CO_2] and three different temperature regimes in controlled environment conditions (Glasshouses). The hypotheses for this experiment were: i) there is genotypic variation in growth and photosynthesis in response to the combined effects of elevated [CO_2] and temperature; ii) Mature cacao plants would remain responsive to an increase in atmospheric [CO_2] in a future warmer climate; iii) The enhancement in growth at elevated [CO_2] and temperature is less pronounced in mature cacao plants compared to juvenile cacao plant responses in growth chambers.

5.2   Materials and Methods

5.2.1   Plant material and experimental design

Three-year-old cacao trees of six different genotypes (CCN 51, SCA 6, ICS 6, IMC 20, PA 7 and T85/799) were used in this experiment. Initially, the plants were obtained from *in vitro* propagation using the somatic embryogenesis method at Nestlé Research Centre in Tours, France described in detail in Chapter 3. In February 2017, young plants were received and transferred to controlled glasshouses at the Crops and Environment Laboratory (CEL), the University of Reading. In March 2017, all the plants were first planted into 10 L pots, and in April 2018 were subsequently re-potted into 50 L pots filled with a growth substrate comprising a mixture of sand, gravel, and vermiculite (1:2:2 vol:vol:vol). From March 2017 until treatments were initiated, the plants were grown in glasshouses at ambient [CO_2] and day and night temperatures of 31°C and 21°C respectively. Supplementary lighting was used when irradiance fell below 148 µmol. m^{-2} s^{-1} and to maintain a day length of 12 hours (details are provided in Chapter 2). Shade screens (50%) were used to minimize excessive light during sunny days and closed at irradiances above 648 µmol m^{-2} s^{-1}. The plants were watered six times per day with Long Ashton nutrient solution modified for use in cacao (End, 1990).

The experiment started on 17-09-2019 and ran for 378 days until 29-09-2020. The six mature cacao genotypes were arranged into six controlled glasshouse compartments (each one measured 10m x 6m x 3.8 m). Each glasshouse was divided into two sections in order to create two East-West blocks to allow for a potential light gradient across each experimental area. Replicates, which varied in number for each genotype (from eight to three) due to some limitation on tree availability, were allocated randomly within each block in each glasshouse. The treatments consisted of a combination of three temperature regimes and two CO_2 concentrations and were assigned in each of the six glasshouses during the experimental period (Figure 5.1). The [CO_2] treatments comprised two levels: ambient (a target of 400ppm) and elevated (a target of 700ppm). Temperatures were set to three different

maximum/minimum regimes following a daily sine wave temperature profile, between a minimum temperature at 06:00 h and a maximum temperature at 14:00 h. The three temperature regimes were: T1 (control temperature), T2 (control temperature + 2.5°C) and T3 (control temperature + 5.0°C). The mean daily maximum/minimum control temperature corresponds to the current average in the cacao-growing regions in Ghana during the last 42 years. Within each temperature treatment, three different temperature phases were applied to simulate seasonal temperature shifts experienced throughout the year in Ghana (West Africa): phase 1 (dry season) from June to September, phase 2 (major rainy season) from October to January, and phase 3 (minor rainy season) from February to May. Maximum and minimum temperature values for each of the phases in each of the experimental temperature regimes are presented in Table 5.1. The actual average temperature, $[CO_2]$ (include day and night values) and relative humidity recorded within each glasshouse during the experimental period is summarised in Table 5.2. Regarding ambient $[CO_2]$, values were slightly higher than the target.

T1 a[CO$_2$]				T3 a[CO$_2$]				T3 e[CO$_2$]			
I		**II**		**I**		**II**		**I**		**II**	
IMC20	ICS6	CCN51	T85/799	PA7	ICS6	ICS6	T85/799	T85/799	IMC20	PA7	CCN51
T85/799	PA7	IMC20	SCA6	SCA6	CCN51	T85/799	CCN51	SCA6	CCN51	SCA6	T85/799
CCN51	SCA6	PA7	ICS6	CCN51	PA7	CCN51	SCA6	ICS6	T85/799	CCN51	PA7
ICS6	T85/799	CCN51	CCN51	T85/799	ICS6	SCA6	ICS6	CCN51	ICS6	ICS6	CCN51
SCA6	CCN51	SCA6	IMC20	SCA6	CCN51	CCN51	IMC20	T85/799	CCN51	SCA6	IMC20
PA7	CCN51	PA7	CCN51	CCN51	SCA6	IMC20	PA7	IMC20	PA7	IMC20	SCA6
CCN51	IMC20	T85/799	T85/799	ICS6	T85/799	T85/799	CCN51	PA7	ICS6	CCN51	ICS6
	ICS6	SCA6		IMC20	IMC20			CCN51	SCA6	T85/799	

I	**II**		**I**		**II**		**I**	**II**			
CCN51	SCA6		CCN51	ICS6	IMC20	ICS6		SCA6	CCN51		
SCA6	CCN51		PA7	CCN51	ICS6	SCA6		CCN51	CCN51		
CCN51	SCA6		ICS6	T85/799	T85/799	PA7		CCN51	SCA6		
SCA6	CCN51		IMC20	PA7	CCN51	CCN51		SCA6	CCN51		
CCN51	CCN51		SCA6	CCN51	ICS6	IMC20		SCA6	CCN51		
CCN51	CCN51		T85/799	T85/799	PA7	T85/799		CCN51	SCA6		
	SCA6		CCN51	SCA6	SCA6	CCN51		CCN51			
			SCA6	IMC20	CCN51						

T2 e[CO$_2$]				**T1 e[CO$_2$]**				**T2 a[CO$_2$]**			

Figure 5.1 Arrangement of climatic treatments ($[CO_2]$ x Temperature) and six mature cacao genotypes (CCN 51, SCA 6, ICS 6, IMC 20, PA 7 and T85/799) across 6 glasshouses. Each box represents a glasshouse compartment. Black dashed lines show the blocks within each glasshouse. Temperature treatments are T1 (control temperature), T2 (control temperature + 2.5°C), and T3 (control temperature + 5.0°C). $[CO_2]$ treatments are a$[CO_2]$ (ambient) and e$[CO_2]$ (elevated).

Table 5.1 Maximum and minimum set temperatures within the greenhouse experiment, the Control simulated the conditions in the cocoa-growing regions of Ghana: phase 1 from June to September, phase 2 from October to January, and phase 3 from February to May.

Temperature	Control temperature			Control temperature + 2.5°C			Control temperature + 5°C		
	Phase 1	Phase 2	Phase 3	Phase 1	Phase 2	Phase 3	Phase 1	Phase 2	Phase 3
Maximum	33.5	29.8	30.9	36.0	32.3	33.4	38.5	34.8	35.9
Minimum	22.1	21.8	21.6	24.6	24.3	24.1	27.1	26.8	26.6

Table 5.2 Climatic glasshouses conditions during the experimental period (mean over 378 days from 17-09-19 to 21-07-20). T1 a[CO_2]= control temperature and ambient [CO_2], T1 e[CO_2]= control temperature and elevated [CO_2], T2 a[CO_2]= control temperature + 2.5°C and ambient [CO_2], T2 e[CO_2]= control temperature + 2.5°C and elevated [CO_2], T3 a[CO_2]= control temperature + 5.0°C and ambient [CO_2], and T3 e[CO_2]= control temperature + 5.0°C and elevated [CO_2].

	Mean daily temperature (°C)	[CO_2] (ppm)*	Relative Humidity (%)
T1 a[CO_2]	26.2	474.7	58.1
T1 e[CO_2]	26.5	663.3	60.7
T2 a[CO_2]	28.3	483.8	56.4
T2 e[CO_2]	28.1	708.2	58.8
T3 a[CO_2]	31.1	414.8	61.1
T3 e[CO_2]	30.9	680.8	58.7

*[CO_2] values were slightly above the set target

5.2.2 Plant growth measurements

Non-destructive growth measurements. The stem girth (cm) of each tree was measured at 20 cm from the top of the substrate using a flexible metric tape on 17-09-2019, which corresponded to the day on which treatments were imposed. A second value was recorded on 10-08-2020 (328 days from the imposition of treatments and before the final plant harvest). The initial stem girth was subtracted from the final measurement to calculate the stem girth increment (cm) during the experimental period. Once a week, two tagged branches of each tree were monitored from Nov 2019 to May 2020. The flushing (hereafter called inter-flush, days) period was calculated as the time elapsed between the appearance of the last leaf of one flush and the appearance of the first leaf of the following flush. Once the flush was developed, number of leaves per flush and flush length (cm) were recorded.

Destructive measurements. From 10-08-2020 to 21-08-2020, all the cacao trees of three genotypes (ICS 6, IMC 20 and PA 7) from treatment combinations T1a[CO_2], T1e[CO_2], T3a[CO_2], and T3e[CO_2] were destructively harvested. The trees were cut at the top of the substrate and divided into mature and

immature leaves (leaves that have not fully hardened), branches and trunk and fresh weights were recorded using a balance (CBK 32, *Adam Equipment*, Milton Keynes, UK). Leaf area (cm^2) was obtained using a WD3 WinDIAS leaf image analysis system (*Delta T Devices*, Cambridge, UK). For this, a sub-sample was measured and total leaf area calculated from the ratio of the subsample leaf fresh weight to the total leaf fresh weight. Roots were removed from the substrate, washed and the fresh weight was also recorded. All the samples were oven-dried to a constant weight at 70°C and dry weights were recorded to obtain aboveground dry biomass (g), root dry biomass (g) and total plant dry biomass (g).

5.2.3 Measurements at leaf level

Stomatal parameters. On 24-04-2020 before the destructive harvest, using clear fingernail polish and transparent adhesive tape, leaf imprints were made from the abaxial surface of one fully expanded and hardened leaf from each tree in each glasshouse treatment combination. The imprints were viewed at 400x magnification with a *Leitz Dialux 20* light microscope with a *Leica DFC450* digital camera attached and *Leica Application suite* software version 4.6.2 (*Leica Microsystems*, Wetzlar, Germany). Three digital images were recorded from random areas of each imprint. Number of stomata and epidermal cells were counted using *ImageJ* version 2.2 analysis software (Rueden *et al.*, 2017). The number of stomata per image was converted to stomatal density (SD, stomata mm^2), and the stomatal index (SI) which relates the number of stomata per unit area (SD) to the number of epidermal cells per unit area (ECD), was calculated as SI= [SD/ (ECD+SD)]*100 (Salisbury, 1927).

Instantaneous gas exchange parameters. Leaf gas exchange measurements were conducted between 17-02-2020 and 21-02-2020 on all the genotypes in each treatment combination of temperature and [CO_2] using a portable *LC pro*-SD infrared gas analyser (IRGA) fitted with an artificial light attachment and an internal CO_2 source (*ADC BioScientific*, Great Amwell, Herts., UK). The measurements were carried out between 09:00 to 13:00 on an exposed youngest fully expanded and hardened leaf from each of four plants per genotype. For each measurement, the IRGA leaf cuvette was held at the [CO_2] (either 400 or 700 ppm for ambient and elevated CO_2), and temperature treatment (maximum growth temperature) associated with each growth condition. The irradiance was set at 696 µmol m^{-2} s^{-1} photosynthetically active radiation (PAR) which is saturating for cacao (Baligar *et al.*, 2008; Lahive *et al.*, 2018). Net photosynthetic rate (*Pn*), stomatal conductance (*gs*,) and leaf transpiration (*E,*) measurements were recorded after 10 min of IRGA stabilization. Intrinsic water-use efficiency (iWUE) was calculated as the ratio between *Pn* and *gs*.

Additionally, from 29-09-2020 to 03-10-2020 after 378 days of long-term treatment exposure, photosynthetic acclimation to elevated [CO_2] and temperature was tested in trees of CCN 51. A young fully mature and fully hardened leaf from each replicate tree of CCN 51 was used for the measurements

(n=4). Gas exchange measurements were carried out at an irradiance of 696 µmol m^{-2} s^{-1} between 09:00 to 13:00 hr on trees grown at T1a[CO_2], T1e[CO_2], T3a[CO_2] and T3e[CO_2]. For each glasshouse treatment combination ([CO_2] x Temp), photosynthetic rates were recorded at both elevated (~700ppm) and ambient (~400 pm) [CO_2] and both control (31°C) and maximum set temperature (36°C) after 10 min of the IRGA stabilization. Measurement CO_2 and temperature was controlled within the IRGA chamber. The same leaves for gas exchange measurements were harvested and oven-dried at 70°C to constant weight before being ground to a fine powder. Ground dried samples (~0.2 g) were analysed for carbon and nitrogen content using a *LECO CNH628* Series Elemental Analyser (*LECO Corporation*, Michigan, US).

Light- response curve parameters. Between 19-03-2020 and 03-04-2020, photosynthetic light-response curves were measured on one young, fully expanded and hardened leaf from four plants of the genotypes CCN 51, ICS 6, SCA 6 and T85/799 at the growth conditions of T1a[CO_2], T1e[CO_2], T3a[CO_2] and T3e[CO_2], with a portable infrared gas analyser (IRGA) fitted with an artificial light attachment and an internal CO_2 source (*LC pro*-SD *, ADC BioScientific*, Great Amwell, Herts., UK). The leaf inside the IRGA cuvette was allowed to stabilize at the highest irradiance for 20 min before photosynthesis was measured at eight light intensities (Q) of 696, 435, 348, 261, 174, 87, 44 and 0 µmol m^{-2} s^{-1} PAR. Each of the light intensities was held for 5 minutes before a measurement was made. During measurements the temperature and [CO_2] within the cuvette was set to the growth [CO_2] and maximum growth temperature of the glasshouse within which measurements were being made. Measurements were made between 09:00 and 14:00 hr. A non-rectangular hyperbola was fitted to the photosynthetic light response curve data (Prioul and Chartier, 1977) in the form: Pn= {∅ Q + A$_{max}$ - √ [(∅ Q + A$_{max}$) − 4 ∅ Q k A$_{max}$]/2 k} − Rd, where Pn is net photosynthesis rate, k is the convexity, ∅ apparent quantum yield, Q is irradiance, A$_{max}$ is light-saturated gross photosynthetic rate (hereafter referred to as light-saturated photosynthesis rate) and Rd is apparent dark respiration. Curve fitting was carried out using the Microsoft Excel spreadsheet tool provided by Lobo *et al.* (2013), and the parameters of A$_{max}$, ∅, Rd, light compensation point (LCP) and light saturated point (LSP) were extracted from fitted curves.

Chlorophyll parameters. Chlorophyll content was measured on the same leaf used for the instantaneous gas exchange measurements using a CL-01 field-portable chlorophyll meter (*Hansatech Instruments Ltd.*, Norfolk, UK). Chlorophyll meter units obtained were transformed to chlorophyll content (ChlCont) through the conversion for cacao: ChlCont = (1.945 × chlorophyll meter reading) + 11.392 provided by Daymond *et al.* (2011). Subsequently, chlorophyll fluorescence (maximum quantum efficiency of photosystem II - *Fv/Fm*, ratio and the performance index - *PI*) was measured on the same leaf following dark-adaption using clips for at least 30 min, using a portable chlorophyll fluorimeter (Handy PEA, Hansatech Instruments Ltd, Norfolk, UK).

5.2.4   Data analysis

Glasshouses were blocked (as shown in the Figure 5.1), and balanced (for dry biomass, leaf area, light response parameters and acclimation) and unbalanced (others parameters under study) ANOVA were used to test the effects of genotypes, $[CO_2]$, two temperatures (T1 and T3), and their interactions. Normal distribution (Shapiro-Wilk) and equal variances (Levene's test) were verified before fitting the ANOVA models. Transformation (log or square-root) was carried out in order to normalise and homogenise data on stem diameter increment, net photosynthesis rate, intrinsic water use efficiency, leaves per flush, leaf transpiration rate, chlorophyll content, *Fv/Fm*, quantum efficiency, dark respiration, light compensation point and light saturation point. Bonferroni post hoc test was used to compare means where ANOVA detected significant effects (P value < 0.05). Statistical analyses were carried out using GENSTAT 19th edition software (*VSN International Ltd.*, Hemel Hempstead, UK) graphs were produced using R version 4.0.4 (R Core Team, 2021).

5.3   Results

5.3.1   Plant growth parameters

***Stem girth increment**.* Genotypes showed significant differences in stem diameter increment (P<0.001; Figure 5.2) ranging from 1.0 (±0.2) cm in T 85/799 to 2.0 (±0.1) cm in CCN 51. Additionally, an effect of $[CO_2]$ among the genotypes (P<0.05) was observed such that stem diameter increased by 49%, 67% and 155%, at elevated $[CO_2]$ compared to ambient $[CO_2]$ in CCN 51, PA 7 and IMC 20 respectively, whilst there was no differences between $[CO_2]$ in ICS 6, SCA 6 and T 85/799. No significant effects of temperature or other treatment interactions were observed for stem diameter increment.
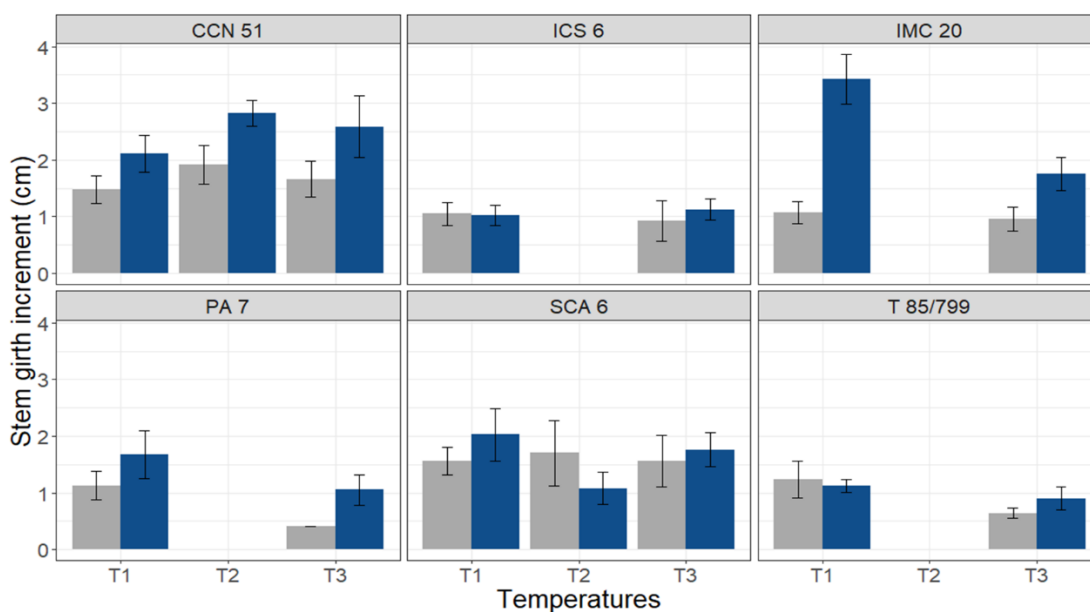
Figure 5.2 Effect of the [CO$_2$] and temperature on stem girth increment of six mature cacao genotypes. Error bars show the standard error of the mean. [CO$_2$] treatments are ambient (grey bar) and elevated (blue bar). Temperature treatments are T1 (control temperature), T2 (control temperature + 2.5°C), and T3 (control temperature + 5.0°C) as summarised in Table 5.2.

Leaves per flush. Significant differences were observed between genotypes in number of leaves per flush ($P<0.001$; Figure 5.3). Overall, there were 13% more leaves per flush at elevated [CO$_2$] compared to ambient [CO$_2$] ($P<0.01$). A significant interaction between temperature treatments and genotypes was observed ($P<0.05$). Leaves per flush increased by 30%, 32% and 50% at T3 compared with T1 in ICS 6, PA 7 and T85/799 respectively. However, temperature did not affect number of leaves per flush in CCN 51, IMC 20 and SCA 6.
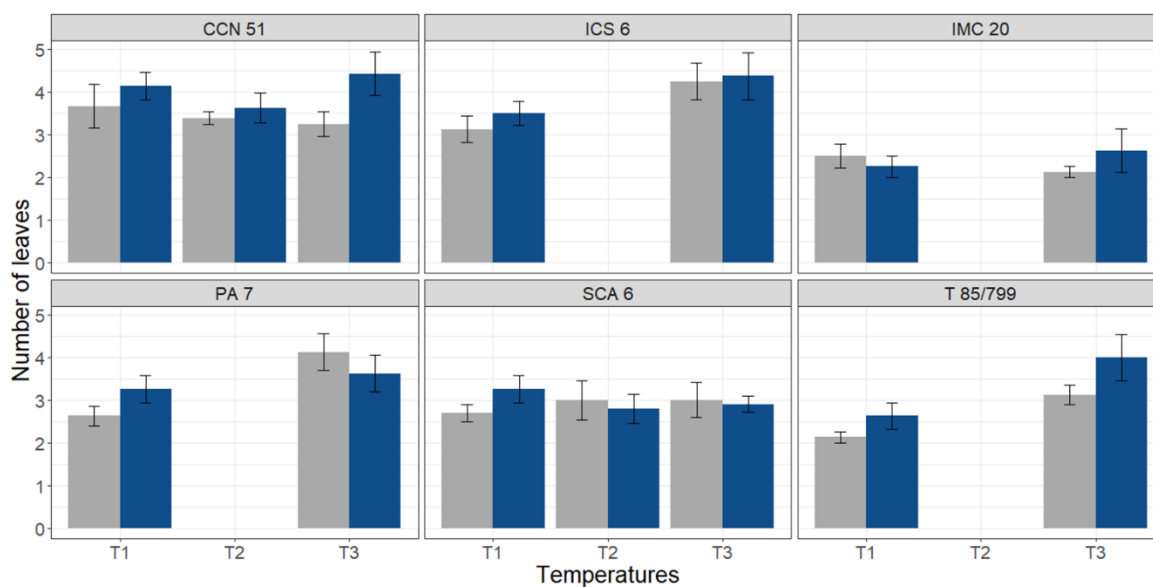
Figure 5.3 Effect of the $[CO_2]$ and temperature on leaves per flush of six mature cacao genotypes. Error bars show the standard error of the mean. $[CO_2]$ treatments are ambient (grey bar) and elevated (blue bar). Temperature treatments are T1 (control temperature), T2 (control temperature + 2.5°C), and T3 (control temperature + 5.0°C) as summarised in Table 5.2.

Inter-flush period. The response of inter-flush period to $[CO_2]$ and temperature varied among the genotypes under study ($P<0.001$) (Figure 5.4). For ICS 6, under elevated $[CO_2]$, the inter-flush period decreased by 25% compared to ambient $[CO_2]$ at T1 whereas it increased by 17% at T3. Conversely, in IMC 20 compared to ambient $[CO_2]$, the inter-flush period increased by 15% in response to elevated $[CO_2]$ at T1 while it decreased by 32% at T3. In T85/799, irrespective of the temperature, inter-flush period decreased by 19% under elevated $[CO_2]$. No independent or interactive effects of temperature and elevated $[CO_2]$ were observed in CCN 51, PA 7 and SCA 6.
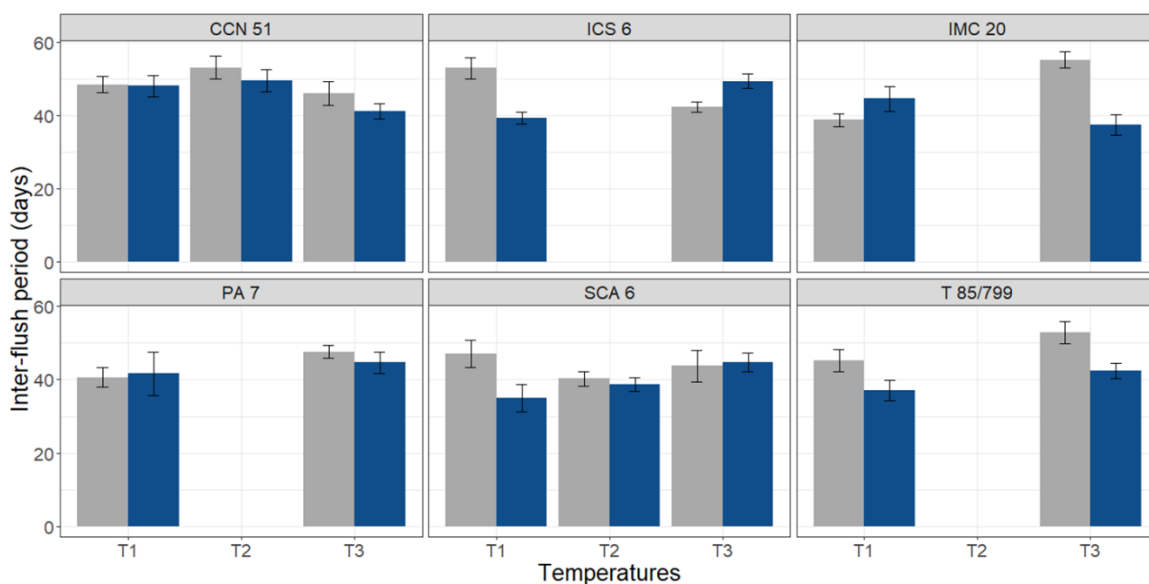
Figure 5.4 Effect of the [CO_2] and temperature on inter-flush period of six mature cacao genotypes. Error bars show the standard error of the mean. [CO_2] treatments are ambient (grey bar) and elevated (blue bar). Temperature treatments are T1 (control temperature), T2 (control temperature + 2.5°C), and T3 (control temperature + 5.0°C) as summarised in Table 5.2.

Flush length. The effects of [CO_2] and temperature on flush length varied among the genotypes under study (*P*<0.01; Figure 5.5). A 5.0°C increase above the control temperature (T3) decreased flush length by 32% in CCN 51 but increased flush length by 50% in T85/799. Irrespective of the temperatures, flush length increased by 38% at elevated [CO_2] in CCN 51. No change in flush length was observed between [CO_2] treatments at T1 for ICS 6, whereas it was 20% longer in elevated [CO_2] than ambient [CO_2] at T3. For IMC 20, under elevated [CO_2], flush length was 29% shorter at T1 compared to ambient [CO_2] whilst it increased by 70% at T3 in the elevated [CO_2] treatment. There were no significant changes in flush length neither under temperature nor [CO_2] treatments in PA 7.
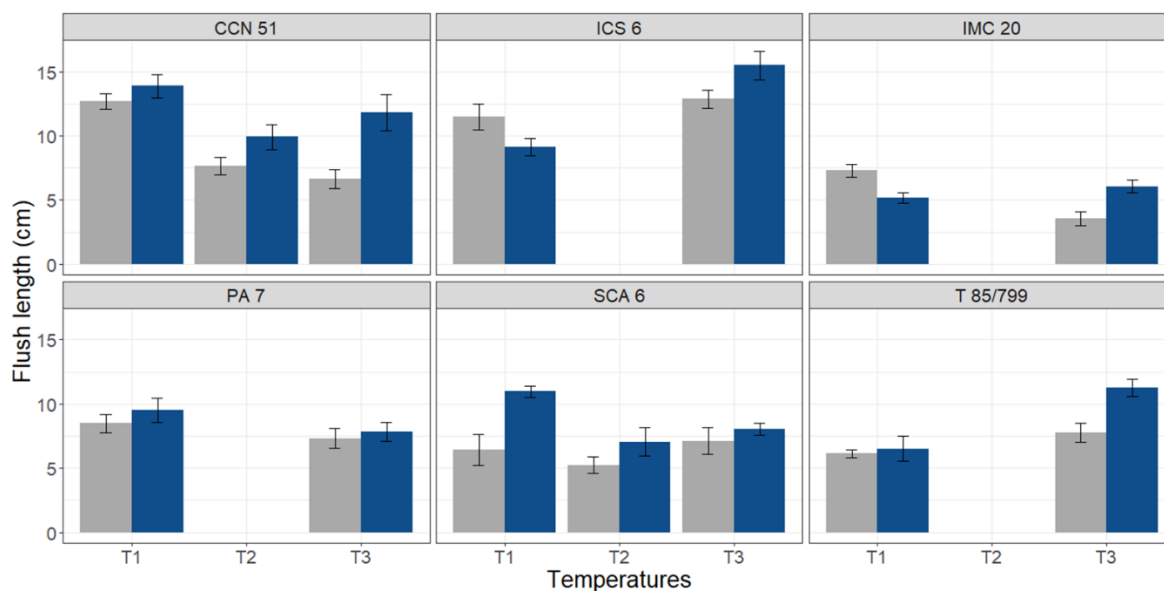
Figure 5.5 Effect of the [CO_2] and temperature on flush length of six mature cacao genotypes. Error bars show the standard error of the mean. [CO_2] treatments are ambient (grey bar) and elevated (blue bar). Temperature treatments are T1 (control temperature), T2 (control temperature + 2.5°C), and T3 (control temperature + 5.0°C) as summarised in Table 5.2.

Aboveground dry biomass. The influence of both temperature and elevated [CO_2] on aboveground dry biomass varied according to genotype ($P<0.01$ and $P<0.05$ respectively) (Figure 5.6). With the temperature increase from T1 to T3, aboveground dry biomass declined in both IMC 20 (21%) and PA 7 (35%), however, aboveground dry biomass of ICS 6 was unaffected. Under elevated [CO_2], aboveground dry biomass increased by 32% compared with ambient [CO_2] in IMC 20 while [CO_2] did not significantly influence biomass in PA 7. However, for ICS 6 aboveground dry biomass declined in the elevated [CO_2] treatment at T1.
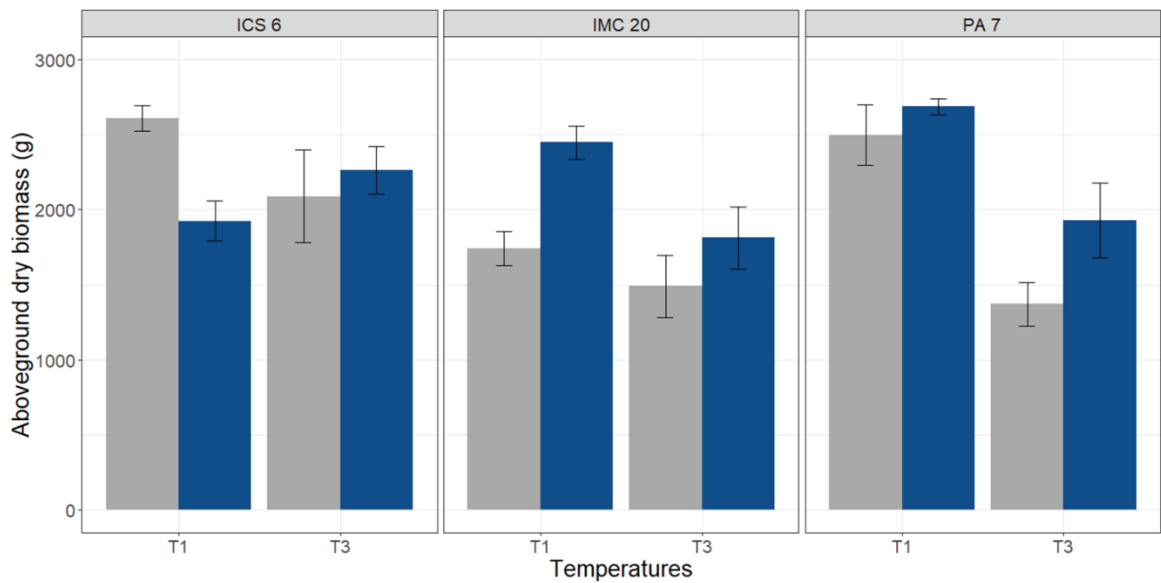
Figure 5.6 Effect of the $[CO_2]$ and temperature on aboveground dry biomass of three mature cacao genotypes. Error bars show the standard error of the mean. $[CO_2]$ treatments are ambient (grey bar) and elevated (blue bar). Temperature treatments are T1 (control temperature), and T3 (control temperature + 5.0°C) as summarised in Table 5.2.

Root dry biomass. Across the temperature and $[CO_2]$ treatments, root dry biomass was significantly ($P<0.001$) greater in PA 7 (1391 (±68) g) compared to ICS 6 and IMC 20 (1019 (±60) g and 804 (±66) g respectively) (Figure 5.7). On average, root dry biomass decreased by 23% in the T3 temperature treatment compared to the control temperature ($P<0.001$) which was more evident in ICS 6 and PA 7. No significant effects of $[CO_2]$ or other treatment interactions on root dry biomass were observed.
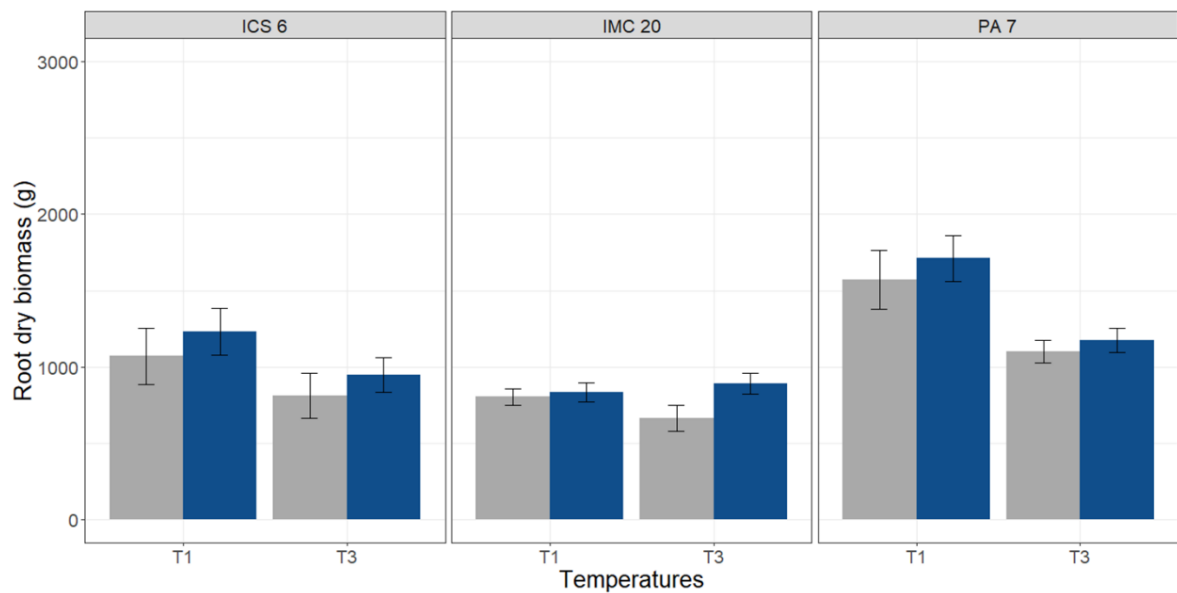
Figure 5.7 Effect of the [CO_2] and temperature on root dry biomass of three mature cacao genotypes. Error bars show the standard error of the mean. [CO_2] treatments are ambient (grey bar) and elevated (blue bar). Temperature treatments are T1 (control temperature), and T3 (control temperature + 5.0°C) as summarised in Table 5.2.

Total dry biomass. Significant differences were observed between genotypes in total dry biomass ($P<0.001$; Figure 5.8). Total dry biomass increased by 27% in IMC 20 under elevated [CO_2] whereas no clear effects of CO_2 enrichment were observed in ICS 6 and PA 7 ($P<0.05$) There was also a significant interaction between temperature and genotypes ($P<0.01$). The effect of an increase in temperature on total dry biomass was evident in PA 7, decreasing by 34% whereas no significant reductions were observed in ICS 6 and IMC 20, although there was a trend towards biomass reduction at the higher temperature in all genotypes.
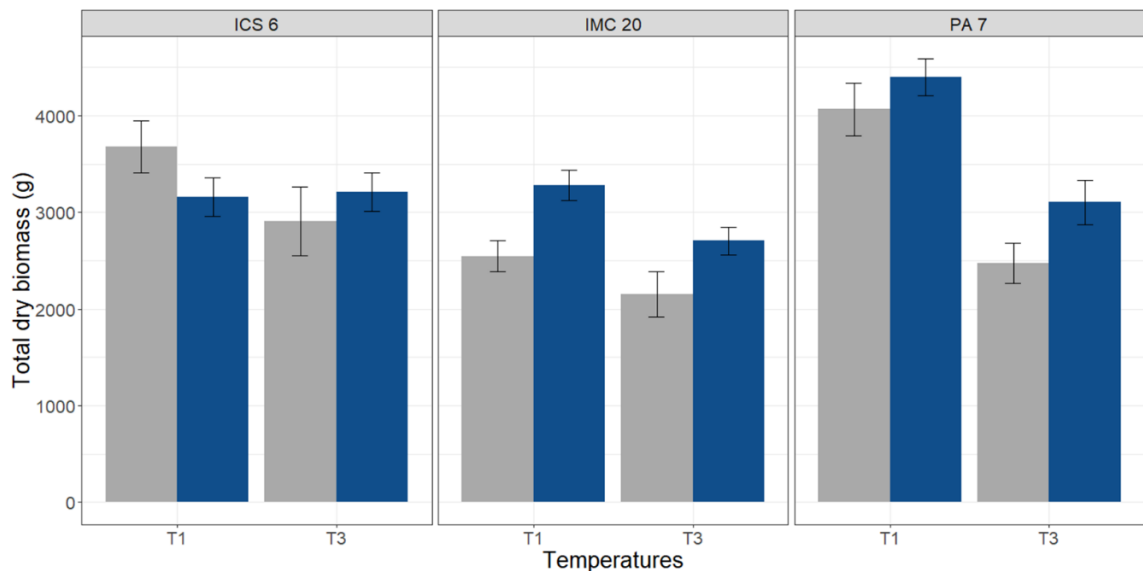
Figure 5.8 Effect of the [CO_2] and temperature on total dry biomass of three mature cacao genotypes. Error bars show the standard error of the mean. [CO_2] treatments are ambient (grey bar) and elevated (blue bar). Temperature treatments are T1 (control temperature), and T3 (control temperature + 5.0°C) as summarised in Table 5.2.

Leaf area. The effects of temperature and elevated [CO_2] on total leaf area per plant varied among the genotypes ($P< 0.01$; Figure 5.9). ICS 6 and PA 7 showed a similar response pattern. At ambient [CO_2] a reduction in total leaf area per plant was observed under T3, whereas at elevated [CO_2] there was an increase in leaf area at the higher temperature such that leaf area in the elevated [CO_2] /high temp environment was similar to that of trees in the ambient [CO_2] /control temperature environment. IMC 20 showed a different response pattern; at the control temperature, leaf area increased by 32% under elevated [CO_2] whilst no impact of [CO_2] elevation was observed at the high temperature.
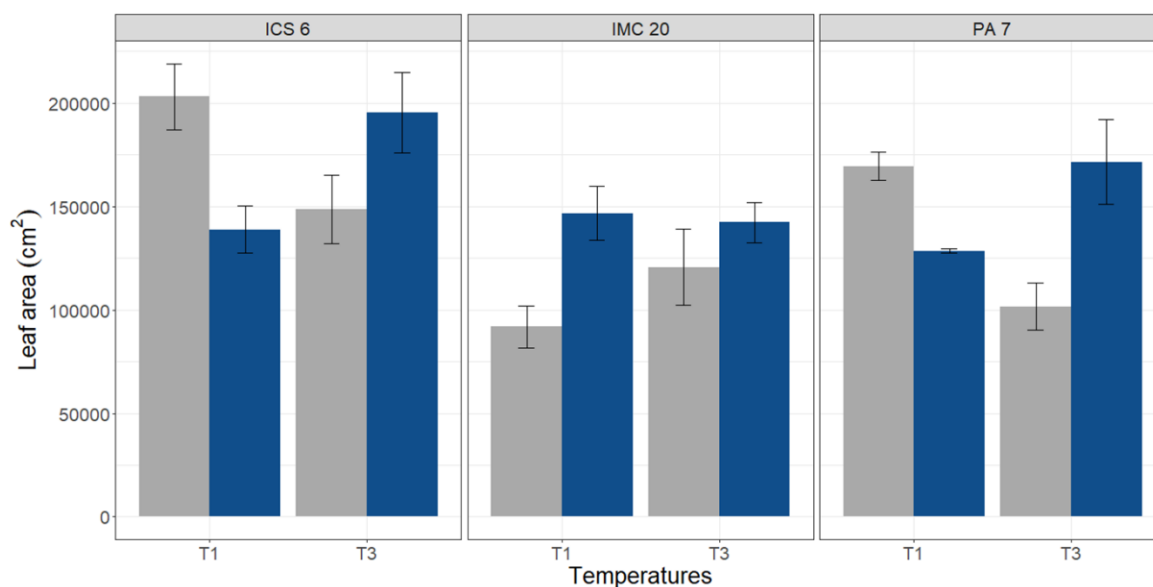
Figure 5.9 Effect of the $[CO_2]$ and temperature on leaf area of three mature cacao genotypes. Error bars show the standard error of the mean. $[CO_2]$ treatments are ambient (grey bar) and elevated (blue bar). Temperature treatments are T1 (control temperature), and T3 (control temperature + 5.0°C) as summarised in Table 5.2.

5.3.2   Stomatal parameters

Stomatal density. Significant differences were observed between genotypes in stomatal density ($P<0.001$; Figure 5.10). The response of stomatal density to $[CO_2]$ also varied amongst genotypes ($P<0.05$). Stomatal density increased significantly under elevated $[CO_2]$ in CCN 51 (16%), ICS 6 (22%) and SCA 6 (12%) ($P<0.05$) whereas there was no change in IMC 20, PA 7 and T85/799. There was also a significant interaction between $[CO_2]$ and temperature ($P<0.01$) on stomatal density. In general, stomatal density tended to increase with temperature in both ambient and elevated $[CO_2]$ environments. However, the increase due to elevated $[CO_2]$ was more evident (+ 15%) in plants grown at T3 whereas not significant changes were observed at T1.
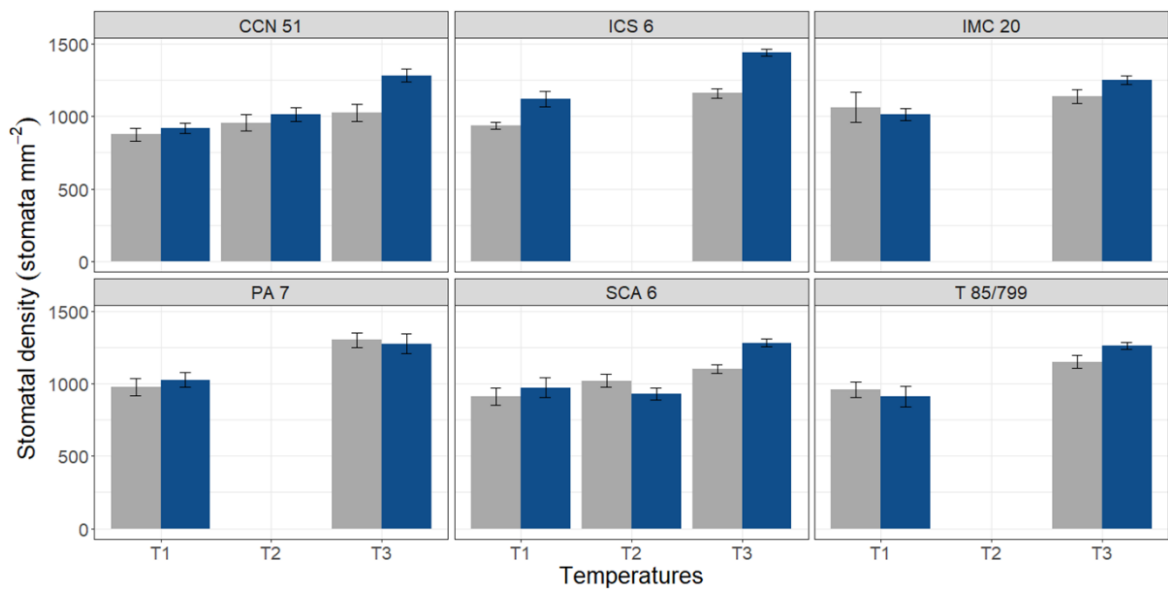
Figure 5.10 Effect of the [CO_2] and temperature on stomata density of six mature cacao genotypes. Error bars show the standard error of the mean. [CO_2] treatments are ambient (grey bar) and elevated (blue bar). Temperature treatments are T1 (control temperature), T2 (control temperature + 2.5°C), and T3 (control temperature + 5.0°C) as summarised in Table 5.2.

Stomatal index. Significant differences were observed between genotypes in stomatal index ($P<0.001$; Figure 5.11). There was an interaction between [CO_2] and genotype in response to stomatal index ($P<0.05$). Stomatal index increased significantly under elevated [CO_2] in ICS 6 (16%), IMC 20 (6%), and SCA 6 (10%) ($P<0.05$) whereas no change was observed in CCN 51, PA 7 and T85/799. There was also a significant interaction between [CO_2] and temperature ($P<0.01$) in stomatal index. In general, stomatal index also tended to increase with temperature in both ambient and elevated [CO_2] treatments. The increase due to elevated [CO_2] was more evident (+ 11%) in plants grown at T3 whereas not significant changes were observed at T1.
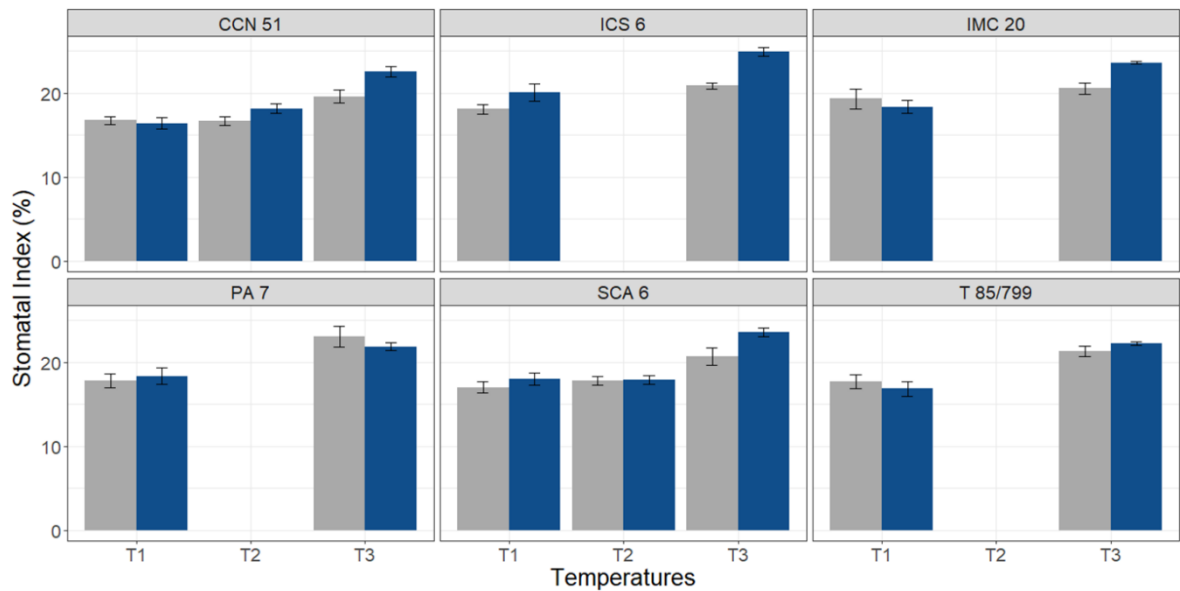
Figure 5.11 Effect of the [CO_2] and temperature on stomata index of six mature cacao genotypes. Error bars show the standard error of the mean. [CO_2] treatments are ambient (grey bar) and elevated (blue bar). Temperature treatments are T1 (control temperature), T2 (control temperature + 2.5°C), and T3 (control temperature + 5.0°C) as summarised in Table 5.2.

5.3.3   Instantaneous gas exchange parameters

Leaf transpiration rate. For all treatments, the leaf transpiration rate increased with an increase in temperature. On average a 35 % increase in transpiration from 0.71 (±0.04) to 0.96 (±0.04) mmol m^{-2} s^{-1} was observed with a temperature increase from T1 to T3 (*P*<0.001) (Figure 5.12). There were no genotypic differences, [CO_2] effects or their interactions on leaf transpiration rate.
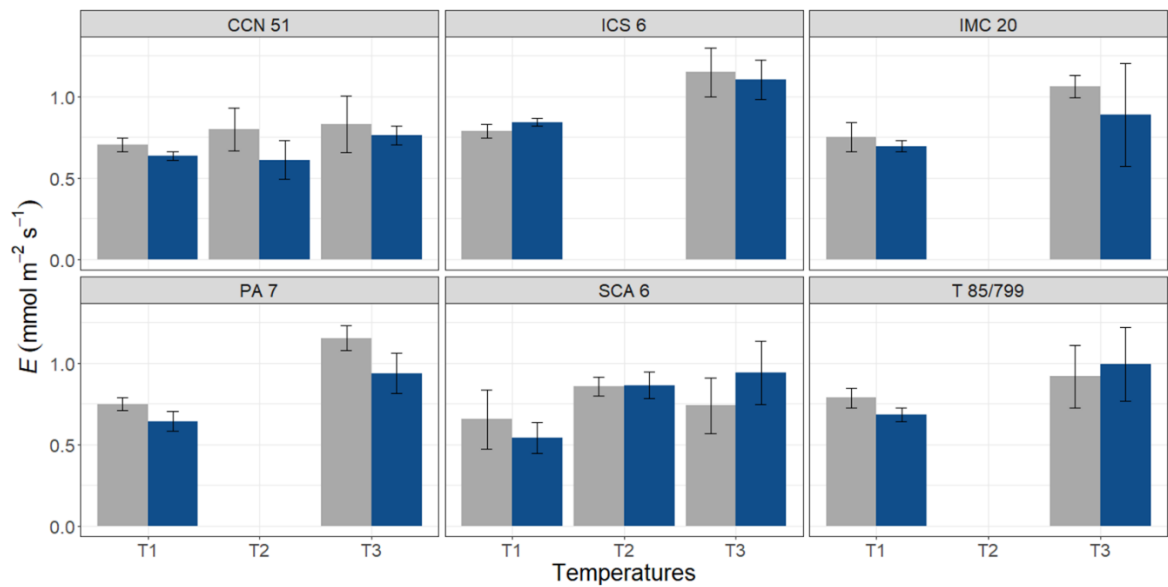
Figure 5.12 Effect of the [CO_2] and temperature on leaf transpiration rate of six mature cacao genotypes. Error bars show the standard error of the mean. [CO_2] treatments are ambient (grey bar) and elevated (blue bar). Temperature treatments are T1 (control temperature), T2 (control temperature + 2.5°C), and T3 (control temperature + 5.0°C) as summarised in Table 5.2.

Stomatal conductance*.* For all treatments, stomatal conductance decreased with an increase in temperature. On average a 17 % decrease in stomatal conductance from 0.03 (±0.001) to 0.02 (±0.001) mol m^{-2} s^{-1} was observed with temperature increases from T1 to T3 ($P<0.05$) (Figure 5.13). There were no effects of the genotypes or [CO_2] and their interactions on stomatal conductance.
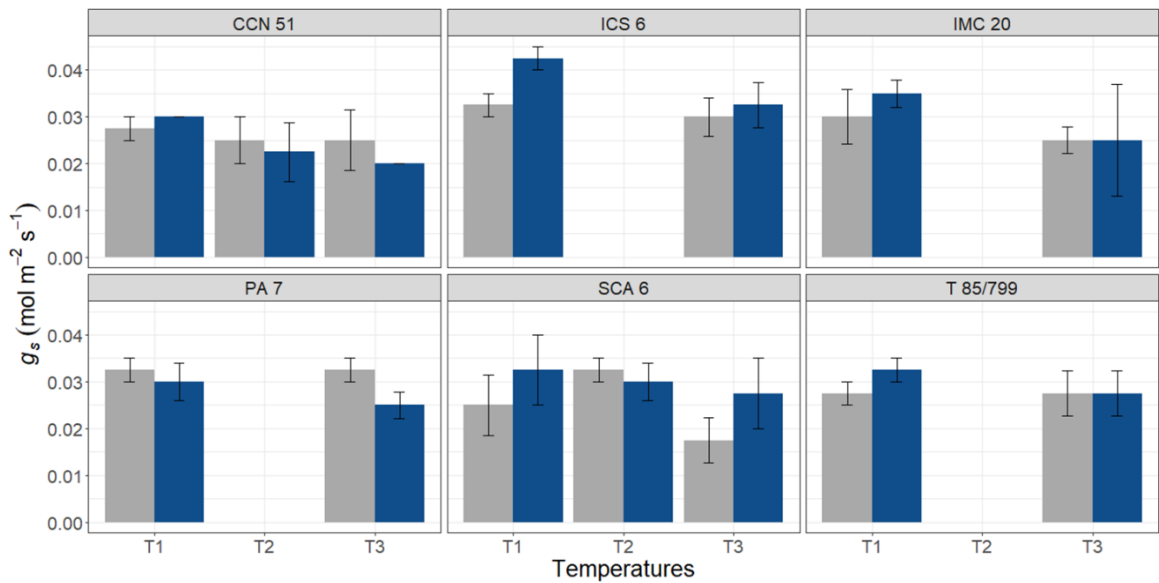
Figure 5.13 Effect of the [CO_2] and temperature on stomatal conductance of six mature cacao genotypes. Error bars show the standard error of the mean. [CO_2] treatments are ambient (grey bar) and elevated (blue bar). Temperature treatments are T1 (control temperature), T2 (control temperature + 2.5°C), and T3 (control temperature + 5.0°C) as summarised in Table 5.2.

Net photosynthetic rate. Across the genotypes and temperature treatments, net photosynthetic rate increased by 51% (from 2.87 (±0.11) to 4.33 (±0.11) µmol m^{-2} s^{-1}) in plants grown under elevated compared with ambient [CO_2] ($P<0.001$; Figure 5.14). Conversely, net photosynthetic rate decreased by 10% (from 3.78 (±0.11) to 3.42 (±0.11) µmol m^{-2} s^{-1}) in T3 compared to T1 ($P<0.05$). No significant effects of genotypes or other interactions on net photosynthetic rate were observed.
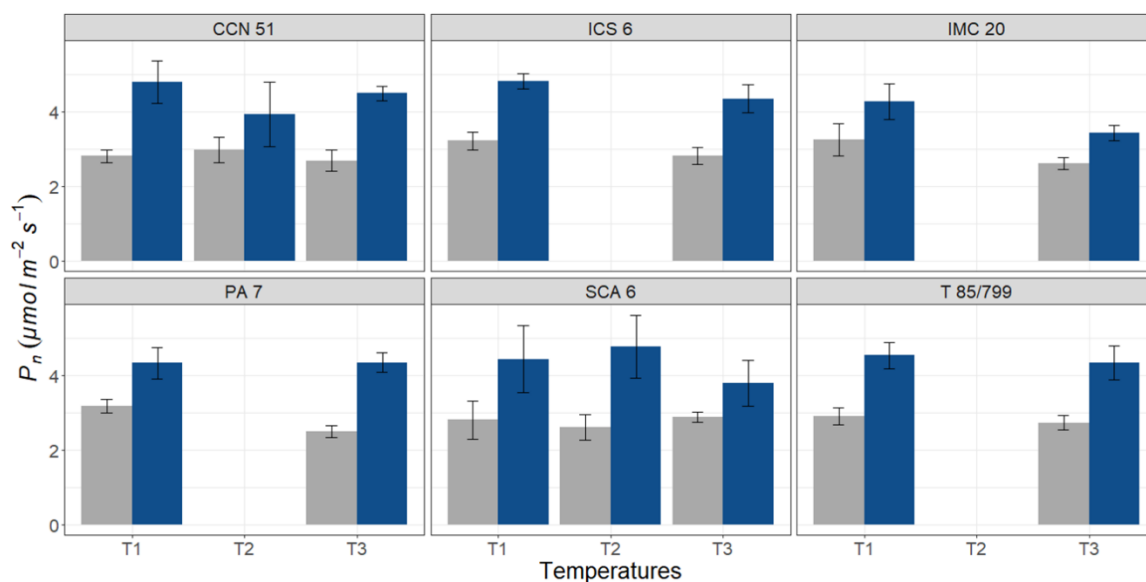
Figure 5.14 Effect of the $[CO_2]$ and temperature on net photosynthetic rate of six mature cacao genotypes. Error bars show the standard error of the mean. $[CO_2]$ treatments are ambient (grey bar) and elevated (blue bar). Temperature treatments are T1 (control temperature), T2 (control temperature + 2.5°C), and T3 (control temperature + 5.0°C) as summarised in Table 5.2.

Intrinsic water use efficiency. For all the treatments, intrinsic WUE increased with an increase in temperature. On average an increase in intrinsic WUE of 24% was observed with a temperature increase from T1 to T3 ($P<0.01$; Figure 5.15). Overall, intrinsic WUE also increased by 42% in plants grown under elevated $[CO_2]$ ($P<0.001$). There were no effects of the genotype or interactions between factors on intrinsic WUE.
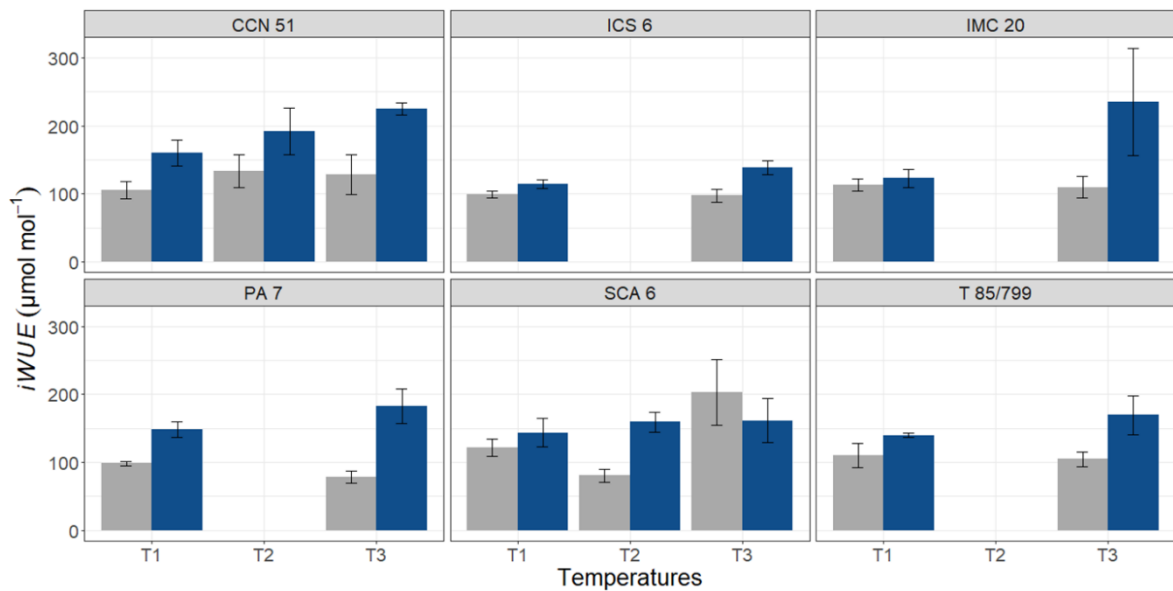
Figure 5.15 Effect of the [CO_2] and temperature on intrinsic water use of six mature cacao genotypes. Error bars show the standard error of the mean. [CO_2] treatments are ambient (grey bar) and elevated (blue bar). Temperature treatments are T1 (control temperature), T2 (control temperature + 2.5°C), and T3 (control temperature + 5.0°C) as summarised in Table 5.2.

5.3.4 Light- response curve parameters

Quantum efficiency. There was an overall increase of 39% in quantum efficiency (Ø) in plants under elevated [CO_2] compared to ambient [CO_2] (from 0.04 (±0.002) to 0.06 (±0.002) mol mol^{-1}) ($P<0.001$; Figure 5.16). Although the interaction between genotypes and [CO_2] was not significant, the increase was more evident in CCN 51, SCA 6 and PA 7. No significant effects of genotypes, temperature treatments or other interactions on quantum efficiency were observed.
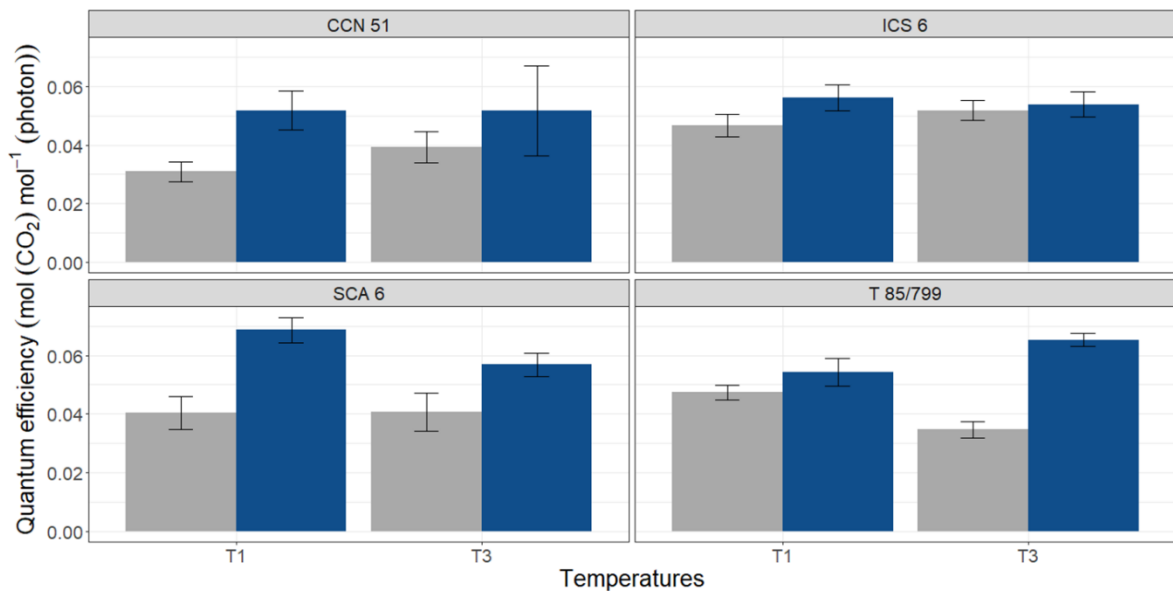
Figure 5.16 Effect of the [CO_2] and temperature on apparent quantum efficiency (∅) of four mature cacao genotypes. Error bars show the standard error of the mean. [CO_2] treatments are ambient (grey bar) and elevated (blue bar). Temperature treatments are T1 (control temperature) and T3 (control temperature + 5.0°C) as summarised in Table 5.2.

Dark respiration rate*.* Dark respiration rate increased on average by 96% (from 0.44 (±0.03) to 0.87 (±0.03) µmol m^{-2} s^{-1}) under elevated [CO_2] ($P<0.001$; Figure 5.17). There was also a significant interaction between temperature and [CO_2] ($P<0.05$). Increasing temperature did not influence the dark respiration rate in the ambient [CO_2] treatment for all the genotypes. However, under elevated [CO_2], dark respiration rate increased overall by 21% with a temperature increase from T1 to T3, being more evident in ICS 6, SCA 6, and T 85/799. There were no effects of the genotype or other interactions on dark respiration rate.
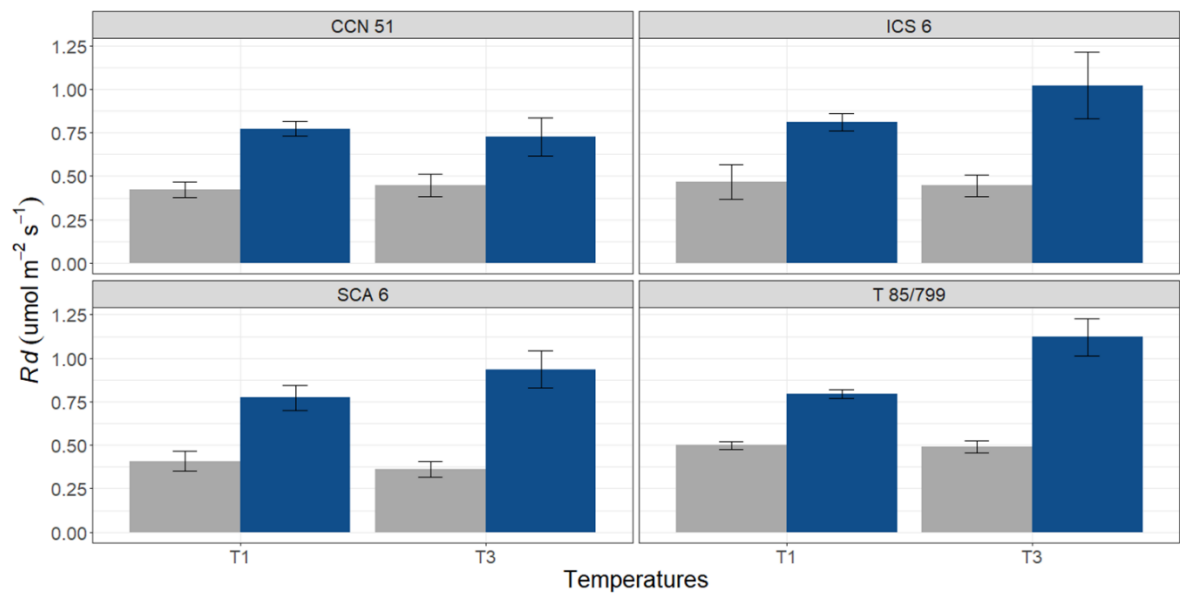
Figure 5.17 Effect of the [CO_2] and temperature on dark respiration rate (*Rd*) of four mature cacao genotypes. Error bars show the standard error of the mean. [CO_2] treatments are ambient (grey bar) and elevated (blue bar). Temperature treatments are T1 (control temperature) and T3 (control temperature + 5.0°C) as summarised in Table 5.2.

Light compensation point. There was a significant interaction between the [CO_2] and temperature treatments in light compensation point ($P<0.05$; Figure 5.18). At ambient [CO_2], temperature did not influence light compensation point whereas at the elevated [CO_2], light compensation point increased by 33% at T3. No significant effects of genotypes or others interactions on light compensation point were observed.
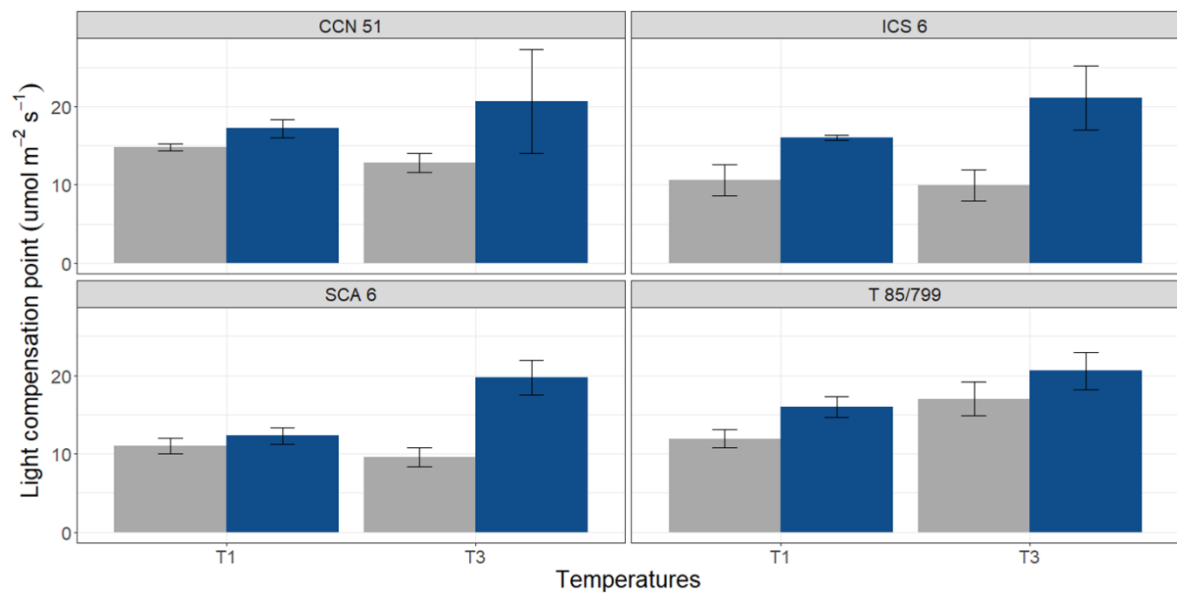
Figure 5.18 Effect of the [CO_2] and temperature on light compensation point of four mature cacao genotypes. Error bars show the standard error of the mean. [CO_2] treatments are ambient (grey bar) and elevated (blue bar). Temperature treatments are T1 (control temperature) and T3 (control temperature + 5.0°C) as summarised in Table 5.2.

Light saturation point. Light saturation point varied among genotypes ($P<0.05$) ranging from 412.9 (±49.2) µmol m^{-2} s^{-1} in SCA 6 to 616.5 (±49.2) µmol m^{-2} s^{-1} in CCN 51 (Figure 5.19). A slight increase in light saturation point was observed at elevated [CO_2] which was on the borderline of significance ($P = 0.051$) in ICS 6. There were no effects of temperature or others interactions on the light saturation point.
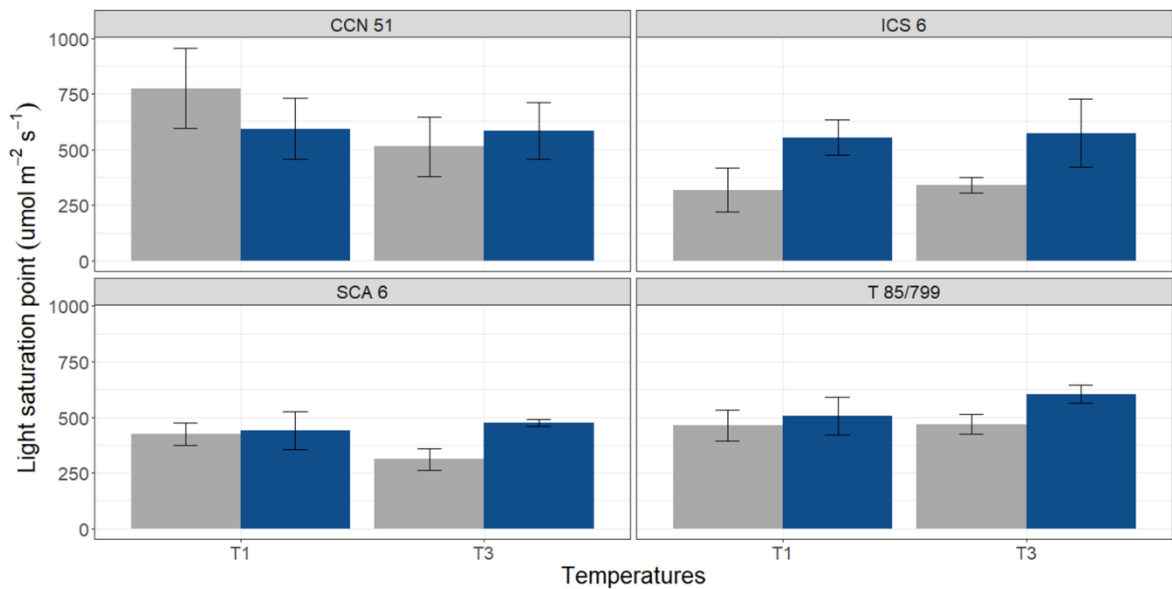
Figure 5.19 Effect of the $[CO_2]$ and temperature on light saturation point of four mature cacao genotypes. Error bars show the standard error of the mean. $[CO_2]$ treatments are ambient (grey bar) and elevated (blue bar). Temperature treatments are T1 (control temperature) and T3 (control temperature + 5.0°C) as summarised in Table 5.2.

Light-saturated photosynthesis rate. Overall, light-saturated photosynthetic rate increased by 66% (2.56 (±0.19) to 4.24 (±0.18) µmol m^{-2} s^{-1}) in trees grown at elevated $[CO_2]$ (P <0.001; Figure 5.20). There was a slight but significant decrease of 15% in the light-saturated photosynthesis rate from 3.66 (±0.18) µmol m^{-2} s^{-1} at T1 to 3.12 (±0.18) µmol m^{-2} s^{-1} at T3 (P <0.05). No significant effects of genotypes or other interactions on light-saturated photosynthesis rate were observed.
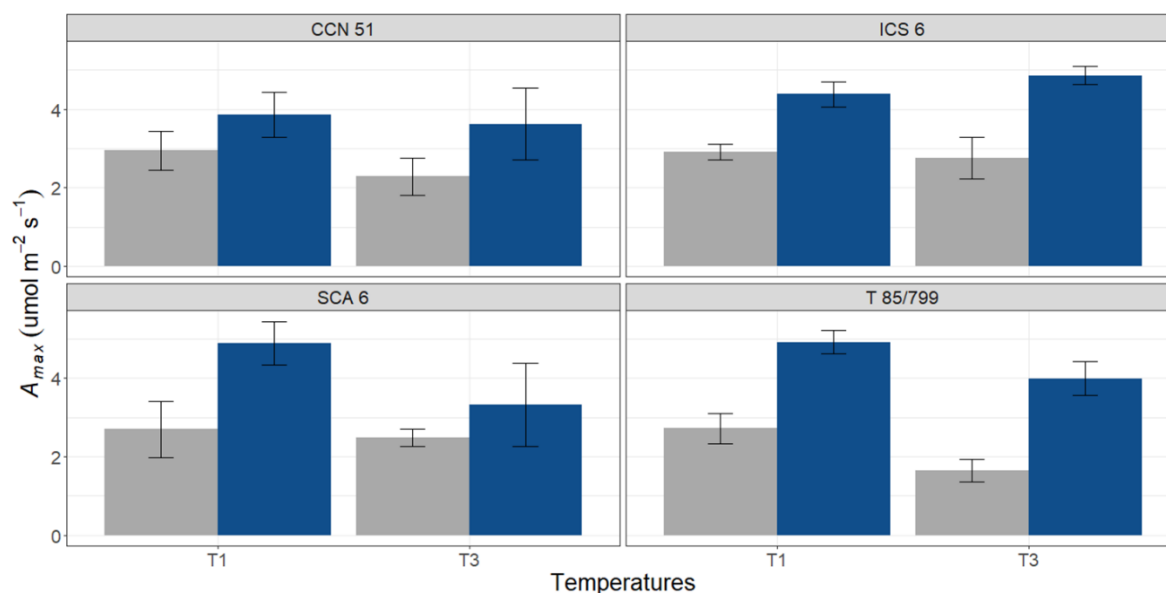
Figure 5.20 Effect of the [CO_2] and temperature on light-saturated photosynthesis rate (A_{max}) of four mature cacao genotypes. Error bars show the standard error of the mean. [CO_2] treatments are ambient (grey bar) and elevated (blue bar). Temperature treatments are T1 (control temperature) and T3 (control temperature + 5.0°C) as summarised in Table 5.2.

5.3.5   Chlorophyll parameters

Chlorophyll content. Significant differences were observed between genotypes in chlorophyll content (*P*<0.01; Figure 5.21) being lowest in T85/799 (54.25 (±4.04) µg cm^{-2}) and highest in SCA 6 (71.62 (±4.02) µg cm^{-2}). Increases in temperature from T1 to T3 resulted in a decrease by 18% in chlorophyll content (*P*<0.001). Overall, chlorophyll content declined by 16% in plants grown under elevated [CO_2] (*P*<0.01). No significant interactions among treatments on chlorophyll content were observed.
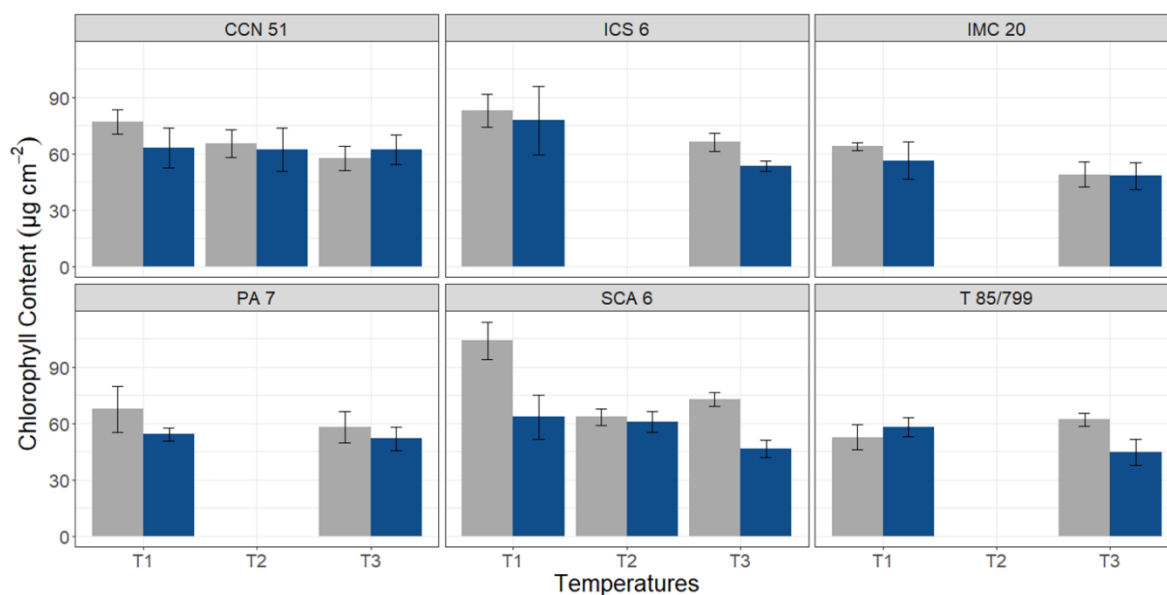
Figure 5.21 Effect of the [CO_2] and temperature on chlorophyll content of four mature cacao genotypes. Error bars show the standard error of the mean. [CO_2] treatments are ambient (grey bar) and elevated (blue bar). Temperature treatments are T1 (control temperature), T2 (control temperature + 2.5°C), and T3 (control temperature + 5.0°C) as summarised in Table 5.2.

Maximum quantum efficiency of PS II and Performance index. No effects of temperature, [CO_2] or genotype were observed on *Fv/Fm* whilst the effect of genotype on *PI* was on the borderline of significance (P=0.066; Figure 5.22) and was lowest in T85/799 (3.3 (±0.2)) and highest in ICS 6 (4.2 (±0.2)). No other significant main factor or interactions on *PI* were observed.
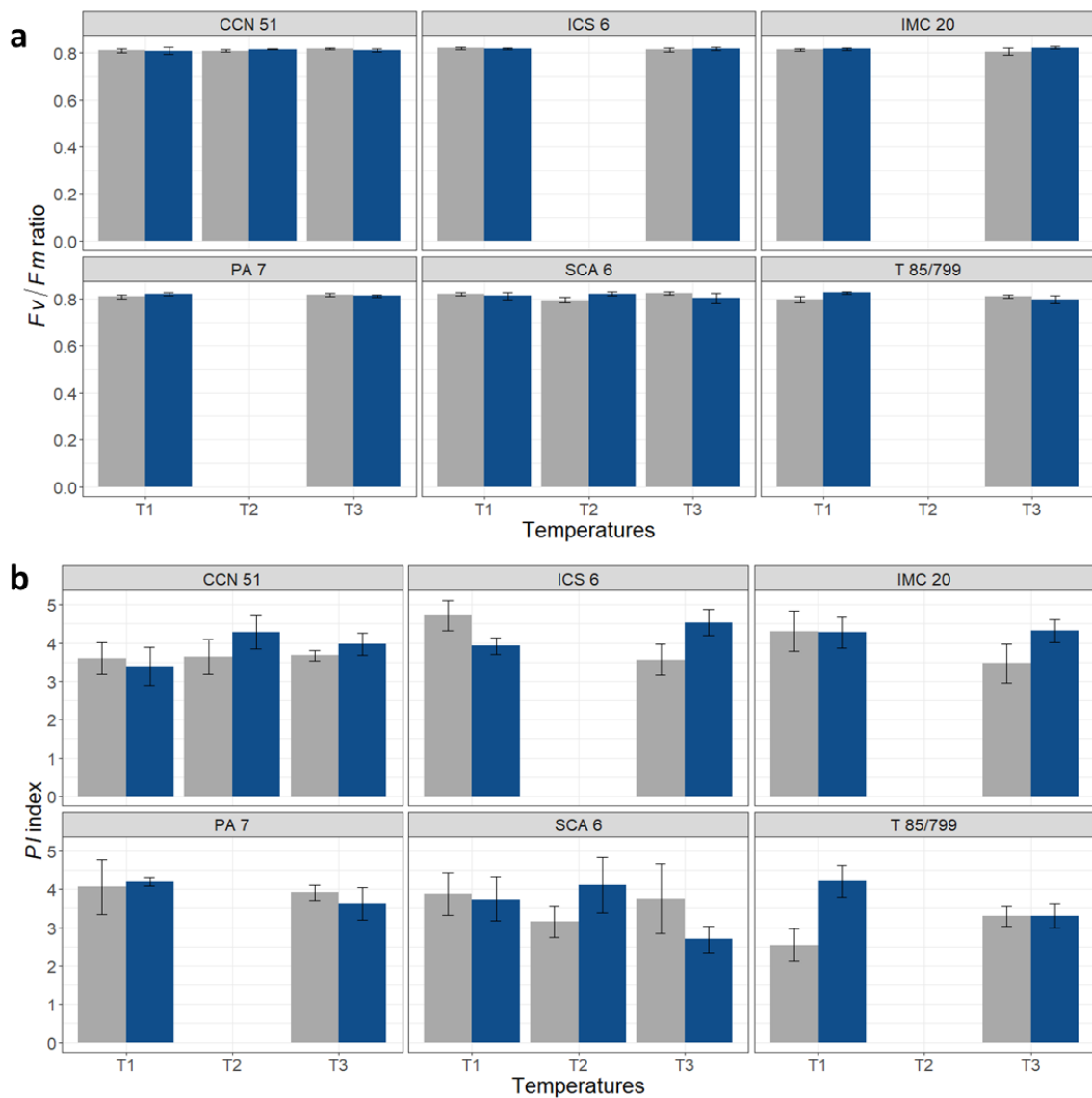
Figure 5.22 Effect of the $[CO_2]$ and temperature on maximum quantum efficiency of PS II (a) and Performance index (b) of four mature cacao genotypes**.** Error bars show the standard error of the mean. $[CO_2]$ treatments are ambient (grey bar) and elevated (blue bar). Temperature treatments are T1 (control temperature), T2 (control temperature + 2.5°C), and T3 (control temperature + 5.0°C) as summarised in Table 5.2.

5.3.6   Photosynthetic acclimation

Photosynthetic acclimation to elevated $[CO_2]$. Long-term effects of elevated $[CO_2]$ growth conditions on downregulation of photosynthesis and stomatal conductance was explored at each temperature growth condition. The response of photosynthesis to instantaneous elevated $[CO_2]$ was dependent on growth temperature and $[CO_2]$ environment ($P<0.01$; Figure 5.23a). Under the control growth temperature of T1, for plants that had been grown at elevated $[CO_2]$ the photosynthesis rate was lower (4.71 (±0.21) $\mu mol\ m^{-2}\ s^{-1}$) than for plants that have been grown at ambient $[CO_2]$ (6.37 (±0.21) $\mu mol\ m^{-2}\ s^{-1}$) and exposed to a short-term increase in $[CO_2]$. In contrast, under higher temperature growth conditions of

T3, in plants that had been grown at elevated [CO_2] the photosynthesis rate was higher (9.32 (±0.32) µmol m^{-2} s^{-1}) than for plants that had been grown at ambient [CO_2] (6.78 (±0.32) µmol m^{-2} s^{-1}) and exposed to instantaneous increase in [CO_2]. The response of stomatal conductance to an instantaneous increase in [CO_2] was also dependent on growth temperature and [CO_2] ($P<0.001$ Figure 5.23b). In plants grown under the control temperature of T1, an instantaneous increase in [CO_2] increased stomatal conductance by 72% in trees grown at ambient [CO_2] whereas following long-term growth at elevated [CO_2], stomatal conductance declined by 46% with an instantaneous increase in [CO_2]. However, in plants grown under the higher temperature of T3, no significant differences in stomatal conductance were observed in response to an instantaneous increase in [CO_2] neither for trees grown at ambient nor elevated [CO_2] conditions.
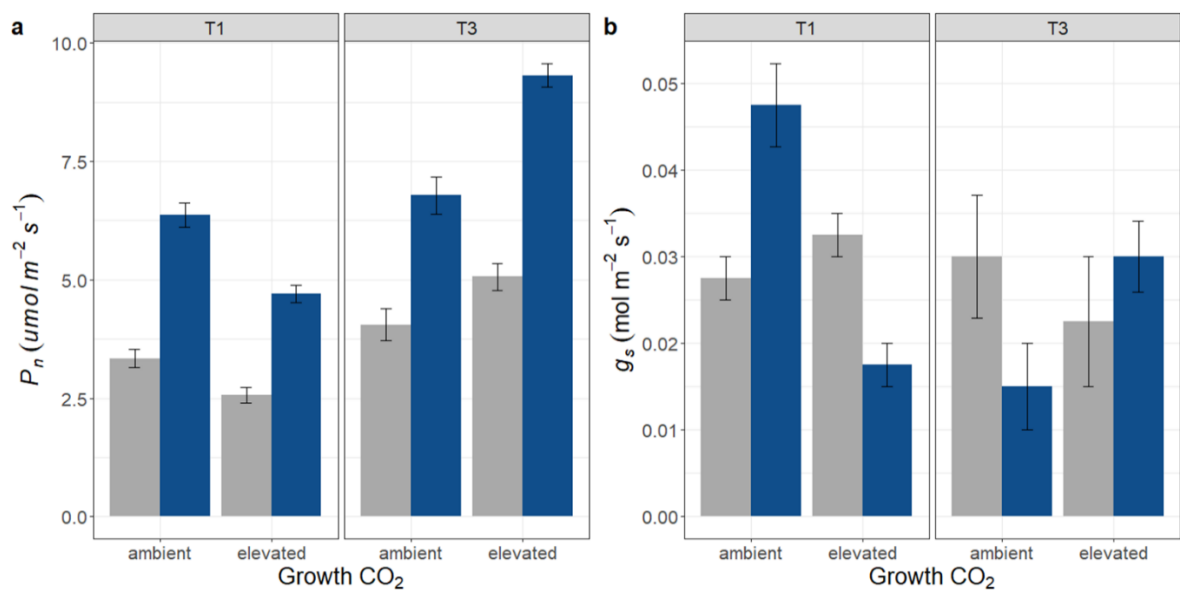
Figure 5.23 Effect of an instantaneous increase of [CO_2] on photosynthesis rate (a) and stomatal conductance (b) of CCN 51 mature trees grown under long-term conditions of two [CO_2] environments and two temperature treatments. Error bars show the standard error of the mean (n=4). Growth [CO_2] conditions are ambient and elevated. Growth temperature conditions are T1 (control temperature) and T3 (control temperature + 5.0°C) as summarised in Table 5.2. Instantaneous [CO_2] are ambient (grey bar) and elevated (blue bar).

Photosynthetic acclimation to elevated temperature. Long-term effects of elevated temperature growth condition on leaf photosynthesis and stomatal conductance was explored at each [CO_2] growth environment. The response of photosynthesis to an instantaneous increase in temperature from 31 to 36°C was dependent on the [CO_2] and temperature growth condition ($P<0.001$; Figure 5.24a). For plants grown at T1 and ambient [CO_2], an instantaneous increase in temperature from 31 to 36°C resulted in a decline in photosynthesis by 30%, whereas following long-term growth at T3, photosynthesis rates did not differ when measured at 31 or 36°C. In contrast, in plants grown at T1 and elevated [CO_2], an

instantaneous increase in temperature from 31 to 36°C resulted in a significant increase (48%) in the photosynthetic rate of trees. However, photosynthesis rate did not differ when measured at 31 or 36°C in trees grown at T3, being higher than the rates of trees grown at T1. On the other hand, there was a decrease in stomatal conductance with a short-term increase in temperature (36°C) in trees grown under ambient $[CO_2]$ and control temperature (T1). However, an overall significant increase of 72% in stomatal conductance in trees grown in long-term elevated temperature was observed ($P<0.001$; Figure 5.24b).
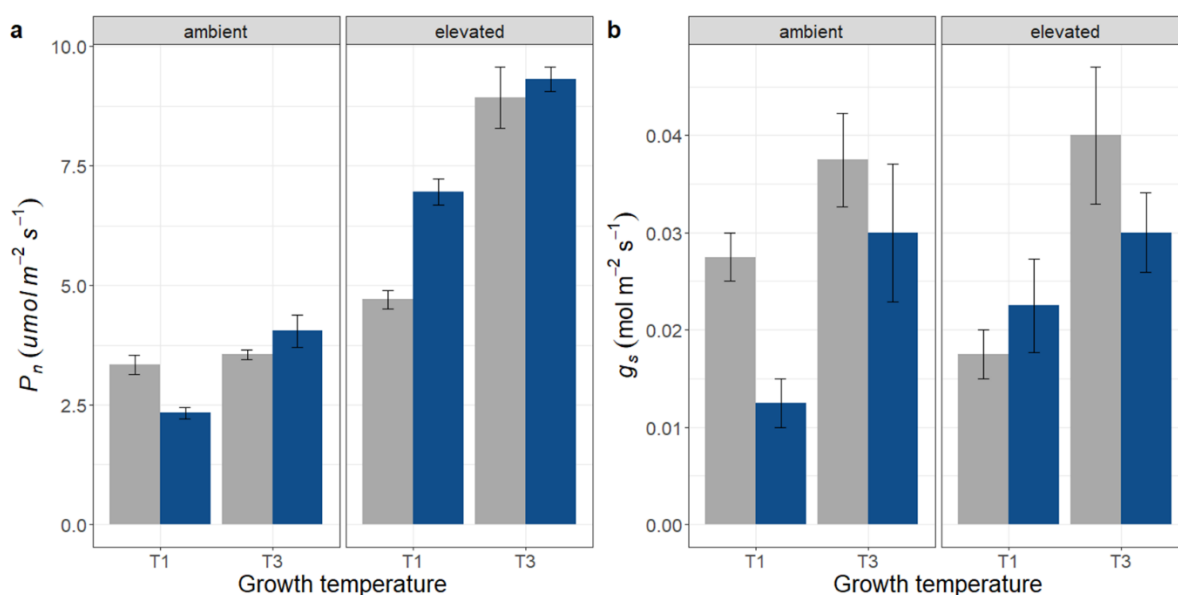
Figure 5.24 Effect of an instantaneous increase in temperature on net photosynthesis rate (a) and stomatal conductance (b) of CCN 51 mature trees grown under long-term conditions of two $[CO_2]$ environments and two temperature treatments. Error bars show the standard error of the mean (n=4). Growth $[CO_2]$ conditions are ambient and elevated. Growth temperature conditions are T1 (control temperature) and T3 (control temperature + 5.0°C) as summarised in Table 5.2. Instantaneous temperatures are T1 (grey bar) and T3 (blue bar).

Leaf carbon and nitrogen concentration. Overall, leaf nitrogen content increased by 8% under elevated temperature (T3) ($P<0.05$; Figure 5.25b). Leaf nitrogen decreased very slightly in response to elevated $[CO_2]$ at each temperature although this was not significant ($P>0.05$). No effects of temperature or $[CO_2]$ on leaf carbon content were observed.
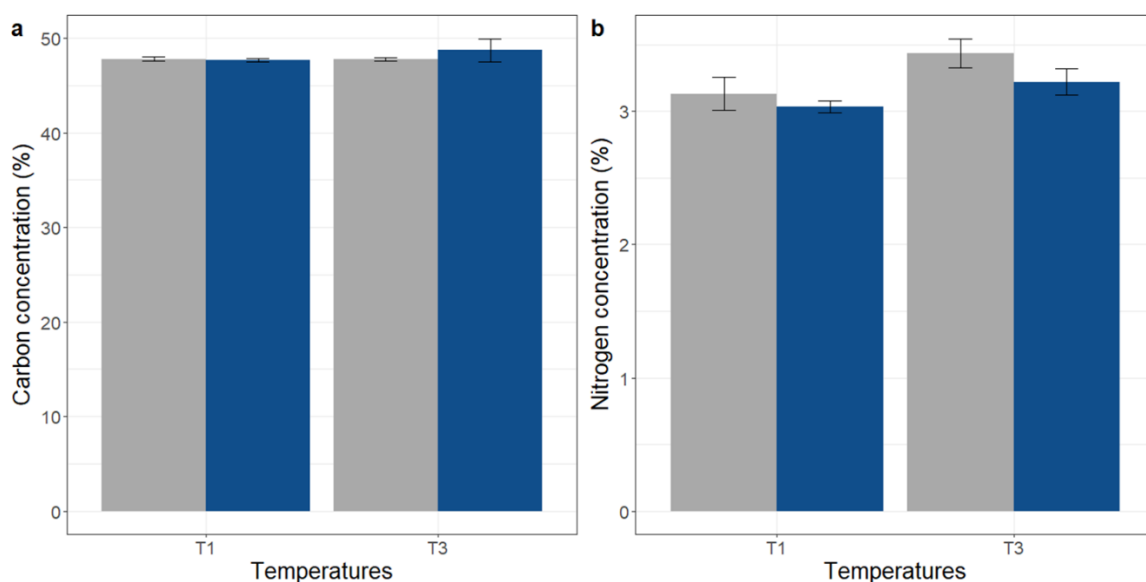
Figure 5.25 Effect of the $[CO_2]$ and temperature on leaf carbon (a) and nitrogen (b) concentration of CCN 51 mature cacao genotype. Error bars show the standard error of the mean (n=4). $[CO_2]$ treatments are ambient (grey bar) and elevated (blue bar). Temperature treatments are T1 (control temperature) and T3 (control temperature + 5.0°C) as summarised in Table 5.2.

5.4   Discussion

In this study, mature trees of six contrasting cacao genotypes (CCN 51, SCA 6, ICS 6, IMC 20, PA 7, and T85/799) were grown for 378 days under glasshouse conditions, and subjected to two CO_2 concentrations and two growth temperatures, in order to explore whether plant growth and physiology of mature trees of different genotypes remained responsive to both elevated $[CO_2]$ and temperature. The key findings were: (i) mature cacao trees showed genotypic variation in growth and physiology responses to combined effects of elevated $[CO_2]$ and temperature, (ii) photosynthesis and therefore growth of mature cacao trees remained responsive to increases in elevated $[CO_2]$ under warming conditions, (iii) the enhancement in growth at elevated $[CO_2]$ and temperature was less pronounced in mature cacao plants compared to juvenile material, (iv) downregulation of photosynthesis in response to elevated $[CO_2]$ was observed under current control temperatures while under control temperature + 5.0°C, photosynthesis acclimated positively, and (v) thermal acclimation of photosynthesis occurred under both ambient and elevated $[CO_2]$.

The different genotypes studied here varied in terms of their growth response to long-term exposure to elevated $[CO_2]$ and temperature. For example, when considering growth responses to elevated $[CO_2]$ stem diameter increased in CCN 51, PA 7 and IMC 20, inter-flush period was shorter in all the genotypes except ICS 6, and flush extension increased in CCN 51, SCA 6 and T 85/799. In juvenile cacao plants, stem diameter also increased under elevated $[CO_2]$ by 9% (Chapter 3) and 28% (Chapter 4) in combination

with high temperatures, while there was no significant effects of elevated [CO_2] on inter-flush period (Chapter 3). Increased stem diameter (by 22%) and no changes in the inter-flush period were also noted previously on a wide set of mature cacao plants subjected to elevated [CO_2] under controlled conditions (Lahive *et al.*, 2021). The authors highlighted that differences in the growth responses to [CO_2] elevation, could be related to differences in cocoa genetic materials, which could be exploited to select materials suited to a changing climate. Physiological plasticity within species determines the capacity of plant species to adapt to climate change (Huang *et al.*, 2015). Here it has been shown that there is genotypic variability in the plasticity of cacao in response to atmospheric [CO_2] increases and warming scenarios.

Irrespective of temperature, a positive independent effect of elevated [CO_2] was observed in the number of leaves per flush across all genotypes (increased by 13%). Compared to the previous short-term experiments with juvenile cacao plants, no effects of elevated [CO_2] in leaves per flush were observed, although there was an increase at elevated temperature (Chapter 3). However, more leaves per flush were noted in combination of both elevated [CO_2] and temperatures from 28.5/19.5°C to 36/27°C (Chapter 4). Lahive *et al.* (2021), working with six different mature cacao genotypes grown under elevated [CO_2] at a temperature regime of ~32/19°C, reported slight but non-significant increases in leaves per flush. Earlier studies on trees have shown that seedlings are most responsive to the [CO_2], and as trees develop, they grow more slowly and could be less responsive to CO_2 enrichment (Lee and Jarvis, 1995). However, the increase in number of leaves at elevated [CO_2] in this present study, might be attributed to the need to allocate more carbon in the growing components.

An overall increase in total biomass of 10% was observed in cocoa trees grown at elevated [CO_2] over a period of 378 days. Carbon dioxide enrichment is known to increase plant growth and lead to greater biomass production (Li *et al.*, 2019). A similar increase in dry biomass was also reported by Lahive *et al.* (2021) in mature cacao trees grown for 210 days at elevated [CO_2] in greenhouses, who noted that some genotypes were more reactive to [CO_2] elevation than others. In this current study, the findings on mature trees were consistent with the positive responses of [CO_2] observed for juvenile cacao materials (Chapter 3 and 4). A decline in total dry biomass with a 5° C increase was more evident in PA 7 (-34%) whereas no significant effects were observed in ICS 6 and IMC 20 which suggests differences in genotypic sensitivity to temperature increases. However, the effect of increased temperature was partially mitigated by elevated [CO_2]. CO_2 enrichment has been shown to mitigate adverse effects of heat stress on photosynthesis and plant growth among various species of plants through the inhibition of photorespiration (Idso and Idso, 1994). Similarly, a compensatory effect was also evident in the short-term experiment with juvenile plants (Chapter 3). This finding is consistent with Hebbar *et al.* (2020), who observed that although elevated temperatures reduced total biomass of cacao seedlings, a

combination with elevated [CO_2] was similar to the control treatment. Here, by studying a range of genotypes it has been shown that some are more sensitive to temperature increases than others, but that CO_2 enrichment can partially overcome the negative impacts of warming conditions for more susceptible genotypes.

Whilst the pattern of response to treatments of above ground biomass was similar to that of total biomass, this was not the case for root biomass. Under non-limiting water and nutrient conditions, there were no significant effects of elevated [CO_2] on belowground dry biomass across the genotypes during the experimental period. However, a significant decline (by 23%) of belowground dry biomass was noted under the warming scenario (control +5.0°C). In contrast, there was an increase of 46.3% in root dry biomass in juvenile cacao plants grown under chamber conditions at elevated [CO_2] (Chapter 4). However, in the seedlings a decline of root dry biomass was observed when temperatures increased above 36/27°C. A general improvement of root dry biomass in plants grown at elevated [CO_2] has been reported in woody and herbaceous species under long-term field experiments (De Graaff *et al.*, 2006; Nie *et al.*, 2013). However, it has been noted that the responses could be driven by several interactions with other factors such as soil characteristics, water and nutrient availability (Piñeiro *et al.*, 2017). Wan *et al.* (2004), working with one-year-old shade tolerant maple seedlings grown for four seasons in open-top chambers (OTCs) subjected to both elevated [CO_2] and air temperature, reported that there was a significant decline in root biomass with an increase in temperature, but no clear effects of elevated [CO_2] or interactions between the two factors were noted. It has been suggested that under warming scenarios, reduction in root biomass could be explained due to increases in the maintenance of respiration of plant roots which increases exponentially with temperature (Johnson, 1990; Atkin *et al.*, 2000). Here, the smaller root system under a warming scenario may have implications on how cacao plants could access water under limiting conditions as well as reduced uptake of certain nutrients that may impact plant development.

The response of leaf area to elevated [CO_2] and temperature varied between genotypes such that for ICS 6 and PA 7 a decline in leaf area under the warmer scenario at ambient [CO_2] was counteracted at elevated [CO_2]. In contrast, irrespective of [CO_2] treatment, the elevated temperature did not impact leaf area in IMC 20. Lahive *et al.* (2021) and Handley (2016), working with a broad range of mature cacao genotypes grown under controlled facilities and subjected to elevated [CO_2], reported slight but not significant increases in leaf area to elevated [CO_2]. A similar small but not significant increment in leaf area of young cacao plants of one genotype grown at elevated [CO_2] under OTC were reported by Hebbar *et al.* (2020). However, the same authors observed that leaf area of plants grown under elevated [CO_2] and elevated temperature treatment, was similar to the control chamber. Similar to the experiments on mature plant, in the short-term experiments reported previously (Chapters 3 and 4), the

combined effects of elevated both [CO_2] and temperature in leaf area also varied according to genotype. In those that appeared to be 'susceptible' to warm conditions, elevated [CO_2] ameliorated the negative effect of temperature increases, while in those more 'tolerant', elevated [CO_2] enhanced the leaf area. Here it has been shown that responses of total dry biomass and leaf area, suggest the potential to select materials more tolerant to increased temperatures. However, this compensation effect of [CO_2] on biomass and leaf area in a warming scenario, may have implications for water use and water demand by the plant. As was seen in this study, with no significant effects of elevated [CO_2], leaf transpiration rate increased (discussed later) whereas root dry biomass declined with increases in temperature. So, increases in leaf area due to the compensation effect of elevated [CO_2] under a warming scenario might impact plant development under soil moisture limitations.

The observation of increased stomatal density and stomatal index under elevated [CO_2] in some genotypes was more evident under the warmest condition (5.0°C above control temperature) than control temperature. In contrast, no effect of elevated CO_2 on stomatal density was observed by Lahive *et al.* (2021). This may be due to a shorter period of exposure to the treatment conditions, or different genotypes used in the study, and also the study was not conducted at higher temperatures. Although it has been reported that stomatal density might decrease under [CO_2] enrichment (Woodward and Kelly, 1995), only a 5% decrease in stomatal density due to elevated [CO_2] was observed by (Ainsworth and Rogers, 2007) in a wide variety of species. The magnitude of stomatal density and stomatal index response to [CO_2] increases could be affected by several factors, including experimental facilities, duration of the experiment, intra/inter specific differences and other environmental factors (Xu *et al.*, 2016). Here, the effect of elevated temperature on stomatal density and stomatal index was consistent with the short-term experiments (Chapter 3 and 4); after 7 months of exposure under higher temperatures (up to 5.0°C above control temperature), increases in these parameters were also exhibited in the mature cacao trees. Under well-watered conditions, the enhancement of these traits under warming treatment might help the cocoa trees to regulate leaf temperature through the transpiration process (cooling effect). However, the greater transpiration from leaves could lead to reductions in soil moisture during water deficit which may exacerbate the negative effects of high temperatures.

Despite the increase in stomatal density and index at elevated [CO_2] this did not lead to higher stomatal conductance. This is consistent with the results of Lahive *et al.* (2021). However, in contrast to the present study, Lahive *et al.* (2021) observed significant differences among genotypes. Using growth cabinets and two different juvenile cacao genotypes (PA 107 and SCA 6), no effects of [CO_2] elevation was noted in Chapter 3, whereas stomatal conductance and leaf transpiration rate declined with increases in [CO_2] for T63/971 x T60/887 (Chapter 4) which may suggest genotypic variation in response

to CO_2 elevation. A meta-analysis of 13 long-term field studies of forest species grown under $[CO_2]$ enrichment, showed an average decline of stomatal conductance by 21% being more evident in young trees than old trees (Medlyn *et al.*, 2001). Similarly, reductions in stomatal conductance by 24% (Hebbar *et al.*, 2020) and 38% (Baligar *et al.*, 2021a) have been reported in young cacao materials subjected to CO_2 elevation. Stomatal conductance declined with increases of temperature under the present study. On the contrary, increases in stomatal conductance were observed in young cacao plants grown in the cabinets and subjected to elevated temperature regimes (chapter 3). Differences in this response might be attributed to 'indirect' temperature effects on stomata aperture due to the VPD in the growth chambers being maintained at a non-limiting level, whereas in the glasshouses this was more difficult to control. In a study examining the effect of elevated temperatures on the stomatal mechanisms of coniferous species, Urban *et al.* (2017) reported increases in stomatal conductance when VPD was maintained constant at 1 kPa. In the present study, the reduction in stomatal conductance under elevated temperature did not reflect the increases in transpiration rates. Here, more stomata per unit area may have supported the higher transpiration rate observed. On the other hand, since there were stomatal restrictions under the warming scenario, the transpiration may have also resulted from water loss through the cuticle as it has been previously reported in cacao (Baligar *et al.*, 2008; Lahive *et al.*, 2019).

Photosynthesis rate increased by 51% in mature cacao plants grown under elevated $[CO_2]$ whereas there was a slight (10%) but significant reduction with temperature increases of 5.0°C above control. Since no impacts of elevated temperature on chlorophyll fluorescence were observed (discussed later) the declines in photosynthesis were likely due to reductions in CO_2 uptake through lower stomatal conductance rather than thermal damage to the photosynthetic machinery. However, the effect of elevated $[CO_2]$ remained positive even under control temperature + 5.0°C treatment. A compensatory effect of elevated $[CO_2]$ under warming conditions (~35.3/26.9°C) was also reported in 6-month-old plants by Hebbar *et al.* (2020). In this study, the overall stimulation in photosynthesis rate in mature trees at control temperature + 5.0°C and elevated $[CO_2]$ was similar to that seen in under growth cabinets in the short-term experiments on seedlings at 36/27°C (Chapters 3 and 4). In the present study, the overall increases in final biomass was approximately 20% of the increase in leaf photosynthesis rate. This greater enhancement of leaf-level photosynthesis than growth has been previously observed in mature cacao trees (Lahive *et al.*, 2021). Similar responses have been noted in other species. In a meta-analysis performed in soybean using 111 studies, Ainsworth *et al.* (2002) pointed out that changes in photosynthesis rate does not correlated to the magnitude of changes in total dry biomass and final yield. As seen in this study, overall leaf area remained unchanged with growth at elevated $[CO_2]$ and the significant increases in dark respiration might affect overall carbon gain over time. Leaf dark respiration

is widely recognized as a determinant of growth, maintenance, and carbon cycling in plants (Li *et al.*, 2013). Here, mature cacao trees seemed to be less responsive in total dry biomass accumulation under long-term exposure of elevated $[CO_2]$ compared to the juvenile cacao plants grown under non-limiting conditions and short-term exposure as it has shown in Chapter 4. Although leaf turnover (the fallen leaves) was collected across the experimental period at each treatment combination, this was not accounted in the final harvest due to the complexity to refer values to individual trees, and also for the low proportion of dry biomass provided in the final dry biomass.

The observed increase in iWUE at elevated $[CO_2]$ was associated with the significant increase in leaf photosynthesis rate rather than changes in stomatal conductance. Similar increases in iWUE of juvenile cacao plants grown under short-term at elevated $[CO_2]$ was also observed in chapters 3 and 4 and (Lahive *et al.*, 2021). Increases in iWUE can take place by improvement in leaf-level photosynthesis rates, a decline in stomatal conductance or a combination of the two parameters (Ainsworth and Long, 2005; Ainsworth and Rogers, 2007). On the other hand, the increase in iWUE with increasing temperature was likely due to the reduction in stomatal conductance. According to Hatfield and Dold (2019), as the $[CO_2]$ increases at moderate temperatures, the positive effect on iWUE also increases; however, as temperatures approach the species optimum temperature, the effect diminishes. In this experiment, the positive effect of elevated $[CO_2]$ on iWUE was evident even at warmer conditions (control temperature + 5.0°C) suggesting that the mature cacao trees did not reach supra-optimal temperatures in this study.

The decline in leaf chlorophyll content at higher temperatures and elevated $[CO_2]$ is consistent with studies on woody species such as *Eucalyptus saligna* (Murray *et al.*, 2013), *Quercus gilva* (Jeong *et al.*, 2018) and also in cocoa (Hebbar *et al.*, 2020). Genetic variation in the impacts of temperature on chlorophyll content and fluorescence has been previously reported (Daymond and Hadley, 2004). In this study, the observed decline in chlorophyll content, might be related to the slight reduction in leaf nitrogen concentration under elevated $[CO_2]$ conditions (data presented on one genotype in the acclimation analysis). The fact that no effects of elevated temperature or $[CO_2]$ on chlorophyll fluorescence parameters (*Fv/Fm and PI*) were observed indicates that no important changes in the primary photochemistry process occurred. These results in mature cacao plants are consistent to the findings in juvenile cacao plants grown in the short-term experiment under growth cabinets presented in Chapter 3.

This study has shown that elevated $[CO_2]$ increased the apparent quantum yield (Ø) by 39% in mature cacao plants whereas no effect of temperature was observed. The results suggest that the benefit of elevated $[CO_2]$ on quantum yield observed in juvenile plants (Chapter 4) is maintained in adult plants.

Lahive *et al.* (2021) also observed increases in Ø in a wide set of mature cacao plants grown for an extended period at elevated [CO_2]. It has been shown that increasing [CO_2] increases net carbon uptake in low light environments by increasing Ø (Long and Drake, 1991). Thus, an improvement in Ø for CO_2 fixation would be an important advantage in plants such as cacao which are often cultivated under the shade of other trees. Furthermore, in combination with appropriate light and under non-limiting water and nutrient conditions, crop systems where plants are grown at high density and so there is more self-shading could benefit under elevated [CO_2] scenario. Simultaneous with the increase in Ø was an increase in the light compensation point under elevated [CO_2]. In contrast, decreases of this parameter in plants grown at elevated [CO_2] has been reported in Amelonado cacao seedlings (Lahive *et al.*, 2018), mature cacao plants under long-term exposure (Lahive *et al.*, 2021) and observed in young cacao plants under the short-term experiment in Chapter 4. Furthermore, the impact of temperature in the light compensation point was more evident at elevated [CO_2]. It has been pointed out that increases in respiration rates or reductions in Ø could be responsible for increases in the light compensation point (Givnish, 1988; Lewis *et al.*, 1999). Here, the finding suggests that the increase in the light compensation point in response to elevated temperature and [CO_2] was related to the high respiration rate observed.

Evidence for downregulation of photosynthesis in response to [CO_2] was observed at the control temperature. There are various reasons why downregulation may occur at elevated [CO_2]. Under elevated [CO_2], the rate of photosynthesis rate may decline to balance source-sink ratio when carbohydrates are synthesized faster than sinks capability to utilise them, which results in photosynthetic acclimation (Makino and Mae, 1999; Salazar-Parra *et al.*, 2015). Downregulation has been also attributed to reductions in both activity and amount of ribulose-1, 5-biphosphate carboxylase-oxygenase (Rubisco) which is related to N content, stomatal conductance and chlorophyll content (Drake *et al.*, 1997; Makino and Mae, 1999; Ellsworth *et al.*, 2004; Ainsworth and Long, 2005). Here, following long-term growth at elevated [CO_2], stomatal conductance declined by 46% compared to an instantaneous increase in [CO_2]. Additionally, although CCN 51 mature cacao plants were cultivated under non-limiting water and nutrient conditions, there were slight decreases in leaf N content and significant reductions in leaf chlorophyll content that might have led to the photosynthetic downregulation observed. In contrast, at + 5.0°C above the control no evidence for downregulation was observed. Temperatures above the optimum may weaken photosynthetic capacity in plants and lead to decrease in photosynthesis rates (Hamerlynck *et al.*, 2000). By increasing temperature conditions of plants grown under elevated [CO_2], the photosynthetic performance might be enhanced due to Rubisco kinetic properties, as well as increased sink metabolism (use of photosynthetic final products; Lewis *et al.* 2002). Additionally, nitrogen is an important element that can regulate the photosynthetic process due to its importance in Rubisco activity and the electron transport components (Huang *et al.*, 2017).

Here, it has been shown that although no significant changes in stomatal conductance were observed, leaf N content increased in the mature cacao plants under the warming treatment which may be correlated with the positive impact on the rate of photosynthesis. Similar studies in other species have shown that high temperature may increase photosynthesis rate of *Populus cathayana* Rehd under elevated $[CO_2]$ (Zhao *et al.*, 2012), no downregulation in photosynthesis was observed in rose plants (Urban *et al.*, 2001) and photosynthesis of well fertilized and watered eucalyptus seedlings, remained responsive to increasing atmospheric $[CO_2]$ in future warming scenarios (Ghannoum *et al.*, 2010).

Photosynthetic acclimation to high temperatures was observed at current $[CO_2]$. CO_2 assimilation rates depend on temperature and vary between and within species and is also related to the growth conditions (Berry and Bjorkman, 1980; Yamori *et al.*, 2013). Increasing growth temperature has been found to increase the optimal temperature for photosynthesis (Berry and Bjorkman, 1980). This study has evidenced that after long-term growth at control temperature + 5.0°C, the optimal temperature for leaf photosynthesis appeared to increase suggesting adaptive mechanisms to warming conditions. Similar shifts in optimal temperature for leaf photosynthesis and signs of acclimation were reported in seedlings of three Neotropical species, which differed in light requirements and growth rates, when subjected to different temperature regimes (Slot and Winter, 2017). Furthermore, the present study has shown that photosynthetic acclimation to elevated temperature was greater at elevated $[CO_2]$ grown plants compared to those grown at ambient $[CO_2]$. Previously, it has been reported that elevated $[CO_2]$ increases the thermal point which is the temperature at which carbon uptake is optimized (Sage and Kubien, 2007) and may increase the temperature tolerance of photosynthesis in C3 plants (Wang *et al.*, 2008). Although it has been reported that tropical species are operating at temperatures close to their upper limits (Lloyd and Farquhar, 2008), here the cacao plants showed thermal acclimation which was enhanced in combination with long-term elevated $[CO_2]$ condition.

In conclusion, the results indicated that genotypic variation in growth and physiological responses to CO_2 and temperature increases might be exploited for the identification of traits that will support germplasm screening under future scenarios of climate change. In cacao, breeding has primarily focused on yield and disease tolerance, with a few efforts on climate tolerance (Lahive *et al.*, 2019). Compared to juvenile cacao plants, the growth and physiological parameters of mature cacao trees remained responsive to increases of atmospheric $[CO_2]$ under warmer conditions. However, the growth enhancement in mature cacao plants was lower in magnitude than the stimulation of photosynthesis. Furthermore, mature CCN 51 plants showed photosynthetic adjustments to warming and elevated $[CO_2]$ growth conditions. Although this is a preliminary investigation of photosynthetic acclimation in cacao to future climatic scenarios, it is suggested that this is explored across a broader range of genotypes. Finally, this study was carried out on growth and physiological components of cacao mature plants, and

whether such responses are seen in the reproductive components and pod and final bean yield, will be explored in the next chapter.

6 The effects of elevated [CO_2] and increased temperature on reproductive development and pod components of contrasting mature cacao genotypes (*Theobroma cacao* L.)

6.1 Introduction

In general, the reproductive stage in plants is more sensitive to heat stress than vegetative stages (Vara Prasad *et al.*, 2017). Studies have shown that elevated temperatures may cause early flowering in crops (Jagadish *et al.*, 2016; Tun *et al.*, 2021), as well as changes in floral phenology (Alzate-Marin *et al.*, 2021) and flower development (Drinnan and Menzel, 1995; D. Li *et al.*, 2019). On the other hand, a faster growth and early flowering under elevated [CO_2] conditions due to enhancement in photosynthesis rates might be expected (Rolland *et al.*, 2006). However, it has been shown that this response varied among species. In a review, Springer and Ward (2007) observed that 57% of wild species and 62% of cultivated crops showed an alteration in flowering time when grown under elevated [CO_2] (~700 ppm) with both accelerations and delays reported, with identified genotypic variation within species also evident. Similarly, Jagadish *et al.* (2016), reviewing 40 research studies reported that in 28 cases flowering was earlier and in 12 cases the event was delayed when plants were subjected to CO_2 enrichment. Whilst elevated [CO_2] promotes flowering in some species, this process can be accelerated under warming conditions (Tun *et al.*, 2021). For example, in Asteraceae species early flowering at elevated [CO_2] has been observed which is further accelerated with additional rising temperatures (Johnston and Reekie, 2008). However, temperature effects on flowering have gained most attention and the magnitude of the interactive effects with elevated [CO_2] is still not well understood (Ward *et al.*, 2012).

Under non-limiting water and nutrient conditions, early studies conducted by Sale, (1969) showed how temperature impacts flowering in cacao. Using controlled environment rooms, the author reported a similar time to flowering across temperatures, but flower number per cushion was greater at 26.7°C and 30°C compared to 23.3°C. This finding was consistent with field observations carried out by Cazorla *et al.* (1989) in Brazil, who reported that flowering was linked with seasonal variations in mean temperature which ranged from 18 to 28°C. The authors also reported a decline in flowering at the lower temperatures. More recently, an analysis of physiological data from international cacao trials, demonstrated increases in flowering intensity in response to temperature and that genetic variation in the flowering response to temperature exists within cacao germplasm (Daymond and Hadley, 2011). To date, research on the impact of elevated [CO_2] on flowering in cacao is scarce. Handley (2016), found no

clear effects of CO_2 enrichment on flower emergence and the number of flowers in six cacao genotypes grown for 22 months under controlled environment conditions.

The availability of viable pollen that germinates, develops, and leads to effective fertilisation determines the quantity of seeds per plant or fruit. Thus, environmental stress during reproductive stages may have a dominant effect on the final yield (Cohen *et al.*, 2021). Inside the flowers, the pollen grains are highly sensitive to temperature increases that may lead to sterility (Saini and Aspinall, 1982), production of fewer pollen grains (Vara Prasad *et al.*, 2006) and low pollen germination (Jagadish *et al.*, 2010). The interactive effects of temperature and elevated [CO_2] on pollen production, viability, and seed-set have been conducted in various types of crops with varied responses. For instance, pollen viability declined above 33°C in bean whilst no effect of [CO_2] was observed (Vara Prasad *et al.*, 2002). In contrast, the negative effect of high temperature on pollen viability was exacerbated under elevated [CO_2] in sorghum (Vara Prasad *et al.*, 2006) wheat (Bokshi *et al.*, 2021), cowpea (Singh *et al.*, 2010) and tropical legumes (Alzate-Marin *et al.*, 2021), whilst a compensation effect of elevated [CO_2] on pollen viability at increased temperatures has been reported in bell pepper (Aloni *et al.*, 2001). Despite the extensive research in annual crops, there is a lack of knowledge in how climatic factors interact to influence reproductive development in perennial tropical crops, such as cacao. However, individually the effects of temperature and elevated [CO_2] on cacao pollen have been reported.

Assessing the pollen performance in 11 genotypes of cacao (type Nacional) and CCN 51 on the Ecuadorian coast, García-Cruzatty *et al.* (2020) observed that monthly pollen production per flower was higher in months with air mean temperature above 26°C. Similarly, Mena-Montoya *et al.* (2020) reported that pollen flow (pollen dispersion) in seven cacao clones increased at warm temperatures (above 28°C). It has been suggested that the amount of pollen reaching the stigma could be linked to pollen production, which might be favoured under warming temperatures (García-Cruzatty *et al.*, 2020). Early studies regarding the effects of elevated [CO_2] on pollen performance were carried out by Aneja and Gianfagna (1992) in Amelonado cacao trees under controlled conditions. The authors noted that very high [CO_2] (~ 85,000 ppm) improved *in vitro* pollen germination which also led to improvements in pod set when pollen was previously incubated in sealed vials for 6 h. The same authors proposed that elevated [CO_2] might alleviate self-incompatibility in cacao (Aneja and Gianfagna, 1992). However, exploring the long-term effects of elevated [CO_2] (targeted to values of 700 ppm) on pollen performance of six cacao genotypes grown under controlled conditions, Handley (2016), found no significant changes in the percentage of pollen germination and a decline in pollen tube length.

Flower and fruit abortion is a physiological phenomenon that affects yield in many crops (Marcelis *et al.*, 2004). Excessive flowers are typically produced in tropical trees and both flower and fruit abortion is a

commonly observed characteristic (Stephenson, 1981). Sedgley and Griffin (1989) defined this response in terms of three periods: (i) within two weeks after anthesis, which also include unfertilized flowers, (ii) within two months after anthesis, where young fruits abort due to poor seed development, and (iii) drop of immature fruits which is related to internal competition for photosynthetic and nutrient resources. However, environmental stresses such as high temperatures, water deficit, and light conditions (Aloni *et al.*, 1996; Marcelis and Baan Hofman-Eijer, 1997; Marcelis *et al.*, 2004) are important factors that might induce abortion. The cacao reproductive system is particularly characterized by high flowering, of which no more than 5% typically develop into mature pods (Aneja *et al.*, 1999). In addition, cacao pod yield is also affected by a physiological mechanism known as "cherelle wilt" in which the young pods (cherelles) stop growing, turn yellow, wilt, and finally change to a brownish colour. Although this phenomenon is similar to fruit drop in other crops, in cacao the wilted cherelles remain attached to the tree (Valle *et al.*, 1990). Generally cherelle wilt is thought to be caused by lack of assimilate availability for a particular pod, which in turn is impacted by competition for assimilates and low assimilation rates (Nichols and Walmsley, 1965; Alvim, 1977). Hasenstein and Zavada (2001) also suggest that pollen incompatibility might play a role. In cacao, increases in temperature have been highlighted as one key factor that may limit pod development and seed yield (Alvim *et al.*, 1974; End *et al.*, 1988; Daymond and Hadley, 2008). It has also been proposed that high temperatures may increase the occurrence of cherelle wilt (Hadley *et al.*, 1994a; Daymond and Hadley, 2008) due to the higher assimilate demand resulting from increased respiration. End *et al.* (1988), and Daymond and Hadley (2008) showed decreases in final pod size and bean size in plants subjected to elevated temperatures with some genotype-specific responses. In addition, Hadley *et al.* (1994b) working under controlled conditions which simulated climatic conditions of cacao regions of Brazil, Ghana, and Malaysia, reported that increasing temperature reduced the time it took for pods to reach maturity as well as bean dry weight. Regarding the effects of elevated $[CO_2]$, one study has been carried out examining the effects on pollination, cherelle wilt and pod development on six genotypes grown for two years under controlled glasshouse conditions (Handley, 2016). Shifts in the responses of pollination success across two harvest periods, as well as increases in wilting were observed at elevated $[CO_2]$, suggesting competition among vegetative sinks for internal resources occurred. The author also remarked that when pods overcame the "wilt period" (~ 80 days from pollination according to Nichols (1964), there was a clear positive effect of elevated $[CO_2]$ on pod growth rate and final size.

Understanding the combined effects of elevated temperature and $[CO_2]$ is key to determining agricultural practices or genetic improvements required to sustain cacao productivity in future climate scenarios. To date, information on the interactive effects of these climate factors on reproductive processes and pod components in cacao is still scarce. The present experiment was conducted to

elucidate the effects of elevated $[CO_2]$ and increased temperatures on flowering intensity, pollen viability, pod development, and bean parameters of mature cacao genotypes. The hypotheses explored in this experiment were: (i) the combined effects of elevated $[CO_2]$ and temperature on reproductive development and pod components in cocoa will vary genotypically; (ii) elevated $[CO_2]$ may alter the effect of higher temperature on flowering intensity; (iii) higher growth temperature will negatively impact on pollen germination and pollen tube development but elevated $[CO_2]$ will alleviate some of these negative effects ; (iv) the negative effect of high temperature on the pod set, cherelle wilt and pod development will be in part compensated by elevated $[CO_2]$; (v) resource allocation within the pods and bean biomass per pod will be altered by growth conditions.

6.2 Materials and Methods

6.2.1   Plant material and experimental setup

Three cacao genotypes (CCN 51, SCA 6 and T85/799), which were three years old at the start of the experiment were grown under the experimental arrangement described in Chapter 5 (section 5.2.1). The cacao trees were maintained in the same arrangement showed in Figure 5.1, which consisted of a combination of three temperature regimes: T1 (control temperature), T2 (control temperature + 2.5°C) and T3 (control temperature + 5.0°C), and at two CO_2 concentrations: ambient (a target of 400ppm) and elevated (a target of 700ppm). The experimental period ran from 19-11-2019 to 23-03-2021. On 21-04-20 there was a breakdown in the CO_2 enrichment system in glasshouse number 1 (T2 + elevated $[CO_2]$) which led to restrictions in data collection and the incorporation of the treatment for the analysis. Experimental facilities and control of environmental conditions are described in full in Chapter 2. The climatic average temperature, $[CO_2]$ and relative humidity reached within each glasshouse during the experimental period is summarised in Table 6.1. Regarding to ambient $[CO_2]$, values were slightly higher than the target.

Table 6.1 Climatic glasshouses conditions during the experimental period (average values from 19-11-2019 to 23-03-2021). T1 a[CO_2]= control temperature and ambient [CO_2], T1 e[CO_2]= control temperature and elevated [CO_2], T2 a[CO_2]= control temperature + 2.5°C and ambient [CO_2], T2 e[CO_2]= control temperature + 2.5°C and elevated [CO_2], T3 a[CO_2]= control temperature + 5.0°C and ambient [CO_2], and T3 e[CO_2]= control temperature + 5.0°C and elevated [CO_2].

	Mean daily temperature (°C)	[CO_2] (ppm)*	Relative Humidity (%)
T1 a[CO_2]	25.9	474.9	64.1
T1 e[CO_2]	26.1	687.1	67.1
T2 a[CO_2]	28.1	483.8	62.6
T2 e[CO_2]**	28.8	705.4	63.9
T3 a[CO_2]	30.2	415.3	69.9
T3 e[CO_2]	30.1	679.7	63.5

*ambient [CO_2] values were slightly above the set target

** Data presented until 21-04-20 due to heater breakdown

6.2.2   Flowering measurements

Flowering observations were carried out from 20-01-20 to 20-08-20 on trees of CCN 51 (n=8) and SCA 6 (n=5) per treatment combination. Due to the breakdown in glasshouse 1, flowering analysis was restricted to T1 and T3 temperature conditions at ambient and elevated [CO_2]. A 30 cm section of the main trunk located 20 cm from the top of the growing substrate and a 20 cm section of two lateral branches from the jorquette were marked on each tree. Trunk and branches were old enough to produce flowers regularly. The number of flowers within each section was counted at monthly intervals. Before counting, dead and open flowers from within the selected sections were removed using a soft brush in order to keep unopened buds intact. The following day, the number of mature flower buds and open flowers were counted between 08:00 to 11:00 h. Flowering intensity over time and cumulative during the experimental period was measured as the number of the flower per cm^2 using the surface area of the main trunk and branches calculated from equation 6.1 used by Handley (2016) in cacao.

$$A = \left(\frac{(\pi * D1) + (\pi * D2)}{2} \right) * L \qquad (6.1)$$

Where D1 and D2 are the correspondent diameters of the section and L is the length.

6.2.3   In Vitro pollen measurements

Pollen germination was used as a measure of cacao pollen viability through the "sitting drop culture" method (Shivanna and Rangaswamy, 1992). The treatments assessed were the combination of two CO_2 concentrations (ambient and elevated) and two day/night temperatures (T1 and T3). From each of the

treatment combinations, six fresh open flowers from four trees of each of the genotypes CCN 51, SCA 6 and T 85/799 were collected between 07:00 to 08:00 h during 11-03-2021 to 23-03-2021.The flowers were stored in clean petri dishes with moist filter paper placed in the bottom and sealed with Parafilm® tape. Petri dishes were placed in a polystyrene box for transportation to the laboratory. Pollen extraction was carried out in the Seeds Laboratory located in Agriculture School at the University of Reading, using tweezers, needle/pointed tool and a fine paint brush which had been cleaned with 70% ethanol before each use. From each replicate tree, six flowers were pooled and the pollen was brushed from the anthers onto microscope slides containing two drops of Brewbaker and Kwack's germination medium (Shivanna and Rangaswamy, 1992). The medium consisted of: 8% sucrose, 100 mg L^{-1} boric acid, 300 mg L^{-1} calcium nitrate, 200 mg L^{-1} magnesium sulphate and 100 mg L^{-1} potassium nitrate. Each microscope slide was put in an individual petri dish, where relative humidity was kept high using a moist filter paper in the bottom, and then sealed with Parafilm® tape. Pollen grains were germinated in an incubator at the correspondent maximum growth temperature, either 31 and 36°C, for 6 h in the dark.

Germination was examined immediately after the incubation period, using a *Leitz Dialux 20* light microscope at 10x magnification with a *Leica DFC450* digital camera attached (*Leica Microsystems*, Wetzlar, Germany). Six representative areas from each slide were selected for image capture using *Leica Application Suite* software version 4.6.2. The number of germinated and non-germinated pollen grains from each image were counted using *ImageJ* version 2.2 analysis software (Rueden *et al.*, 2017). Pollen grains were considered germinated when the length of the pollen tube was equal to or longer than the pollen diameter (Kakani *et al.*, 2002). Measurements of pollen tube length were also recorded using *ImageJ*. Mean pollen tube length was calculated as the average of 5 pollen tubes measured from each representative area for the pollen counting.

6.2.4   Pollination and pod growth

Hand pollinations were carried out from 19-11-19 to 14-12-19 on trees of CCN 51 (n=8) and SCA 6 (n=5) within each treatment combination. The pollinations were made using pollen from trees of CCN 51 located and grown in the glasshouse 1 (mean daily temperature of 28.1°C, [CO_2] of 708.2 ppm, and relative humidity of 58.7%) since SCA 6 is self-incompatible. Initially, fresh open flowers were collected before 08:00h in clean petri dishes. Four new opened flowers from each tree were identified in the main trunk section and the staminodes were removed carefully with tweezers in order to uncover the stigma. A CCN 51 flower from the petri dish was randomly selected and anthers were uncovered by removing all petals with tweezers. Subsequently, each of the anthers was applied on top of the pistil of the pre-identified flower several times. Each pollinated flower was labelled with the date. Pollinations were carried out weekly in order to generate at least eight pods per tree. Pollinated flowers were assessed on

a weekly basis to determine the number of successful pollinations (recorded as fruit set; identified by the presence of a swollen ovary). Following the protocol used by Handley (2016), cherelle wilt was recorded on any fruit which had been measured at least once and subsequently turned blackish-brown and stopped growing. The pods wilted and the number of successful pollination were used in order to determine the percentage of pods wilted according to the equation 6.2

PW= W/SP * 100   (6.2)

Where PW is the percentage of pods wilted, W is the pods wilted, and SP is successful pollination. For each successful fruit set, pod length and breadth were measured weekly using a digital calliper. Data was collected until no changes in size were observed, at which point it was assumed that the ripening point was approached (~ 5-6 months). Cacao pods can be regarded as a prolate spheroid (elliptical) shape (Ten Hoopen *et al.*, 2012) and the ellipsoid equation 6.3 was used to calculate pod volume

$$V = \frac{4}{3} * \pi * a * b * c \qquad (6.3)$$

Where *V* is the volume (mm³), *a* and *b* are the equatorial radius (mm), and *c* the polar radius (mm). A four parameter logistic regression was implemented to describe the pod volume (pod size) increases across the experimental period according to equation 6.4. This equation has been used previously for examining cacao pod sizes by Daymond and Hadley (2008).

$$Y = \frac{a+d}{1+\exp(-b(X-c))} \qquad (6.4)$$

Whereby *a* is the upper asymptote, *d* is the lower asymptote, *c* is *X* value with a response half-way between *a* and *d*, while *b* in the correspondent slope around the inflection point.

The logistic regression was performed for individual pods, and the biological parameters maximum pod size (mm³), maximum pod growth rate (mm³ day⁻¹) and time to maximum pod growth rate (days) were obtained (*a*, *b*, and *c* respectively from equation 6.4). Additionally, the parameter time to reach 95% of maximum pod size (days) was calculated from the equation.

6.2.5 Pod harvest

Pods were harvested when they reached maturity (considered when the pod stopped growing and a colour change occurred) and harvests was carried out regularly from 21-04-20 to 31-07-20. Pods were removed from the trees by cutting the peduncle using secateurs and transported to the laboratory for destructive determinations. Initially, the whole pod fresh weight was recorded using a digital scale

(KERN, model PCB 250-3, *KERN & SOHN*, Balingen, Germany). Subsequently, the husk was cut in half lengthwise using a small kitchen knife. Beans, husk and placenta were detached, and the pulp was removed from the beans using a paper towel. Finally, the testa (shell) was removed from each bean using a razor blade. Beans, testa, and placenta fresh weight was registered using the same digital scale (KERN, model PCB 250-3, *KERN & SOHN*, Balingen, Germany) while husk fresh weight was taken using a large balance (CBK 32, Adam Equipment, Milton Keynes, UK). Beans, testa, and placenta were individually wrapped into foil and placed in a paper bag together the husk and labelled. Fresh samples were oven dried at 70°C to a constant weight before dry weight was recorded.

6.2.6   Data analysis

A glasshouse blocking system was considered (as shown in Figure 5.1, Chapter 5) and the effects of genotype, $[CO_2]$, temperature, and their interactions were evaluated using an unbalanced three-way ANOVA prior to testing data homoscedasticity and normality. Number of flowers over the time and treatment effects were tested using repeated measures ANOVA. Variation between time periods was tested for using the three-way ANOVA. Data transformation (log or square-root) was carried out in order to normalize the data of successful pollination, maximum pod size, pod dry weight, husk dry weight, average total beans dry weight and average bean number. Post hoc Bonferroni test ($P < 0.05$) was followed to compare group of means. Kruskal-Wallis one-way ANOVA was applied to data which could not be normalised by transformation. Pod growth logistic regressions were modelled using the *drm* function from R package *drc* (Ritz *et al.*, 2015) which allowed extraction of the four parameters from equation 6.3. Statistical analysis were carried using GENSTAT 19th edition software (VSN International Ltd., Hemel Hempstead, UK) and graphs were obtained using R studio version 4.0.4 (R Core Team, 2021).

6.3   Results

6.3.1   Flowering intensity

Figure 6.1 shows the monthly fluctuation of flowering intensity in CCN 51 and SCA 6 in each of the treatments. A significant effect of time ($P < 0.001$) was evidenced through the repeated measurements ANOVA with flowering intensity ranging from 0.051 (±0.003) flowers per cm^2 to 0.072 (±0.003) flowers per cm^2. At each time and the total data across the time, flowering intensity varied significantly between genotypes ($P < 0.001$). Overall, flowering intensity was higher in CCN 51 than SCA 6 (0.53 ±0.02 flowers per cm^2 and 0.32 ±0.02 flowers per cm^2, respectively). No significant effect of $[CO_2]$, temperature and other interactions were observed neither at each timepoint nor combining across all timepoints.
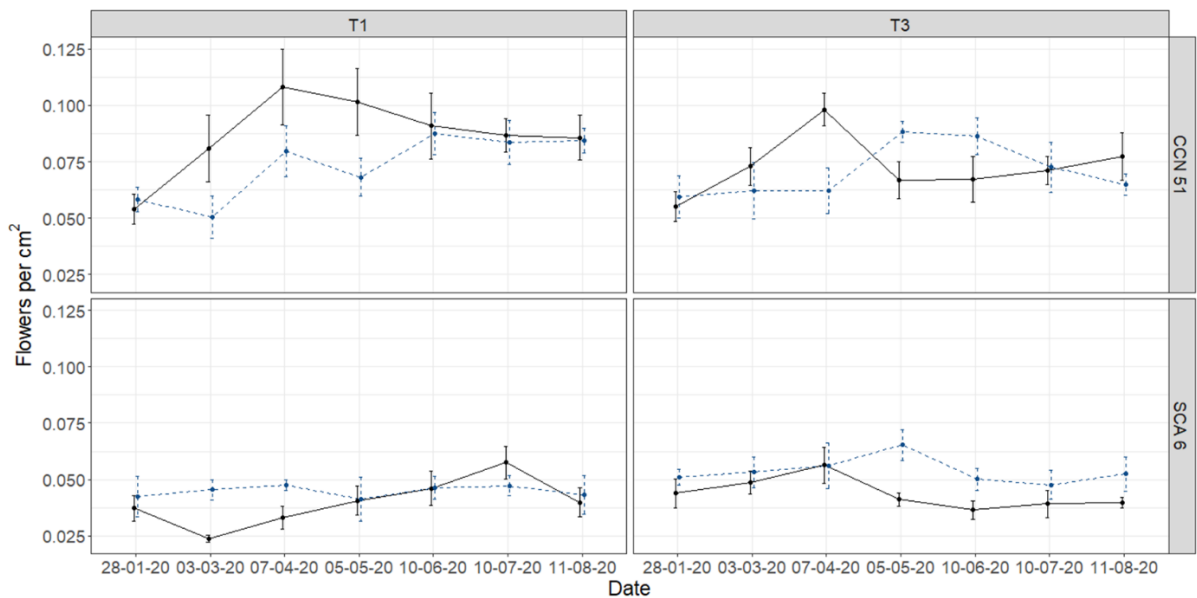
Figure 6.1 Effect of [CO_2] and temperature on flowering intensity of two cacao genotypes over time. Error bars show the standard error of the mean (CCN 51 *n*=8; SCA 6 *n*=5). Temperature treatments are T1 (control temperature) and T3 (control temperature + 5.0°C). [CO_2] treatments are ambient (grey bar) and elevated (blue bar).

6.3.2   Pollen responses

Pollen germination. A significant reduction in percentage pollen germination was observed at elevated growth temperature from 61.4% at T1 to 42.1% at T3 (*P* < 0.01, Kruskal-Wallis one-way ANOVA; Figure 6.2). Pollen obtained from trees grown at ambient [CO_2] had significantly lower germination (44.1%) compared to those collected under elevated [CO_2] (59.1%) (*P* < 0.001, Kruskal-Wallis one-way ANOVA). There was no effect of the genotype on the percentage pollen germination. Although the interaction term could not be tested, the data shows that the decline in pollen germination in the high temperature was larger under ambient [CO_2] treatment compared to the elevated [CO_2] being more evident in T 85/799.
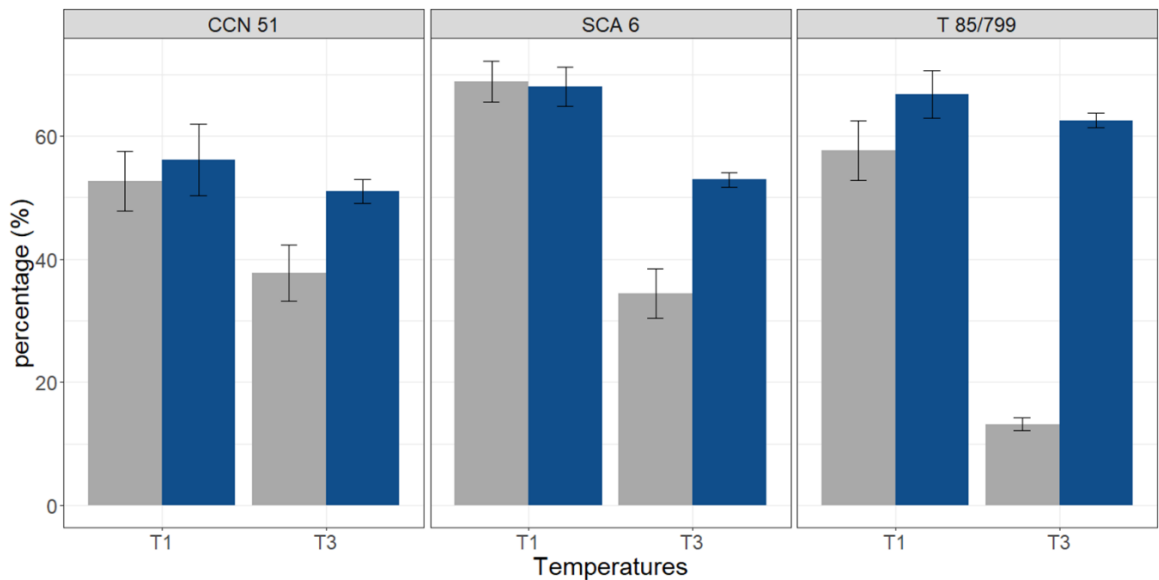
Figure 6.2 Effect of [CO_2] and temperature on percentage of pollen germination of three cacao genotypes. Error bars show the standard error of the mean (n=4). Temperature treatments are T1 (control temperature) and T3 (control temperature + 5.0°C). [CO_2] treatments are ambient (grey bar) and elevated (blue bar).

Pollen tube length. Pollen tube length was significantly higher in pollen collected under elevated [CO_2] (192.9 ± 12.1 µm) than ambient [CO_2] (94.2 ± 9.4 µm) ($P < 0.001$, Kruskal-Wallis one-way ANOVA) (Figure 6.3). Although interactions among main factors were not determined due to the non-normal distribution of the data, the graph shows that the impact of high temperature is much more evident in SCA 6 and T 85/799 under ambient [CO_2].

Figure 6.3 Effect of [CO_2] and temperature on pollen tube length of three cacao genotypes. Error bars show the standard error of the mean (n=4). Temperature treatments are T1 (control temperature) and T3 (control temperature + 5.0°C). [CO_2] treatments are ambient (grey bar) and elevated (blue bar).

6.3.3   Pollination

Pollination success. The percentage of successful and unsuccessful pollinations resulting from the hand pollinations of CCN 51 and SCA 6 genotypes grown under the treatment combinations is presented in Figure 6.4. Pollination success rate was significantly higher in SCA 6 than CCN 51 (53.6 ±3.3% and 41.6 ±5.0% respectively; $P < 0.05$). Pollination success rate also differed with temperature ($P < 0.05$). No difference was seen from T1 to T2 (47.5 ±5.0% and 56.8 ±4.4% respectively), where increases in temperature to T3 caused a decline to 33.1 (±4.4) % in pollination success. Pollination success was significantly higher at elevated $[CO_2]$ (52.3 (±4.1) %) compared to ambient $[CO_2]$ (40.7 (±3.8) %) ($P < 0.01$). No significant interactions among genotypes, temperature and $[CO_2]$ treatments were observed.
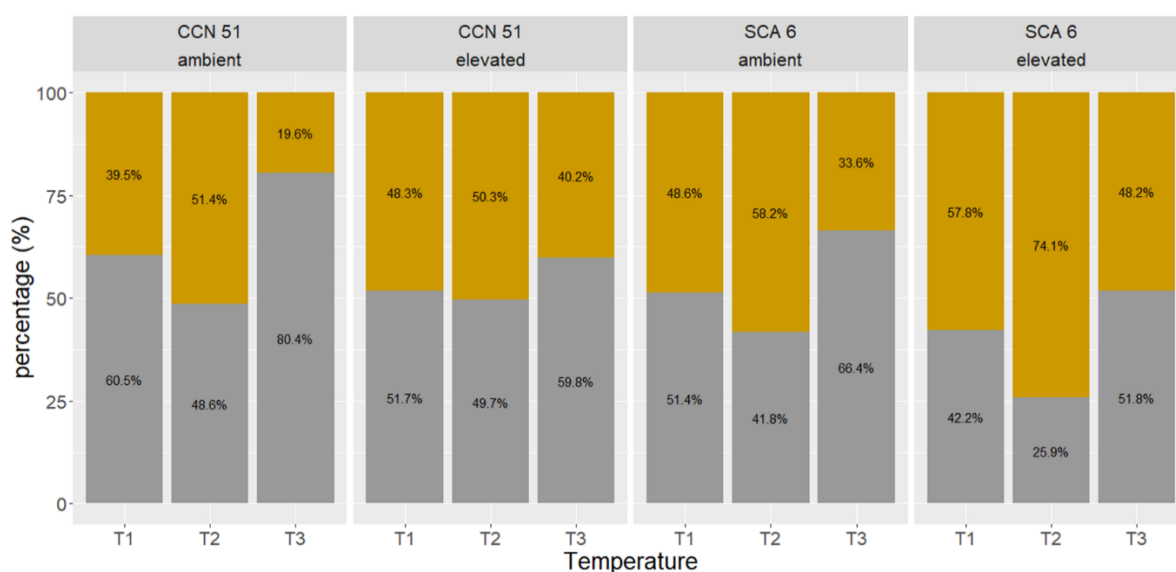
Figure 6.4 Effect of $[CO_2]$ and temperature on pollination success of two cacao genotypes (CCN 51 n=8; SCA 6 n=5). $[CO_2]$ treatments are ambient and elevated. Temperature treatments are T1 (control temperature), T2 (control temperature + 2.5°C), and T3 (control temperature + 5.0°C). Yellow –pollination success; Grey –pollination unsuccessful.

Pod production. Figure 6.5 shows the treatment effects on the percentage of final pods obtained and those wilted during the experimental period. Here, final pods relates to the percentage of pod set that went on to mature. A greater proportion of pods reached maturity in SCA 6 (68.9 ±4.7%) compared to CCN 51 (31.7 ±4.0%), hence, a greater proportion of pods wilted in CCN 51 (68.3 ±4.0%) compared to SCA 6 (31.1 ±4.7%) ($P < 0.001$, Kruskal-Wallis one-way ANOVA). At elevated $[CO_2]$ the proportion of pods reaching maturity was higher than at ambient $[CO_2]$ (53.5 ±4.5% and 38.4 ±4.8%, respectively), whereas there was a significant increase in the percentage of wilted pods of trees grown at ambient $[CO_2]$ (60.6 ±4.8%) compared to those ones grown at elevated $[CO_2]$ (46.7 ±5.5%) ($P < 0.05$, Kruskal-Wallis one-way ANOVA). Regarding temperature effects, a slight reduction in the percentage of final pods was observed

in the T2 temperature treatment (32.8 ±5.2%) which was in the borderline of significance (P = 0.05, Kruskal-Wallis one-way ANOVA) compared to the percentage at T1 (56.8 ±6.5%) and T3 (49.5 ±6.8%), while wilted pods increased from T1 to T2 (43.5 ±6.5% and 67.2 ±5.2% respectively) from which increases in temperature to T3, resulted in a decline of 50.5 (±6.8) %. Interactions among main factors were not determined due to the non-normal distribution of the data.
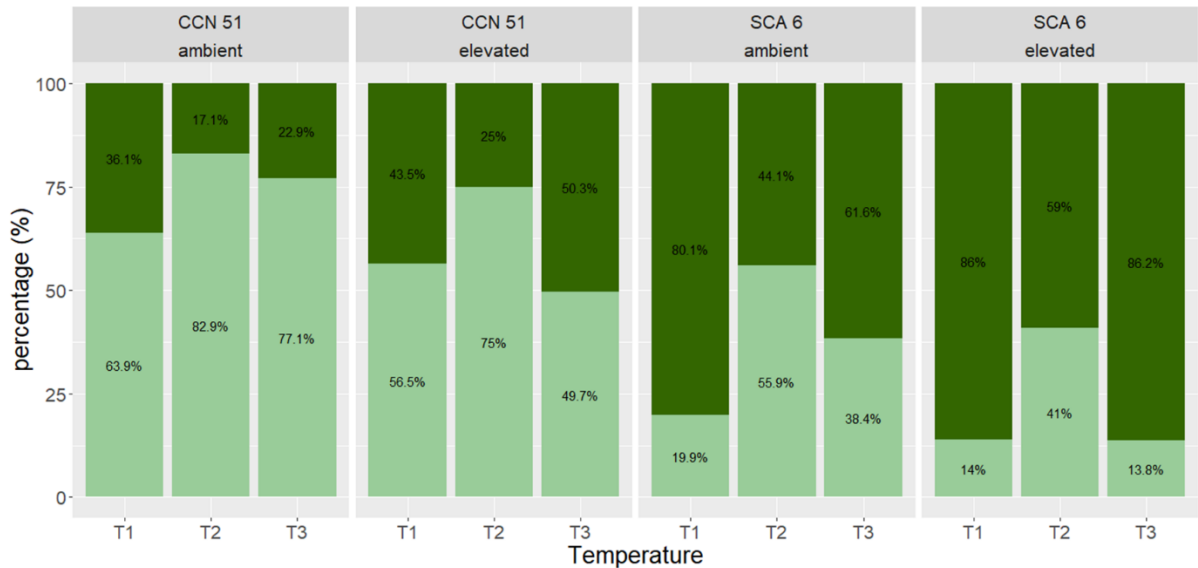
Figure 6.5 Effect of [CO_2] and temperature on pod production of two cacao genotypes (CCN 51 n=8; SCA 6 n=5). [CO_2] treatments are ambient and elevated. Temperature treatments are T1 (control temperature), T2 (control temperature + 2.5°C), and T3 (control temperature + 5.0°C). Dark green – final pods; Light green – wilted pods.

6.3.4   Pod development

The mean regression curves by treatment combination for the genotypes CCN 51 and SCA 6 are shown in Figures 6.6 and 6.7 respectively. Genotypic variation was observed in pod size across time. Pod size declined with an increase in temperature from T1 to T2 in CCN 51 but increased with temperature at elevated [CO_2], whereas in SCA 6 pod size declined when temperature increased to T3 at ambient but remained constant at elevated [CO_2]. The effect of [CO_2] was weaker in SCA 6, so the relative increase in pod size with elevated [CO_2] was not as great as in CCN 51. Pod growth parameters obtained are discussed in more detail below.
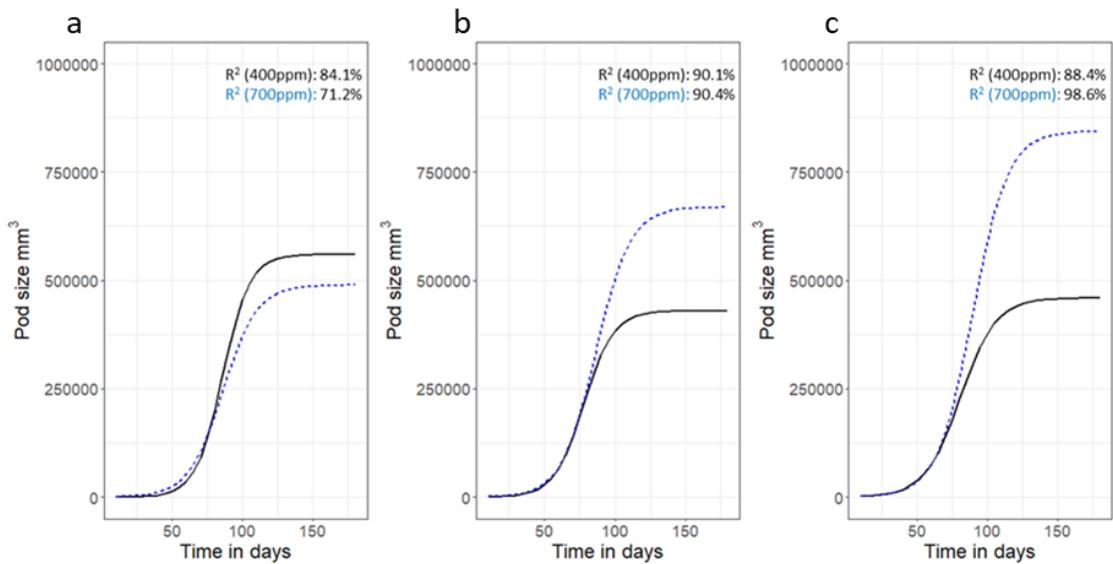
Figure 6.6 Mean regression curves for CCN 51 cacao pods grown under two [CO_2] and three temperature regimes. Curves are based on the logistic equation applied to each treatment combination (n-total=59). Temperature treatments are 31/22°C (a), 33.5/24.5°C (b) and 36/27°C (c). [CO_2] treatments are ambient (black solid line) and elevated (blue dashed line).
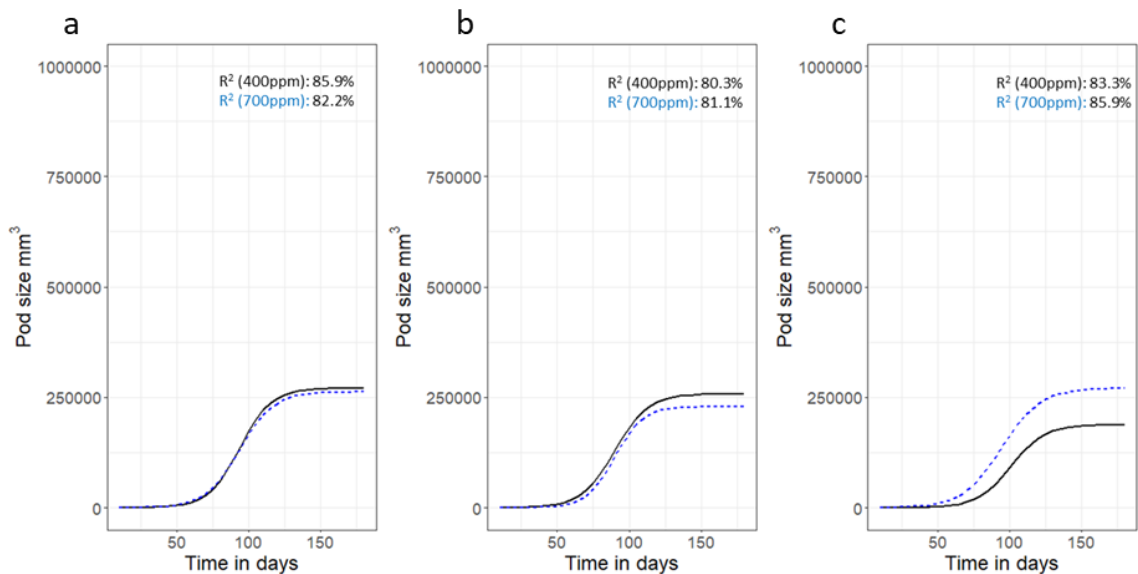
Figure 6.7 Mean regression curves for SCA 6 cacao pods grown under two [CO_2] and three temperature regimes. Curves are based on logistic equation applied to each treatment combination (n-total=98). Temperature treatments are 31/22°C (a), 33.5/24.5°C (b) and 36/27°C (c). [CO_2] treatments are ambient (black solid line) and elevated (blue dashed line).

Maximum pod growth rate. Figure 6.8 shows the effect of temperature and [CO_2] on maximum pod growth rate (parameter "b" estimated from the logistic regression in equation 6.3). Maximum pod growth rate was higher in CCN 51 than SCA 6 (0.096 (±0.003) mm^3 day^{-1} and 0.087 (±0.002) mm^3 day^{-1}

respectively; $P < 0.05$). A significant interaction between temperature, $[CO_2]$ and genotype was also observed ($P < 0.05$). In CCN 51, under elevated $[CO_2]$, an increase in maximum pod growth rate was observed with temperature increases from T1 to T3, whereas at ambient $[CO_2]$ this maximum pods growth rate declined with increases in temperature ranging from 0.113 (±0.006) $mm^3 day^{-1}$ at T1 to 0.092 (±0.006) $mm^3 day^{-1}$ at T3. For SCA 6, at elevated $[CO_2]$, no changes in maximum pod growth rate were observed with increases in temperature. However, at ambient $[CO_2]$, whilst increases in temperature from T1 to T2 did not impact maximum pod growth rate (0.094 ±0.001 $mm^3 day^{-1}$ and 0.097 ±0.002 $mm^3 day^{-1}$ respectively) an increase to T3 resulted in a decline to 0.075 (±0.004) $mm^3 day^{-1}$.
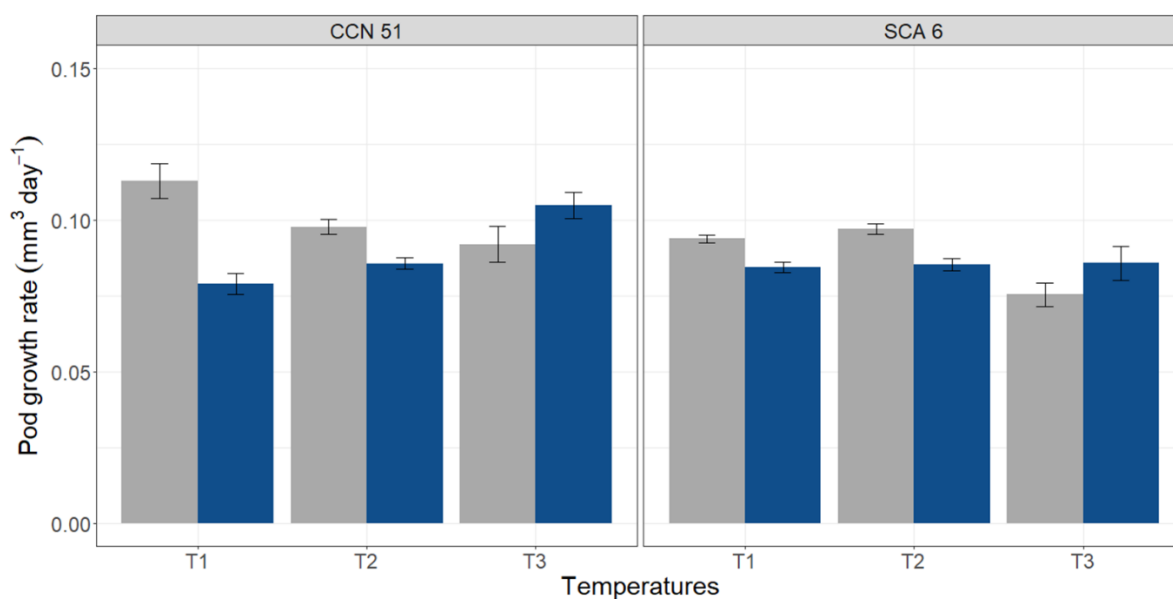
Figure 6.8 Effect of $[CO_2]$ and temperature on maximum pod growth rate of two cacao genotypes. Error bars show the standard error of the mean (CCN 51 n-total=59; SCA 6 n-total=98). Temperature treatments are T1 (control temperature), T2 (control temperature + 2.5°C), and T3 (control temperature + 5.0°C). $[CO_2]$ treatments are ambient (grey bar) and elevated (blue bar).

Maximum pod size. The effects of the treatment combinations on maximum pod size (logistic regression parameter "a" from equation 6.3) are shown in Figure 6.9. Overall, there was a significant effect of genotype on maximum pod size which was higher in CCN 51 (619,079 (±25,903) mm^3) than in SCA 6 (278,753 (±10,944) mm^3) ($P < 0.001$). There was also a significant interaction between temperature and $[CO_2]$ in both genotypes ($P < 0.001$). For CCN 51, at ambient $[CO_2]$, increases in temperature from T1 to T2 reduced maximum pod size from 619,145 (±22,539) mm^3 to 517,957 (±10,421) mm^3, respectively, whilst no change was seen at T3 (516,684 (±26,252) mm^3). Conversely, under elevated $[CO_2]$, increases in temperature increased maximum pod size ranging from 504,133 (±58,718) mm^3 at T1 to 836,913 (±11,632) mm^3 at T3. In SCA 6, at ambient $[CO_2]$, increases in temperature from T1 to T2 did not affect maximum pod size (294,992 (±14,866) mm^3 and 261,480 (±14,928) mm^3, respectively), while

temperature increases up to T3 resulted in a decline of maximum pod size to 215,762 (±23,414) mm^3. Under elevated $[CO_2]$, maximum pod size of SCA 6 declined significantly from T1 (315,937 (±18,616) mm^3) to T2 (241,230 (±3,538) mm^3) whereas at T3 maximum pod size was similar to that of the control temperature (354,888 (±22,010) mm^3).
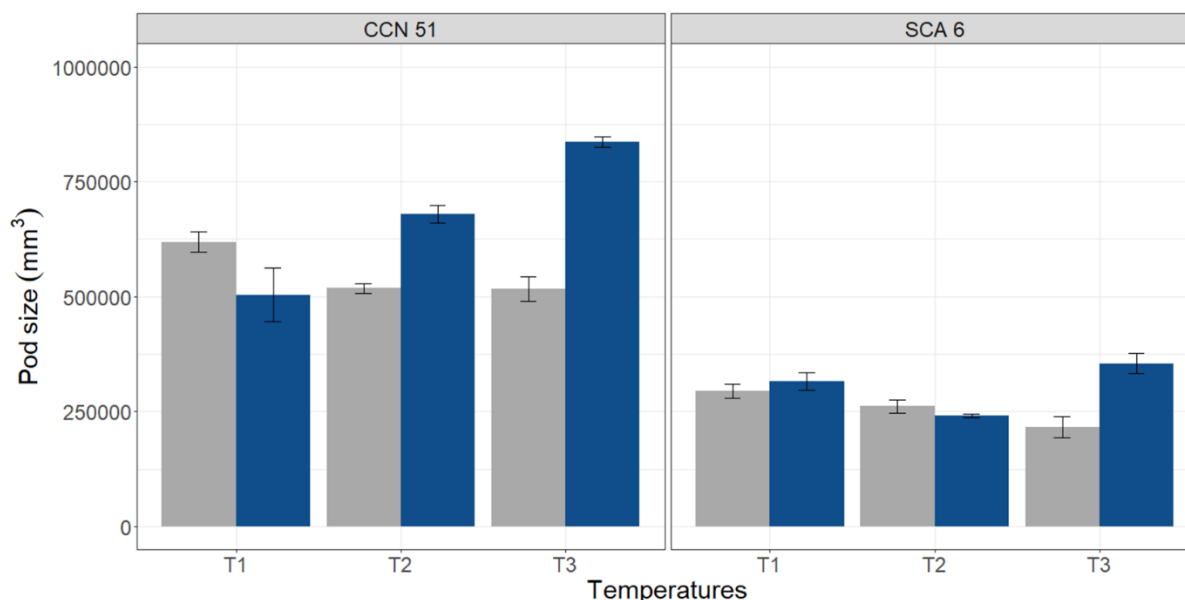
Figure 6.9 Effect of $[CO_2]$ and temperature on maximum pod size of two cacao genotypes. Error bars show the standard error of the mean (CCN 51 n-total=59; SCA 6 n-total=98). Temperature treatments are T1 (control temperature), T2 (control temperature + 2.5°C), and T3 (control temperature + 5.0°C). $[CO_2]$ treatments are ambient (grey bar) and elevated (blue bar).

Time to the maximum pod growth rate. The time taken to reach the maximum pod growth rate varied between genotypes ($P < 0.001$) ranging from 85 (±1) days in CCN 51 to 97 days (±1) in SCA 6 (Figure 6.10). There was a significant interaction between genotype and $[CO_2]$ ($P < 0.001$). The effect of elevated $[CO_2]$ was evident in SCA 6, where time to the maximum pod growth rate decreased by 5%, whereas no effect of CO_2 was observed in CCN 51. There was also a significant interaction between genotype and temperature treatments ($P < 0.001$). For CCN 51, an increase in temperature from T1 to T2 increased time to the maximum pod growth rate by 7%, further temperature increases had no effect. In SCA 6, time to the maximum pod growth rate did not differ significantly when temperature increased from T1 to T2 (94 (±1) days and 91 (±2) days, respectively) while there was a significant increase at T3 to 106 (±2) days. Across the genotypes, there was a significant interaction between temperature and $[CO_2]$ ($P < 0.01$). The time to maximum pod growth rate did not differ between $[CO_2]$ treatments at T1 and T2 whereas there was a significant 6% decline at T3 in the elevated $[CO_2]$ treatment.
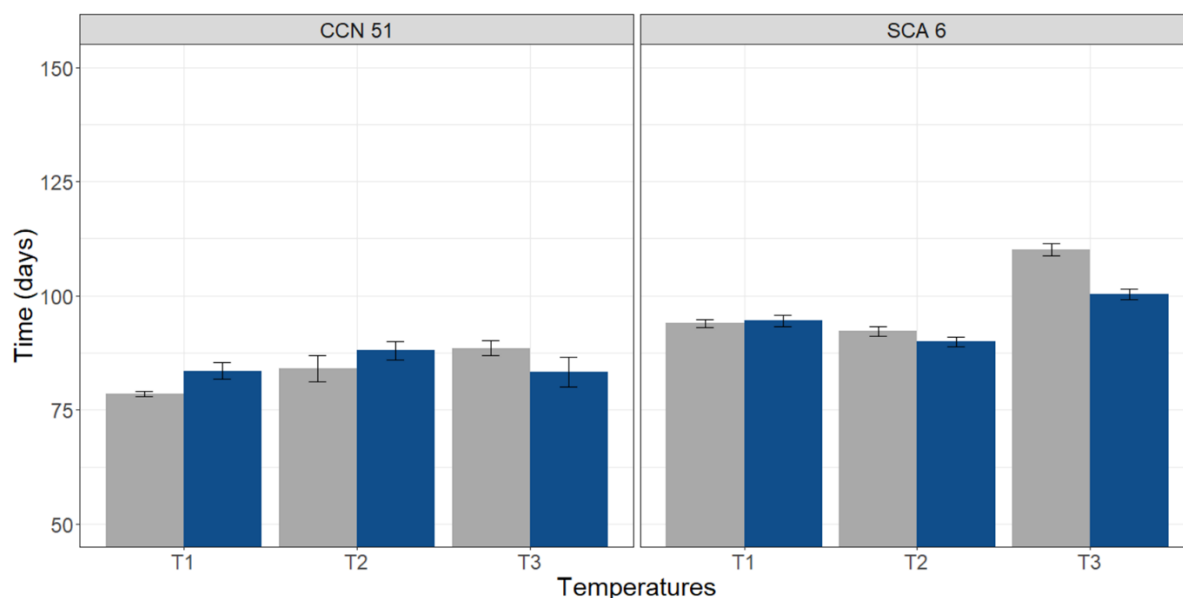
Figure 6.10 Effect of the $[CO_2]$ and temperature on time to maximum pod growth rate of two cacao genotypes. Error bars show the standard error of the mean (CCN 51 n-total=59; SCA 6 n-total=98). Temperature treatments are T1 (control temperature), T2 (control temperature + 2.5°C), and T3 (control temperature + 5.0°C). $[CO_2]$ treatments are ambient (grey bar) and elevated (blue bar).

Time to reach 95% of maximum pod size. Across the temperature and $[CO_2]$ treatments, the time to reach 95% of maximum pod size was significantly higher in SCA 6 (129 (±1) days) than CCN 51 (123 (±2) days) ($P < 0.001$) (Figure 6.11). There was also a significant temperature effect on this parameter ($P < 0.001$), with pods taking 8% longer to reach full size in the T3 treatment compared to the T1 treatment. There was a significant interaction between temperature and $[CO_2]$ ($P < 0.001$). At T1 and T2, time to reach 95% of maximum pod size did not differ between $[CO_2]$ treatments whereas at T3 this parameter declined by 7.8% under elevated $[CO_2]$.
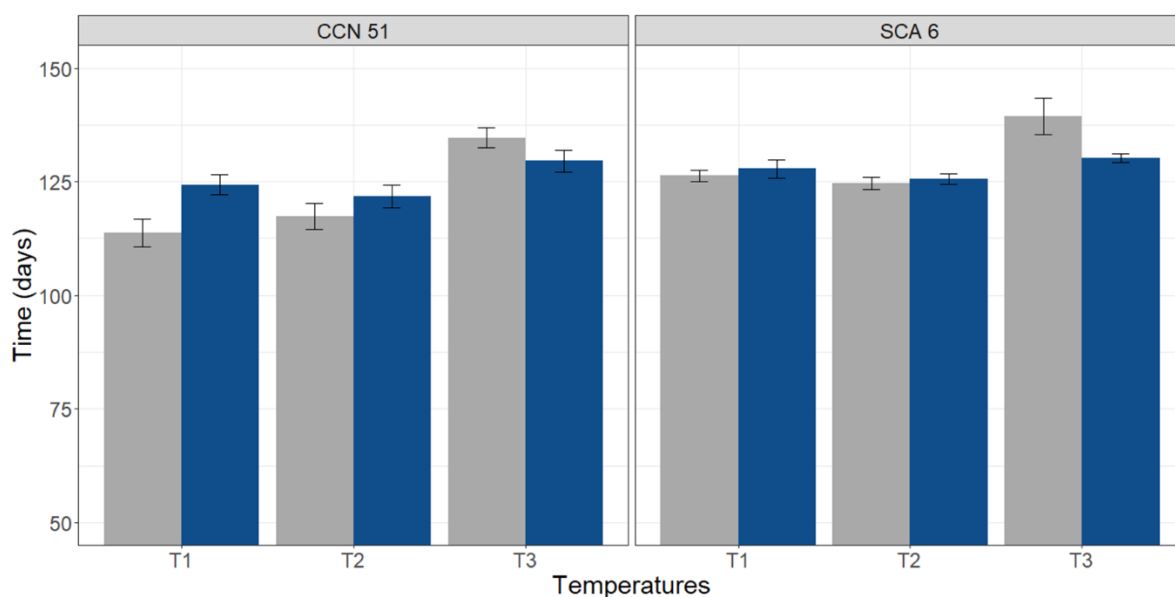
Figure 6.11 Effect of [CO_2] and temperature on time to reach 95% of maximum pod size of two cacao genotypes. Error bars show the standard error of the mean (CCN 51 n-total=59; SCA 6 n-total=98). Temperature treatments are T1 (control temperature), T2 (control temperature + 2.5°C), and T3 (control temperature + 5.0°C). [CO_2] treatments are ambient (grey bar) and elevated (blue bar).

6.3.5   Pod and bean parameters

Pod dry weight. Pod dry weight was significantly higher in CCN 51 (72.4 (±2.95) g) than SCA 6 (36.5 (±1.11) g) ($P < 0.001$) (Figure 6.12). The response to temperature varied in each of the genotypes ($P < 0.05$). In CCN 51, pod dry weight did not differ between the T1 and T2 treatments (65.4 (±5.2) g and 68.6 (±5.2) g, respectively), whilst pod dry weight increased in the T3 treatment to 78.9 (±3.6) g. Conversely, in SCA 6, pod dry weight declined by 11% as temperature increased from T1 to T2, further temperature increases did not affect pod dry weight. Elevated [CO_2] did not influence pod dry weight at T1, but at higher temperatures pod dry weight was significantly higher (by 23.75 and 54.4% at T2-especially in CCN 51- and T3 respectively) in the elevated [CO_2] condition compared to the ambient [CO_2] treatment ($P < 0.01$).
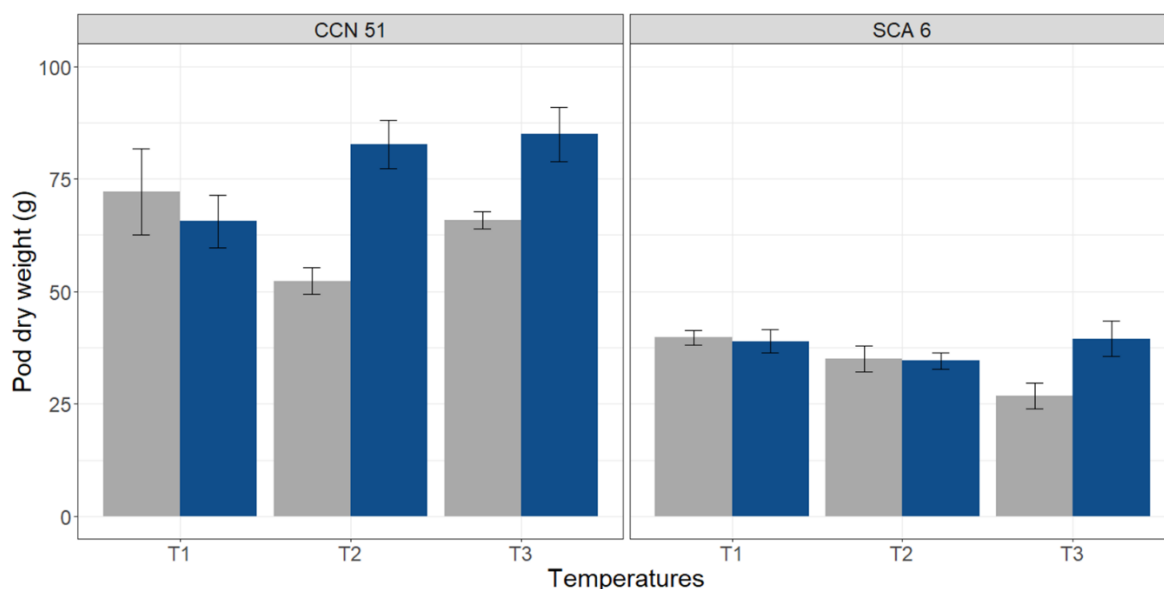
Figure 6.12 Effect of $[CO_2]$ and temperature on pod dry weight of two cacao genotypes. Error bars show the standard error of the mean. Temperature treatments are T1 (control temperature), T2 (control temperature + 2.5°C), and T3 (control temperature + 5.0°C). $[CO_2]$ treatments are ambient (grey bar) and elevated (blue bar).

Husk dry weight. Husk dry weight was significantly higher in CCN 51 (39.9 (±1.6) g) than SCA 6 (21.8 (±0.6) g) ($P < 0.001$) (Figure 6.13). There was also a significant interaction between temperature, $[CO_2]$ and genotypes ($P < 0.01$). In CCN 51, elevated $[CO_2]$ did not influence husk dry weight at T1, but at higher temperatures husk dry weight was significantly higher (by 38.2% and 27.3% at T2 and T3 respectively) at elevated compared with ambient $[CO_2]$. In SCA 6, elevated $[CO_2]$ did not influence husk dry weight at T1 and T2 whereas husk dry weight was significantly higher by 61.7% at T3 in the elevated $[CO_2]$ condition compared to the ambient $[CO_2]$.
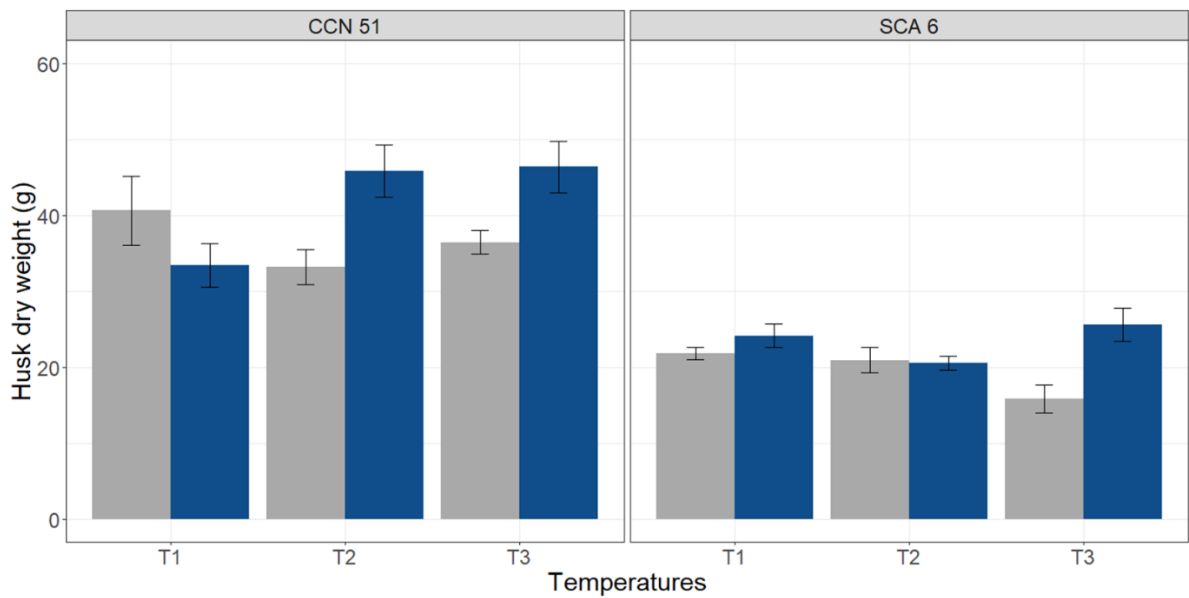
Figure 6.13 Effect of [CO_2] and temperature on husk dry weight of two cacao genotypes. Error bars show the standard error of the mean. Temperature treatments are T1 (control temperature), T2 (control temperature + 2.5°C), and T3 (control temperature + 5.0°C). [CO_2] treatments are ambient (grey bar) and elevated (blue bar).

Total bean dry weight per pod. Total bean dry weight per pod was higher in CCN 51 (26.8 (±1.2) g) than SCA 6 (10.2 (±0.4) ($P < 0.001$) (Figure 6.14). Although there was no significant interaction between genotypes and CO_2, the elevated [CO_2] treatment resulted in a significant increase of 25.1% in the average total bean dry weight compared to the ambient CO_2 treatment in CCN 51. No significant effects of temperature or other treatments interactions were observed.
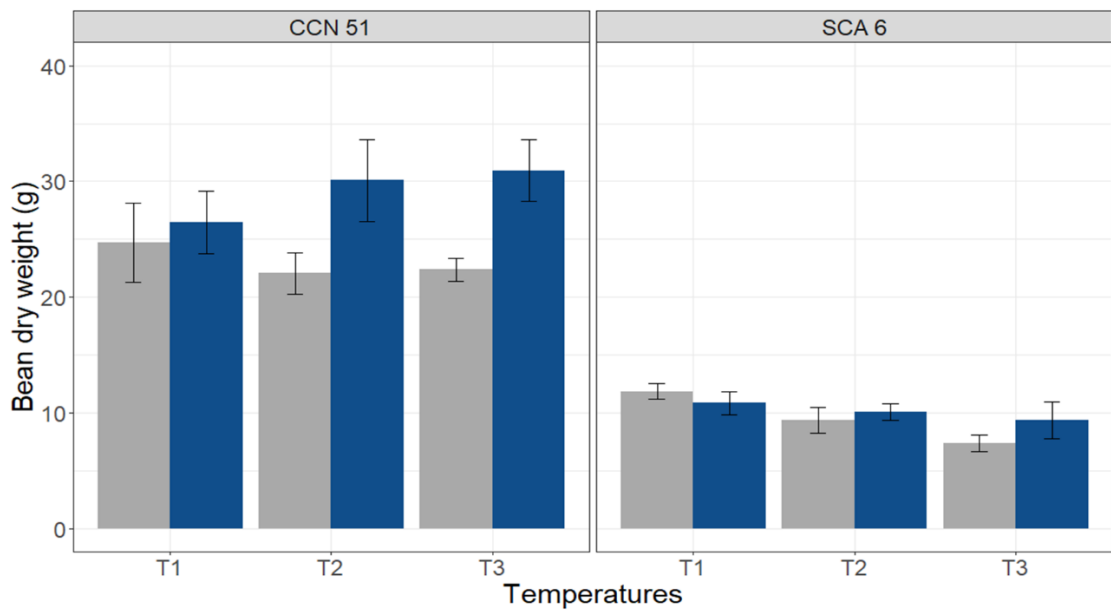
Figure 6.14 Effect of $[CO_2]$ and temperature on the average of total bean dry weight per pod of two cacao genotypes. Error bars show the standard error of the mean. Temperature treatments are T1 (control temperature), T2 (control temperature + 2.5°C), and T3 (control temperature + 5.0°C). $[CO_2]$ treatments are ambient (grey bar) and elevated (blue bar).

Number of beans per pod. Overall, average number of beans per pod ($P < 0.001$) was significantly higher in CCN 51 (47 (±1)) than in SCA 6 (40 (±1)) (Figure 6.15). There was also a significant interaction between $[CO_2]$ and genotypes ($P < 0.05$). For CCN 51, the number of beans per pod did not differ between CO_2 treatments, whilst in SCA 6 there were 9 % fewer beans per pod were produced under elevated CO_2 compared to ambient CO_2. No significant effects of temperature or other treatment interactions on the number of beans per pod were observed.
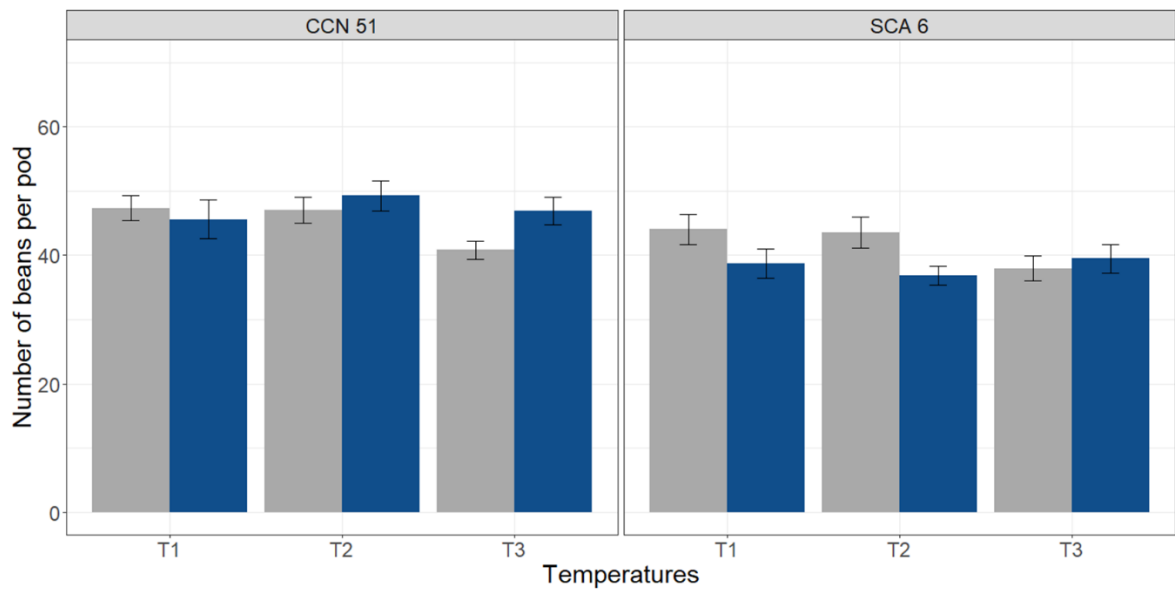
Figure 6.15 Effect of $[CO_2]$ and temperature on the number of beans per pod of two cacao genotypes. Error bars show the standard error of the mean. Temperature treatments are T1 (control temperature), T2 (control temperature + 2.5°C), and T3 (control temperature + 5.0°C). $[CO_2]$ treatments are ambient (grey bar) and elevated (blue bar).

Individual bean dry weight. Individual bean dry weight was significantly higher in CCN 51 (0.58 (±0.02) g) than in SCA 6 (0.25 (±0.01) g) ($P < 0.001$, Kruskal-Wallis one-way ANOVA) (Figure 6.16.). Beans which developed under elevated $[CO_2]$ weighed more than those ones from the ambient $[CO_2]$ condition (0.39 (±0.02) g and 0.31 (±0.02) g, respectively) ($P < 0.05$, Kruskal-Wallis one-way ANOVA). However, temperature did not influence individual bean dry weight. Interactions among main factors were not determined due to the non-normal distribution of the data.
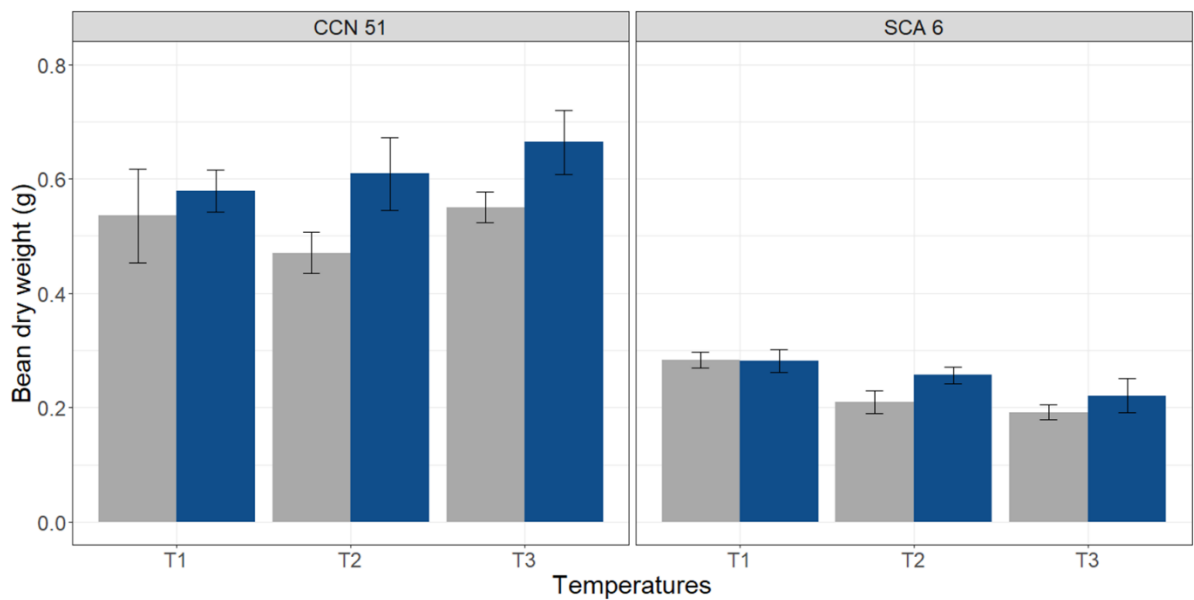
Figure 6.16 Effect of [CO_2] and temperature on the average individual bean dry weight of two cacao genotypes. Error bars show the standard error of the mean. Temperature treatments are T1 (control temperature), T2 (control temperature + 2.5°C), and T3 (control temperature + 5.0°C). [CO_2] treatments are ambient (grey bar) and elevated (blue bar).

Bean to husk ratio. Figure 6.17 shows the effects of [CO_2] and temperature on bean to husk ratio for CCN 51 and SCA 6 which was significantly higher in CCN 51 than SCA 6 (0.69 (±0.03) and 0.47 (±0.01), respectively) ($P < 0.001$). There was a significant effect of the temperature on bean to husk ratio ($P < 0.01$) which was more evident in SCA 6. Bean to husk ratio declined with increasing temperature from 0.57 (±0.02) at T1 to 0.49 (±0.03) at T3. A significant interaction between genotype and [CO_2] was also observed ($P < 0.05$). Bean to husk ratio was not influenced by CO_2 treatment in CCN 51 whereas in SCA 6 it declined by 14% in the elevated CO_2 treatment compared to the ambient CO_2 treatment.
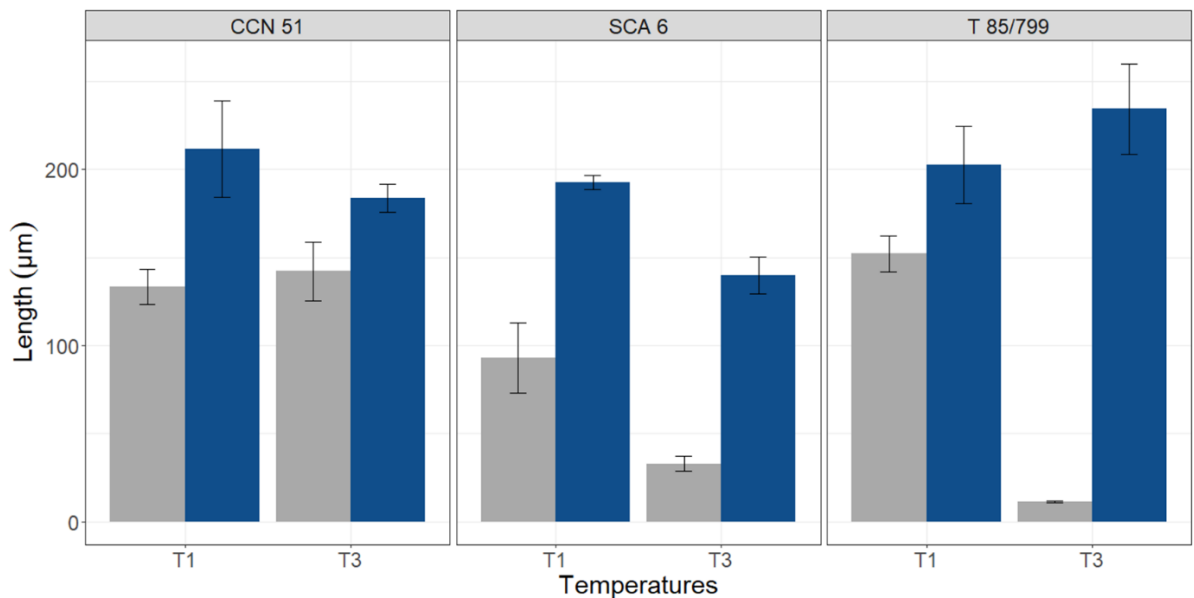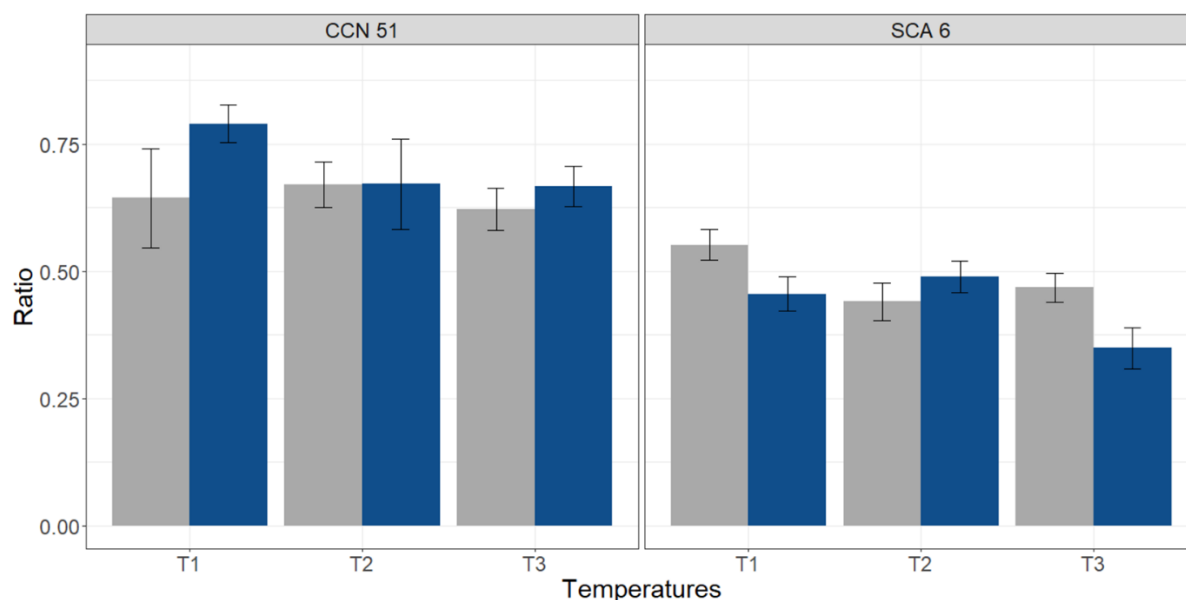
Figure 6.17 Effect of [CO_2] and temperature on bean to husk ratio of two cacao genotypes. Error bars show the standard error of the mean. Temperature treatments are T1 (control temperature), T2 (control temperature + 2.5°C), and T3 (control temperature + 5.0°C). [CO_2] treatments are ambient (grey bar) and elevated (blue bar).

Bean shell percentage. Bean shell percentage which was significantly higher in SCA 6 than CCN 51 (19.4 (±0.3) % and 13.7 (±0.5) %, respectively) ($P < 0.001$, Kruskal-Wallis one-way ANOVA; Figure 6.18). Elevated [CO_2] did not influence bean shell percentage ($P > 0.05$, Kruskal-Wallis one-way ANOVA). There were also significant differences among temperatures ($P < 0.001$, Kruskal-Wallis one-way ANOVA). Bean shell percentage did not differ statistically with increases of temperatures from T1 to T2 (15.9 (±0.4) % and 16.9 (±0.7) %, respectively) but the T3 treatment caused an increase in the bean shell percentage up to 20.8 (±0.9) %. Although interactions among main factors were not determined due to the non-normal distribution of the data, the effects of increases temperatures in the bean shell seemed to be larger in SCA 6 than CCN 51
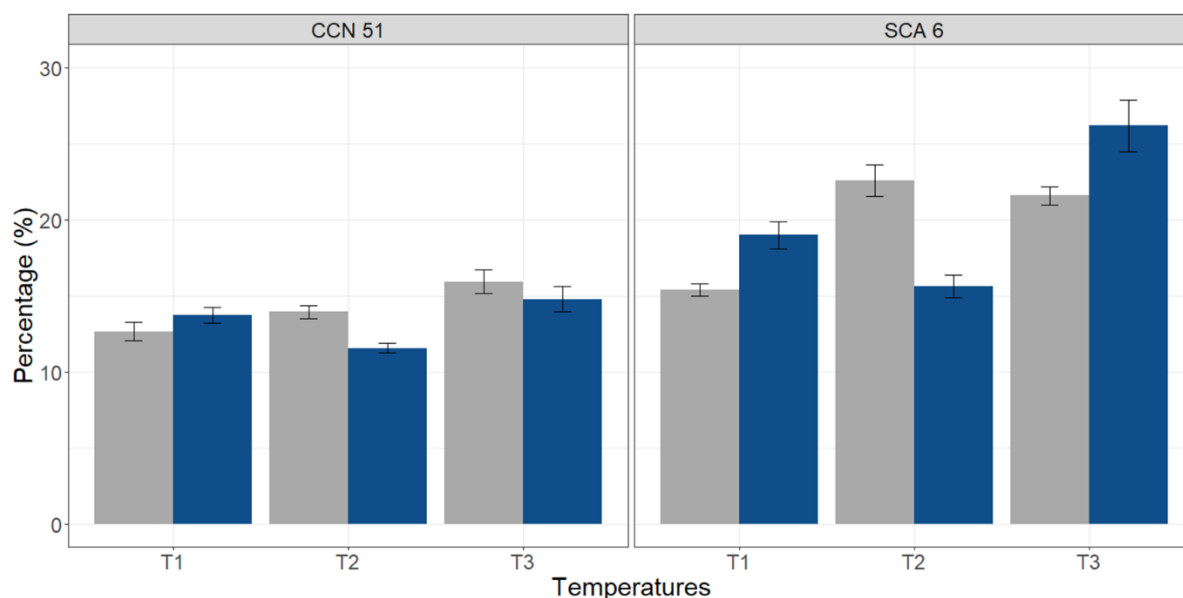
Figure 6.18 Effect of $[CO_2]$ and temperature on bean shell percentage of two cacao genotypes. Error bars show the standard error of the mean. Temperature treatments are T1 (control temperature), T2 (control temperature + 2.5°C), and T3 (control temperature + 5.0°C). $[CO_2]$ treatments are ambient (grey bar) and elevated (blue bar).

6.4   Discussion

Three contrasting mature cacao genotypes (CCN 51, SCA 6, and T85/799) were used in the present experiment with the aim of examining the combined effects of elevated $[CO_2]$ and increased temperature on flowering intensity, pollen viability, pod development and pod components. Trees were maintained under six controlled environment glasshouses and exposed to three day/night temperatures and two $[CO_2]$ treatments for 11 months. To date, this is the first documented report to show the effects of these combined environmental factors on the reproductive development and pod components in cacao. The most important findings were: (i) genotypic differences in the response of reproductive parameters to elevated $[CO_2]$ and temperature were identified, (ii) flower intensity was unaffected by the $[CO_2]$ and temperatures treatments, (iii) elevated $[CO_2]$ alleviated the negative effect of warming conditions on cacao pollen viability, (iv) elevated $[CO_2]$ enhanced pollination success, pod set and reduced wilting under high temperature conditions, and (v) the positive effects of elevated $[CO_2]$ on pod development and pod biomass in the warming scenario was more apparent in CCN 51 than SCA 6.

In a meta-analysis carried out over a wide range of crops and wild species, Jablonski *et al.* (2002) reported that plants growing at elevated $[CO_2]$ produced significantly more flowers than those growing at ambient $[CO_2]$. However, the present study has shown that $[CO_2]$ enrichment did not affect flowering intensity in cacao under the experimental conditions. A similar observation was reported by Handley (2016) who did not observe effects of elevated $[CO_2]$ on flowering intensity of six cacao genotypes across an experimental period of 16 months. It was hypothesized that the effects on flowering would be

quantitatively similar to the effects observed on the photosynthesis rate and vegetative growth seen in Chapter 5. Early studies in Ghana, hypothesised that increased vegetative growth and enhanced flowering can be explained in terms of carbohydrate production (Asomaning *et al.*, 1971). However, according to Valle *et al.* (1990), pod development affects also cacao flowering intensity, because of the competition between fruits and flowers for carbohydrates, despite the relatively low energy expenditure required for the flowering process. In this study, similar to Handley (2016), the flowering data collection was carried out when pods were developing, which may have diminished the effect of elevated [CO_2]. Furthermore, no impact of high temperature was observed on flowering intensity across the experimental period. Cocoa studies exploring the effect of temperature on flowering have used lower temperatures compared to those used in the present experiment. Sale (1969) examining the effects of three temperature regimes on 13 month-old cacao plants of the ICS 95 cacao genotype under growth rooms, reported that flowering was reduced at 23°C while increases were observed at 26.7° and 30°C. Subsequently, a study carried out by Daymond and Hadley (2011) using data from an international clone trial under field conditions in Brazil, showed a high positive correlation between temperatures (ranging from ~20 to 25°C) and flowering intensity. Alvim (1977), observed that the decline in flowering intensity under field studies in Bahia, Brazil coincided with low temperatures (below ~23°C), and also the period of the maximum pod load. The author pointed out the possible internal competition for assimilates, which might affect the flowering responses to temperature changes.

In general, pollen samples from trees grown at the set temperature of 36/27°C showed a decline in pollen germination and pollen length tube compared to those grown at 31/22°C. These parameters were recorded as a measure of pollen viability and the potential of the pollen tube to successfully reach the ovule in the fertilization process. High temperatures may have many different effects on early reproductive processes, including microsporogenesis, megasporogenesis, stigma receptivity, anthesis, pollen germination and pollen tube development, and if any of these processes fail, fertilization will decrease (Jumrani *et al.*, 2018). In several crops elevated temperatures have been observed to adversely affect pollen shed, pollen viability, and pollen tube length (Gross and Kigel, 1994; Peet *et al.*, 1998; Kakani *et al.*, 2005). According to Hedhly *et al.* (2009), usually the male gametophyte is more sensitive to high temperatures than the female gametophyte at all stages of development. The present study has found similar sensitivity in the male gametophyte in cocoa contained in pollen developed under the high temperature treatment. However, percentage germination and tube length both increased to a level similar to the control in pollen obtained from flowers grown in the elevated [CO_2] x high temperature environment. Previously, it has been suggested that elevated [CO_2] may mitigate the effect of high temperatures on pollen performance (Aloni *et al.*, 2001). High levels of atmospheric [CO_2] are associated with changes in the acquisition of resources, including carbon and nitrogen, which improve the

reproductive and vegetative development of biomass in general (Jablonski *et al.*, 2002; Ainsworth and Long, 2005). In cacao, an early study showed that elevated $[CO_2]$ may enhance pollen performance (Aneja and Gianfagna, 1992). The authors reported that pollen did not germinate well *in vitro* unless cacao flowers were pre-treated under enclosed vials with increased CO_2 (~85,000 ppm). Here, although a much lower target for the elevated $[CO_2]$ (~700 ppm) was implemented, pollen viability responded positively to the elevated $[CO_2]$ and therefore this might overcome the negative effects of future warming conditions in cacao.

Manual pollinations were carried out under standardized procedures in order to attribute variation in pollination success to the treatments under study. Genotypic variation in pollination success was observed. Furthermore, the percentage of pollination success achieved was low compared to the percentage of the pollen germination. Usually, in cacao the percentage of flowers which set pods is less than 5% (Aneja *et al.*, 1999) which has been explained by the degree of pollen compatibility and the quantity of pollen grains deposited on the stigma (Falque *et al.*, 1995; De Almeida and Valle, 2007). Reproductive processes affect successful fruit set in plants, and it has been noted that sexual reproduction is more sensitive to high temperatures than vegetative processes (Singh and Jasrai, 2012). Here, as expected, pollination success declined under higher temperature conditions at ambient $[CO_2]$. However, in trees grown under elevated $[CO_2]$, pollination success at high temperature increased by 105% and 44.3% in CCN 51 and SCA 6 compared to those grown at ambient $[CO_2]$. A similar compensation effect was observed by Aloni *et al.* (2001) working on bell pepper pollen germination and carbohydrate changes under high temperature (32/26°C- max/min). The authors noted that increases in assimilate availability due to CO_2 enrichment mitigated the inhibition of sucrose and starch metabolism, thereby increasing their use for germination under high temperatures stress. It is suggested in this study that assimilates from the higher rates of photosynthesis under elevated $[CO_2]$ (Chapter 5), may have alleviated the negative impact of warming conditions on cacao reproductive development under the experimental conditions. Likewise, working with six mature cacao genotypes in controlled glasshouses, Handley (2016) reported that trees grown under long-term $[CO_2]$ enrichment had a significantly higher successful pollination rate in a first cycle of hand pollination, although this was not apparent in the second cycle. The author attributed the positive effect of elevated $[CO_2]$ in the first cycle as being a result of a higher photosynthesis rate observed in trees growing under $[CO_2]$ enrichment. However, after prolonged long-term conditions, trees had larger biomass, and the reduction in successful pollination suggested allocation of assimilates towards the vegetative components. This may support the claim that in cocoa, vegetative components are stronger assimilate sinks than the reproductive components (Alvim, 1977).

The observation of a decline in wilting by 23% in trees grown under elevated [CO_2] compared to those grown at ambient [CO_2] could be might be explained by the enhancement of photosynthesis leading to greater assimilate availability. Comparing pod set, wilting and yield between naturally pollinated, hand pollinated and de-podded cacao trees, Valle *et al.* (1990) reported that final pod set is regulated by assimilate production, with cherelle wilt as the physiological process whereby trees may balance resources. Increases in temperature have been linked to higher rates of wilting, in part due to increased carbohydrate demand through increases in pod respiration rates (Hadley *et al.*, 1994a). Working with five cacao genotypes growing under semi-controlled glasshouses, Daymond and Hadley (2008) observed increases in cherelle wilt at higher temperatures and genotypic variation in assimilate competition among vegetative and reproductive components. Here, wilting did not differ considerably with increases in temperature ranging from 43.5% to 50.5% at set maximum temperatures of 31/22°C and 36/27°C, respectively while there was a slight reduction of 10% in photosynthesis rate observed in Chapter 5. However, due to a lower pod set at elevated temperature this may have impacted on the number of wilted pods.

Overall, maximum pod growth rate and maximum pod size responded similar to the treatment combinations of temperature and [CO_2]. Under ambient [CO_2], increases in temperature reduced maximum pod growth rate and maximum pod size. End *et al.* (1988) and Daymond and Hadley (2008) also reported a decline in cacao final pod size with increasing temperature. However, the highest temperature in these two studies was not as high as the present study. The reduction in these parameters may have resulted from the slight decline in photosynthesis rates as was noted in Chapter 5. Declines in final fruit size in response to high temperatures has been reported in several crops (Lopez and Dejong, 2007; Menzel, 2021). However, under elevated [CO_2], maximum pod growth rate and maximum pod size increased in CCN 51 and both were more stable in SCA 6 with an increase in temperature, which can be related to the high assimilation rates at elevated [CO_2]. This finding is in agreement with Handley, (2016), who observed an enhancement of these parameters after two periods of pod growth in cacao trees grown for 22 months under [CO_2] enrichment. The author suggested that after long-term exposure, the trees were able to allocate more assimilates to the reproductive components, and also pointed out that this positive effect on fruit development may vary among genotypes. It has been reported that [CO_2] enrichment improved fruit size and fresh weight in Japanese pear (*Pyrus serotina* Reheder cv. Kosui) and yield in Valencia Orange (*Citrus sinensis* (L.) Osbeck) after short-term exposure (Downton *et al.*, 1987; Ito *et al.*, 1999). Assimilation of [CO_2] may be affected by several factors and water availability has been observed to be an important factor affecting this process (Sousa Pereira *et al.*, 2017). Here, cacao trees were grown under non-limiting water and nutrient

conditions and this may have resulted in the rapid response of pod development in response to CO_2 enrichment.

The time to reach maximum pod growth rate and maximum pod size increased in trees grown at highest set temperature of 36/27°C. Earlier studies in cacao, have shown decreases in fruit maturation time under warming (~25°C) in the field (Alvim *et al.*, 1974) and in greenhouse conditions (~27-28°C) (End *et al.*, 1988; Daymond and Hadley, 2008). However, the temperature regimes based on mean temperatures of these studies may be considered sub-optimal conditions for cacao according to Balasimha *et al.* (1991) and the results observed in Chapter 4. Conversely, when trees were subjected to the two higher temperature regimes (set to 33.5/24.5°C and 36/27°C), pod growth rate declined which is consistent with the observed delay in time to the reach the final pod size. This might suggest that temperatures above 31/22°C could represent supra-optimal conditions for cacao pod development. Nonetheless, under elevated [CO_2], days to reach maximum pod growth rate and maximum pod size were less variable at the 31/22°C and 33.5/24.5°C . However, at the highest set temperature of 36/27°C, elevated [CO_2] seemed to compensate the negative effect by reducing the time for pod development, which may have been caused by the extra assimilates obtained under the CO_2 enrichment conditions.

Genotypic variability in the response of pod and bean biomass to the combined effects of elevated [CO_2] and increased temperature observed here, confirm that physiological studies have the potential to support selection of material for cacao yield improvement under projected scenarios. It has been noted that in the current century, increases in temperature may result in yield reductions which vary from 2.5% to 10% among a number of crops (Hatfield *et al.*, 2011). Daymond and Hadley (2008) reported a negative relationship between increased temperature and pod and bean dry weight. However, the authors observed that some genotypes were more sensitive to temperature increases than others. In the present study, an overall detrimental effect of increased temperature on pod dry weight, husk dry weight, bean dry weight per pod and individual bean dry weight were observed. As discussed previously, increases in temperature reduced photosynthesis rate in cacao trees (Chapter 5), which may have an impact on the assimilate distribution to the reproductive components. Optimum and critical temperature are specific for crops, from which changes in thresholds may affect reproductive success and final yield (Vara Prasad *et al.*, 2017). Therefore, from this study, temperatures above 31/22°C appear to be supra-optimal for the development of cacao pod components. However, a compensatory effect of [CO_2] enrichment was observed in pod and husk dry weight, bean dry weight per pod and individual bean dry weight at the highest temperature, which was more pronounced in CCN 51 than SCA 6. In cacao, vegetative components compete with reproductive components for assimilates (Mckelvie, 1956; Bastide *et al.*, 2009) and the competitive balance may be affected by environmental factors. The increase in photosynthesis rates due to [CO_2] enrichment in mature cacao trees (Chapter 5), was

expected to support the assimilate demand for reproductive development. Similarly, Handley (2016) reported increases in pod, husk and individual bean weight in trees grown at elevated [CO_2] for two years under controlled glasshouses conditions. The author observed a positive trend after a second pod production cycle, suggesting that the amount of assimilates invested in reproductive growth would have increased as the trees matured and vegetative growth slowed. Here, the effect of elevated [CO_2] on pod parameters was more evident under warming conditions for both genotypes. Hamilton *et al.* (2008), showed that high [CO_2] improved the thermo-tolerance of photosynthesis in C_3 species. The mitigating effect of elevated [CO_2] on the apparent adverse effects of supra-optimal temperatures on photosynthesis were observed in tropical *Coffea arabica* and *C. canephora* plants under well-watered conditions (Rodrigues *et al.*, 2016). As was seen in coffee, the improvement in cacao pod parameters at temperatures above the control suggests that [CO_2] enrichment might strengthen cacao performance at supra-optimal temperatures under non-limiting water and nutrient conditions.

Contrasting differences between genotypes in bean to husk ratio, average bean number per pod and bean shell percentage were observed. Bean to husk ratio and the number of beans per pod were higher in CCN 51 than SCA 6, whereas, bean shell percentage was higher in SCA 6 than CCN 51. The genotypes under study differ in their morphology and yield components (Turnbull and Hadley, 2022). SCA 6 has a low vigour, small pods and beans, while CCN 51, which is widely cultivated in cacao-producing countries, is a vigorous genotype, with larger pods and bigger beans. Overall, bean to husk ratio declined when the temperature increased by 5°C above the control treatment, driven by a proportional decline in both husk and bean dry weight under warming conditions. However, the response to elevated [CO_2] was a reduction in bean to husk ratio in SCA 6 whereas no changes in CCN 51 were observed. Previously, bean to husk ratio has been identified as an important assimilate partitioning component which contributes to variability in cacao yield (Daymond *et al.*, 2002). Differential responses in assimilate partitioning towards the beans may be a potential trait in order to identify materials performing better under abiotic stresses.

The trend of a decline in the number of beans per pod at the highest temperature is consistent with a number of other studies in annual crops (Gross and Kigel, 1994; Vara Prasad *et al.*, 2008) and tropical fruits (Utsunomiya, 1992; Chu and Chang, 2020). According to Falque *et al.* (1995), there is a positive relationship between pollination intensity (number of pollen grains received per stigma) and fruit and seed set in cacao. In the present study, across the treatment combinations, a standard methodology was applied at each attempt in order to ensure the same pollen volume was delivered during the hand pollinations. As discussed previously, negative effects of increased temperatures were observed in pollen viability which may have resulted in reduced fertilization events and lower number of beans under the warmest scenario. On the other hand, CCN 51 trees grown under elevated [CO_2] did not show

changes in number of beans while SCA 6 saw a 10% decline, reflected in the reduced bean to husk ratio. However, Handley (2016) did not observe significant changes in bean number of mature genotypes grown under $[CO_2]$ enrichment across two harvest periods during 22 months. Differences may have resulted either due to different genotypic responses or the lack of enough harvested pods across the treatments as was reported by the author.

Shell percentage is one of the primary properties of cacao beans which is of commercial interest and a low shell percentage is preferred by the processing industry. In this study, there was a genotypic difference in bean shell percentage, which was higher in SCA 6 than CCN 51. Furthermore, shell percentage increased with increasing temperature up to 36/27°C, and the response was stronger in SCA 6. A negative relationship between average bean dry weight and bean shell percentage was shown in this experiment, which is consistent with Toxopeus and Wessel (1970) who also highlighted that bean shell percentage may also be affected by environmental factors. A variety that is viable in one region, may not be in another, given the effect of higher temperatures on reducing bean weight and increasing shell percentage (Daymond and Hadley, 2008).

To conclude, this study has shown within the ranges of temperature and $[CO_2]$ tested it was not possible to see any effect on flowering intensity. However, this was not reflected in the other reproductive components which were more responsive. For most of the reproductive components studied here, where temperature appeared to have a negative effect this was partially mitigated by an increase in $[CO_2]$. This positive effect of elevated $[CO_2]$ on reproductive development is comparable to growth and photosynthesis responses under $[CO_2]$ enrichment (Chapter 5). Pod growth and pod components parameters were more responsive to elevated $[CO_2]$ under warm conditions in CCN 51 than SCA 6. Despite the small number of genotypes used in this study the contrasting genetic variation has shown a potential opportunity for selecting genotypes better suited to a changing climate.

7   General Discussion

7.1   Introduction

Among several abiotic factors, atmospheric [CO_2], temperature and rainfall patterns are projected to change in the near future. Since the industrial revolution, the [CO_2] has increased from 280 ppm to more than 400 ppm, and could reach levels up to 970 ppm (under IPCC scenario: RCP 8.5) by the end of the century (IPCC, 2014). As a result of increases in greenhouse gases and, in particular, increased [CO_2], the projected rise in global air temperature could range from 1.5°C to 5.8°C by 2100 (IPCC, 2021). Furthermore, the increase in [CO_2] and temperatures also impacts the hydrological cycle leading to changes in precipitation that may change the frequency and intensity of droughts, forest mortality and water availability (Allen *et al.*, 2010; Phillips *et al.*, 2010; Swann *et al.*, 2016). Climate change impacts are especially significant in tropical regions, where resource-poor farmers face particular risks and where forests play a crucial role in global carbon cycling and biodiversity (Kooperman *et al.*, 2018). Although photosynthesis may react more positively to CO_2 enrichment scenarios in tropical species than temperate species (Cernusak *et al.*, 2013), they may be more sensitive to warming because they evolved in an area with relatively small temporal variations in temperature (Sheldon, 2019). Cacao is cultivated in developing countries in the humid tropics, supporting the income of millions of people in rural areas. However, challenges such as pests and diseases, ageing plantations, unsuitable planting materials and effects of climate change can all impact cocoa yields and could mean that the supply of cacao beans may not keep pace with the growing demand (Lahive *et al.*, 2019).

Based on predicted temperature information, initial models of climate change impacts on cacao in West Africa suggested that some areas in Ghana and Côte d'Ivoire will become unsuitable for cacao production by 2050 (Läderach *et al.*, 2013; Schroth *et al.*, 2016). These findings led to great discussion across the cacao value chain about the actual responses of cacao crops to climate change scenarios. A recent land-surface model incorporating changes in temperature and the seasonal cycle of precipitation as well as cacao-specific, physiological parametrization (based on experimental data obtained under controlled conditions), predicted that elevated [CO_2] could ameliorate the impacts of high temperatures and changes in precipitation on cacao net primary productivity (NPP) in West Africa (Black *et al.*, 2021). However, models need to be validated with experimental field data to examine if changes in NPP may be reflected in yield changes. In addition, the identification of genotypes better adapted to a changing environment is an important route toward mitigation of climate change in the field.

The majority of research examining the impacts of climate on cacao physiology has focussed on single environmental factor changes. Adverse effects of water deficit have been demonstrated (for example,

Deng *et al.*, 1990; Mohd Razi *et al.*, 1992; Dos Santos *et al.*, 2014; Ávila-Lovera *et al.*, 2016) and cocoa responses to temperature gained attention in early studies (Sale, 1968; Balasimha *et al.*, 1991; Hadley *et al.*, 1994b, 1994a). More recent studies have shown the positive effects of elevated $[CO_2]$ on the growth and physiology of cacao seedlings (Baligar *et al.*, 2005, 2008; Lahive *et al.*, 2018). Recently, studies to examine the interaction between elevated $[CO_2]$, temperature and water deficit in cacao have been carried out under controlled facilities (glasshouses and OTCs) (Lahive *et al.*, 2018, 2021; Hebbar *et al.*, 2020). However, studies on this interaction between temperature and elevated $[CO_2]$ remain limited, particularly in terms of understanding the role of plant development stage, the specific impacts on reproductive development and the degree to which genotypic variation influences responses. This study aimed to close some of these knowledge gaps through the investigation of the growth and photosynthetic responses of young cacao plants and mature trees to the short and long-term effects of the climate change variables $[CO_2]$, high temperature and soil water deficit. This was done through a series of four experiments in controlled environmental facilities (growth cabinets and glasshouses) at the University of Reading. The study also examined how genetic variation affects the physiological responses to these treatments.

7.2 Response to elevated $[CO_2]$

The increase in photosynthesis at elevated $[CO_2]$ results in an increase in carbohydrate production which is normally reflected in greater plant biomass (Thompson *et al.*, 2017). In this study, as expected, cacao growth responded positively to CO_2 enrichment. Elevated $[CO_2]$ enhanced plant growth parameters in seedlings (short-term; Chapters 3, 4) and mature trees (long-term; Chapter 5). However, cacao seedlings were more responsive to CO_2 enrichment than mature cacao trees. This is consistent with early studies carried out by Lee and Jarvis (1995), who reported that responses of seedlings are much larger than those of mature trees (*Fagus sylvatica* L. and *Picea sitchensis*) to increases in $[CO_2]$. Here, although studies on juvenile material may provide an important insight into how CO_2 enrichment impacts on plant performance in the short-term, the incorporation of long-term research with mature trees is required to examine the role of ontogeny, as well as enabling research on the response of reproductive components to environmental variation. The present study also demonstrated genotypic variation in growth parameters such as total dry biomass to $[CO_2]$ elevation. Similarly, Baligar *et al.* (2021a, 2021b) reported genotypic differences in total dry weight of juvenile cacao plants, and Lahive *et al.* (2021) showed significant differences in tree total biomass in a set of mature cacao genotypes subjected to CO_2 enrichment environment. In this study, juvenile plants of PA 107 and T 63/971 x T 60/887 grown under short-term $[CO_2]$ elevation (~90 days) and mature trees of the genotype IMC 20 exposed to elevated $[CO_2]$ for 378 days were more responsive than the other genotypes tested. Such intraspecific variation

in cacao could be exploited in breeding programmes to select varieties that perform better under future climate scenarios.

The stimulation in photosynthesis rates observed here (47 to 68% in seedlings (Chapters 3 and 4) and 51% (Chapter 5) in mature trees) is similar to that reported in a meta-analysis by Curtis and Wang (1998) and Ainsworth and Long (2005) (55% and 47% increase, respectively). The results obtained here are also consistent with the enhancement of the photosynthesis rate of juvenile cacao seedlings from 33 to 56% in response to elevated [CO_2] (Baligar *et al.*, 2008, 2021a; Lahive *et al.*, 2018; Hebbar *et al.*, 2020), and increases by 35 and 43% at the leaf and canopy level respectively of mature cacao trees grown under long-term CO_2 enrichment (Lahive *et al.*, 2021). Despite the positive impact on photosynthesis, the stimulation in growth parameters in mature trees was not as great as in juvenile plants. This discrepancy in response between young and mature plants may be due to the fact that in mature trees there is a large proportion of older and more shaded leaves within the canopy, it is expected that not all leaves will respond as positively to elevated [CO_2] as the young leaves sampled during measurements. However, in young plants, as all leaves are relatively young and there is not much self-shading a greater proportion of the leaves may respond similarly to that used for gas exchange measurements. In addition, in mature trees assimilates might be conducted towards reproductive sinks and maintenance of woody biomass, whereas young plants utilized the available extra assimilates in the growing components (Lahive, 2015). However, a further factor is the observation of acclimation. Although initial stimulation of photosynthesis due to elevated [CO_2] is normally seen, after a prolonged period of time the plants exhibit downregulation of photosynthesis (also known as photosynthetic acclimation). This occurrence has been seen in other species in both FACE studies (Ainsworth and Long, 2005) and growth chambers (Zheng *et al.*, 2019). Here, a preliminary observation on mature plants of the clone CCN 51 showed photosynthetic acclimation after 378 days of exposure to elevated [CO_2]. The photosynthetic rate at elevated [CO_2] was 26% lower in trees grown under long-term [CO_2] elevation in comparison to the photosynthetic enhancement measured in trees exposed to an instantaneous increase in [CO_2]. This downward acclimation was accompanied by a slight decrease in leaf N content and a significant reduction in leaf chlorophyll content. Further research is needed to identify if photosynthetic acclimation to long-term exposure to elevated [CO_2] persists under field conditions, and whether acclimation is observed in some genotypes more than others.

From a physiological point of view, increased water use efficiency (WUE) may represent one of the most significant plant responses to CO_2 enrichment (Long *et al.*, 2004). Here, leaf level WUE was primarily measured from instantaneous leaf gas exchange parameters. Elevated [CO_2] enhances intrinsic water use efficiency (iWUE) by improving photosynthesis rate and/ or decreasing stomatal conductance (Ainsworth and Long, 2005; Ainsworth and Rogers, 2007). In this study, under elevated [CO_2], there was

an enhancement of iWUE driven by increased photosynthetic rates (Chapters 3 and 5) and a decline in stomatal conductance (Chapter 4). These findings are consistent with the improvement of iWUE under elevated $[CO_2]$ observed previously in young cacao seedlings (Baligar *et al.*, 2008, 2021a; Lahive *et al.*, 2018; Hebbar *et al.*, 2020) and mature genotypes (Lahive *et al.*, 2021) grown under controlled environment conditions. Measuring wood carbon isotope ratio ($\delta^{13}C$) in 10-year growth increments from tropical trees of *Cedrela odorata* L. and *Swietenia macrophylla* King in Brazil growing under non-limiting water conditions, Hietz *et al.* (2005) reported that increases in iWUE was probably related to increases in assimilation rates rather than to a decline in stomatal conductance. Similarly, Lahive *et al.* (2018, 2021), and Hebbar *et al.* (2020) concluded that the higher iWUE at elevated $[CO_2]$ observed in cacao plants was due to a higher photosynthesis rate rather than a decline in water loss through reduced transpiration. Therefore, uses of iWUE as a trait to select cacao genotypes resilient to water deficit should consider if the improvement of iWUE under elevated $[CO_2]$ is due to both increases in photosynthesis rate and declines in stomatal conductance.

There was a positive effect of elevated $[CO_2]$ on pollen germination, pollen tube length, successful fertilisation as well as pod number. Under high levels of $[CO_2]$, reproductive and vegetative development generally increase due to changes in the acquisition of resources such as carbon and nitrogen (Jablonski *et al.*, 2002; Ainsworth and Long, 2005). A meta-analysis carried out on 79 species showed that growth under elevated $[CO_2]$ conditions resulted in a significant improvement in plant reproductive responses (Jablonski *et al.*, 2002). Moreover, here a positive effect of elevated $[CO_2]$ on pod growth, pod and bean total dry weight, and average bean dry weight was also observed. Similarly, elevated $[CO_2]$ resulted in improvement of yield in strawberry plants (*Fragaria x ananassa* Duch.) by increasing total fruit number per plant, average fruit fresh weight and dry matter content (Sun *et al.*, 2012), higher yield in tomato (*Lycopersicon esculentum* Mill) (Mamatha *et al.*, 2014), improvement in fruit set of Sour Orange (C*itrus aurantium* L.) (Kimball *et al.*, 2007) and enhancement of harvestable bean yield in coffee (*Coffea arabica*) (Ghini *et al.*, 2015). It has been suggested that under CO_2 enrichment, the production of more assimilates due to enhanced photosynthesis may result in greater allocation towards plant reproductive components, which may impact fruit set and yield. Exploring source-sink imbalances in cv. 'Okitsu' of Satsuma mandarins (*Citrus unshiu* (Mak.) Marcl), Iglesias *et al.* (2003) reported that sucrose supplementation by stem injection improved fruit set by 10%. In cacao, increases in pod size and maximum rate of pod growth in response to elevated $[CO_2]$ were noted only in the second year by Handley (2016). The author suggested that the delayed response of pod growth to elevated $[CO_2]$ may have been because the extra assimilates generated from high photosynthetic rates during the first year were allocated towards the vegetative sinks rather than to pod development. In this research, the delay in response was not observed which could be related to genotypic differences or the

age of the trees. The internal competition for carbohydrates between growing pods and vegetative sinks can lead to cherelle wilt in cacao (Alvim, 1977). Here it was shown that the 23% reduction in cherelle wilt in trees grown under elevated $[CO_2]$ compared to those grown at ambient $[CO_2]$ coincided with the enhancement of photosynthesis (Chapter 5). Despite the overall improvement in pod development and bean yield under elevated $[CO_2]$, variation in the responses of the two studied genotypes was observed. The response to the CO_2 enrichment was more evident in CCN 51 than SCA 6 for pod size, pod dry weight, total bean dry weight, and individual bean dry weight. Genetic variability in morphological and physiological traits in cacao, and the efficiency in the partitioning of dry matter to yield components was suggested as a factor that may be exploited in breeding programmes (Daymond *et al.*, 2002). This study has shown that the variation in the responsiveness to elevated $[CO_2]$ in terms of pod and bean biomass may help in the selection of resilient materials under new climatic scenarios.

7.3   Response to temperature

This study has demonstrated that in the absence of other stresses, the optimum temperature for cacao may be higher than previously reported. Based on field experiments, Balasimha *et al.* (1991), observed that the optimum temperatures for photosynthesis in cacao were achieved between 31-33°C, above which the assimilation rate decreased significantly during the dry season in India. The reduction in photosynthesis at supra-optimal temperatures has been attributed to the direct effect of temperature increase and/or the concomitant increase in vapour deficit pressure (VPD) as temperature increases, which leads to stomatal closure (DaMatta, 2007). However, modest increases in VPD can result in higher transpiration loss through increased evaporative demand (Grossiord *et al.*, 2020). In this study in environmentally controlled growth cabinets where VPD stress was avoided, cacao seedlings showed a sustained increase in photosynthesis with temperature up to 36/27°C (Chapters 3 and 4), suggesting a higher temperature optimum for photosynthesis in cacao when air humidity is maintained at non-stressful levels. However, this assumption needs to be tested further. Therefore, agronomic practices that raise humidity such as maintenance of overhead shade trees might improve microclimate conditions within the cacao crop and ameliorate the impacts of warming (Niether *et al.*, 2018; Blaser-Hart *et al.*, 2021). Changes in growth temperature may cause plants to show phenotypic plasticity in their photosynthetic performance (Hikosaka *et al.*, 2005). It has been observed that the optimal temperature for photosynthesis can change with a change in growth temperature, which can enhance photosynthetic efficiency in a warmer environment (Yamori *et al.*, 2013). In this study, photosynthetic acclimation to elevated growth temperature (~36/27°C) was observed in mature trees of CCN 51. Despite a decline in photosynthesis in response to a short-term increase in temperature from 31 to 36°C, following long-term growth (378 days) at 36°C, that decline was no longer observed, suggesting positive adjustment/acclimation to temperature. Although this is the first attempt to quantify

temperature acclimation in cacao and was carried out on a single genotype, the result is an important starting point but further exploration of the extent to which cacao can acclimate to long-term temperature changes and the genotypic variation in this response is needed.

Despite the sustained positive effect of temperature (up to 36/27°C) on photosynthetic rates in juvenile cacao, growth responses appeared to be genotype specific. PA 107 and T 63/971 x T 60/887 were more responsive than SCA 6 to increases in temperature (Chapters 3 and 4). In contrast, a greater reduction in dry biomass and leaf area was observed in mature trees compared with seedlings in response to increases in temperature up to ~36/27°C, with the clones PA 7 and ICS 6 being more sensitive than IMC 20 (Chapter 5). Cacao genotypes varied widely in their response to temperature regimes, pointing to the need and possibility of identifying plant material appropriate to local growing conditions. The fact that the magnitude of the change in photosynthesis does not predict changes in biomass in mature trees has been suggested to be affected by factors such as dark respiration, which may impact the carbon gained through the photosynthetic process (Lahive et al., 2021). In this study at ambient [CO_2], dark respiration increased slightly with increases in temperature whereas photosynthesis declined by 15% which may have resulted in the lower biomass observed under warming conditions. Although some dry biomass could have been lost through leaf fall, it was not accounted in the final harvest. Additionally, the decline in leaf area in response to temperature increase may have reduced the overall canopy photosynthesis which may have compounded declines in biomass. Furthermore, the assimilate demand for reproductive development (in terms of flower production) may have further reduced assimilate availability to the vegetative components considered in the total dry biomass.

In this study, the responses of leaf level iWUE to increases of temperature varied between juvenile (Chapters 3 and 4) and mature cacao plants (Chapter 5). In young materials iWUE declined up to 36/27°C because stomatal conductance increased more than photosynthesis (Chapter 3), and above 36/27°C photosynthesis declined to a greater extent than stomatal conductance (Chapter 4); however, in mature trees, iWUE declined because the decline in stomatal conductance was larger than photosynthesis (Chapter 6). Here, changes in the stomatal mechanism may have resulted from changes in VPD, which was not under full control in the experiment on mature trees. Stomatal aperture and conductance typically decrease with rapid increases in VPD which negatively impacts plant functioning (Grossiord et al., 2020). It has been suggested that climate change could lead to changes in VPD through increases in air temperature and reductions in relative humidity, which are important factors in the exchange of water vapour and CO_2 at the leaf surface (Ficklin and Novick, 2017). The lack of control of VPD in the glasshouse conditions may have had a negative impact on stomatal control in the high temperature treatment. This could be seen as a more realistic scenario for what may occur in the field. However, the additional VPD control implemented in the growth cabinet experiments is unique as it

allowed us to explore the direct effect of temperature on cacao physiology. Farming practices such as shade, irrigation, and mulching techniques may ameliorate water use in limited water environments and improve air humidity resulting in a better cacao performance under future climatic conditions (Lahive *et al.*, 2019).

The adverse effects of a 5°C temperature increase on reproductive development (pollen viability, pollination, and pod set) and final yield (pod and bean weight) was more evident in SCA 6 than CCN 51 (Chapter 6). A similar sensitivity to temperature was observed in SCA 6 seedlings in relation to biomass and leaf area production (Chapter 3) which indicates genotypic variation amongst cacao germplasm in terms of their sensitivity to a warming environment. This finding could infer the potential to identify temperature resilient material at the juvenile stages. Furthermore, the results here illustrate how climate is likely to impact future yield and the importance of studying reproductive traits in addition to vegetative growth parameters. The overall decline in the reproductive performance and bean yield with increases in temperature were consistent with decreases in photosynthetic rates and the more significant decline in growth seen in mature cacao trees (Chapter 5) that may have affected or diminished assimilate distribution toward reproductive sinks. Despite the difficulty of carrying out this type of work in perennial species, this research has provided novel results in the impacts of climate change on the reproductive development in cacao. However, to improve the understanding of how climate impacts cacao over time, long-term research programmes are essential.

7.4   Responses to water deficit

In this study, cacao physiology and growth responses to water deficit (as well as the combined effects of temperature and elevated $[CO_2]$) of juvenile cacao plants grown in controlled environment growth chambers were examined (Chapter 4). Processes such as photosynthesis and growth are adversely affected by water deficit (Wang *et al.*, 2018). As expected, the imposed water deficit treatment was detrimental to photosynthetic parameters, which resulted in decreases in growth and biomass accumulation. Additionally, the observed reduction in leaf area has been reported as a morphological adaptive mechanism to maintain plant water status by reducing transpirational area (Lahive *et al.*, 2019). This is consistent with previous studies that have shown the adverse effects of water deficit on flushing frequency, leaf number, flowering, dry matter biomass, and leaf area in cacao (Sale, 1970a; Joly and Hahn, 1989a; Mohd Razi *et al.*, 1992; Dos Santos *et al.*, 2018; Lahive *et al.*, 2018). As a result, cacao is typically considered to be sensitive to extreme weather conditions, especially when prolonged water deficit occurs (De Almeida and Valle, 2007). Furthermore, leaf water potential (Ψ_{leaf}) tends to decline in response to water deficit (Balasimha *et al.*, 1991; Ávila-Lovera *et al.*, 2016) which may cause reductions in stomatal conductance and photosynthetic rate (Deng *et al.*, 1990; Mohd Razi *et al.*, 1992). This has

been corroborated in this study. A clear reduction of 44% in Ψ_{stem} of T 63/971 x T 60/887 of seedlings subjected to the water stress treatment may have explained decreases in photosynthesis and stomatal conductance by an average of 35% and 23% respectively.

In the present study, the negative effect of water deficit was combined with reductions in the chlorophyll content and fluorescence parameters (*Fv/Fm* and *PI*) which may also explain the decrease in photosynthetic performance. Stressed leaves usually show alterations in their photochemical processes, which can be used to estimate their photosynthetic performance (Maxwell and Johnson, 2000). Chlorophyll is an important pigment that largely determines photosynthetic capability (Y. Li *et al.*, 2018), and its fluorescence may provide insights into the ability of a plant to tolerate environmental stress and into the extent to which those stresses have been detrimental to the photosynthetic apparatus (Maxwell and Johnson, 2000). Previous studies have incorporated parameters such as *Fv/Fm* as a response to water deficit. However, responses have varied from no changes (Ávila-Lovera *et al.*, 2016; De Almeida *et al.*, 2016), slight decreases (Araque *et al.*, 2012; Hebbar *et al.*, 2020), and significant decreases (Osorio Zambrano *et al.*, 2021) in cacao plants subjected to water deficit which may have resulted due to the intensity of the treatments imposed. In this study, *PI* was identified as being much more responsive to water deficit than *Fv/Fm* (decreasing by 52% and 7%, respectively) suggesting an interesting trait to be included together with *Fv/Fm* as indicators of cocoa response to environmental stresses.

7.5   Combined effects of CO_2, temperature and water deficit

Examining the effects of elevated [CO_2] independently may not provide a true picture of how plants will react to a changing environment. Other aspects such as temperatures, water and nutrient supply are critical components needed to assess and interpret climate change impacts. Temperature and CO_2 are two of the main environmental factors associated with climate change (IPCC, 2014), and together influence the growth and photosynthesis of plants (Dusenge *et al.*, 2019). Despite uncertainty as to how changes in temperature and [CO_2] will affect tree ecophysiology in tropical environments (Chambers and Silver, 2004; Clark, 2004), it has been suggested that the effects of elevated [CO_2] on photosynthesis and growth will be more evident in the tropics than in cooler climates (Hickler *et al.*, 2008; Cernusak *et al.*, 2013; Baig *et al.*, 2015). Here, under non-limiting water and nutrient conditions, CO_2 enrichment and high temperatures increased photosynthesis (up to 36/27°C) in juvenile plants, whereas the effect of elevated [CO_2] remained positive even under a 5°C temperature increase treatment in mature trees. Therefore, CO_2 enrichment could confer a certain degree of tolerance to heat stress in cacao. In coffee, Rodrigues *et al.* (2016) reported that elevated [CO_2] improved photochemical efficiency, energy use and

biochemical functioning which conferred a remarkable resilience to heat stress. However, the underlying mechanism in cacao remains unclear and more research is needed.

Although photosynthesis rates increased (across the genotypes) with elevated $[CO_2]$ at high temperatures, genotypic variation in growth responses such as dry matter biomass and leaf area were noted. Studies in other crops, have shown genotypic variation in response to the combined effects of increased temperature and $[CO_2]$ for example in pea (*Pisum sativum* L.), bell pepper (*Capsicum annuum* L.) and rice (*Oryza sativa* L.) (Ziska *et al.*, 1996; Kumari *et al.*, 2019a, 2019b). Here, it was evident that some cacao genotypes are more sensitive than others to elevated temperatures in terms of growth, but the effect of elevated $[CO_2]$ seemed to partially mitigate the adverse effect of warming in the more susceptible genotypes. For instance, in juvenile plants grown at 36/27°C (max/min), elevated $[CO_2]$ had an additive effect on the positive impact of temperature in PA 107, whereas in SCA 6 elevated $[CO_2]$ compensated for the negative effect of the supra-optimal temperature in terms of leaf area and plant dry biomass. Similarly, in mature trees, a compensatory effect of elevated $[CO_2]$ under the warming scenario was more evident in PA 7 than ICS 6 and IMC 20. Plant growth responses to increasing $[CO_2]$ involves not only photosynthetic responses but also respiratory responses (Morison and Lawlor, 1999). In this study, the discrepancy between photosynthesis stimulation and the differential growth responses observed in cacao plants may have resulted from the stimulation in respiration observed (Chapters 4 and 5). This is consistent with Lahive *et al.* (2021) who reported increases in the dark respiration rate of mature cacao grown at elevated $[CO_2]$, which partially offset the beneficial impacts of $[CO_2]$ elevation on photosynthesis. Here, although dark respiration rates slightly increased with temperature, this was more pronounced at elevated $[CO_2]$ suggesting that CO_2 enrichment may reduce photosynthetic efficiency under warming scenario. On the other hand, with increases in temperature above 36/27°C, the positive effect of elevated $[CO_2]$ on photosynthesis and biomass was overridden by the negative temperature effect (Chapter 4). This implies that the compensatory effect of elevated $[CO_2]$ could be limited in cocoa-growing areas where temperatures frequently reach above 36°C.

In this study, a shift was observed in the optimal temperature for photosynthesis from 36/27°C to 38.5/29.5°C in plants grown at elevated $[CO_2]$ compared to those grown at ambient $[CO_2]$ (Chapter 4). Previous studies have demonstrated that elevated $[CO_2]$ increases the optimum temperature for photosynthesis (Sage and Kubien, 2007) and may improve the heat tolerance of photosynthesis in C_3 plants (Wang *et al.*, 2008; Rodrigues *et al.*, 2016). According to Idso and Kimball (1992), with increases in leaf temperature and $[CO_2]$, sour orange trees (*Citrus aurantium* L.) showed increases in both leaf net photosynthesis and the upper-limiting temperature for growth. Similarly, working in *Coffea arabica* L. and *Coffea canephora*, Ramalho *et al.* (2013) revealed that elevated $[CO_2]$ could alleviate the impact of supra-optimal temperatures on coffee physiological and biochemical parameters. A recent modelling

study carried out in cacao areas of West Africa showed the beneficial effect of elevated [CO_2] and suggested an absence of negative impacts on net primary productivity (NPP) of cacao under warming conditions (Black *et al.*, 2021). However, the authors also pointed out that although NPP is an indicator of vegetative production, more experimental work needs to be carried out regarding physiological responses and how this translates to changes in yield and bean quality. The model, built under known C_3 plant responses, has also suggested that CO_2 increases by the end of the century would increase the optimal temperature for photosynthesis. Here, the experimental data validates the assumptions of the model and confirms that CO_2 may mitigate otherwise supra-optimal temperature effects.

Elevated [CO_2] improved iWUE across the range of temperatures imposed in the experiments, although the benefit of elevated [CO_2] was lower at supra-optimal temperatures. This result is consistent with Hatfield and Dold (2019) who pointed out that increasing [CO_2] at moderate temperatures may increase WUE; however, as temperatures increase above the species optimum the effect might diminish. This study has shown that changes in iWUE under CO_2 enrichment across the temperatures was mediated by changes in photosynthesis rather than significant changes in stomatal conductance. However, more research is required to elucidate the stomatal response to elevated [CO_2] in cacao, as well as identify genetic variability in iWUE under projected climate change.

Elevated [CO_2] has been found to alleviate the adverse effects of high temperatures on photosynthesis and plant growth in different species (Sage and Kubien, 2007; Hamilton *et al.*, 2008). In relation to growth this alleviation is achieved through the maintenance of a positive carbon balance as CO_2 stimulates photosynthesis to a greater extent than high temperature stimulates respiration, thereby counteracting the temperature effect as was seen in Chapter 5. This may have increased the availability of assimilates for reproductive growth (Chapter 6). Although elevated [CO_2] under warming conditions did not show significant effects on reproductive components such as in other crops, for example pepper (*Capsicum annuum* L.) (Kumari *et al.*, 2019a), kidney beans (*Phaseolus vulgaris* L.) (Vara Prasad *et al.*, 2002), peanut (*Arachis hypogaea* L.) (Vara Prasad *et al.*, 2003), strawberry (*Fragaria x ananassa* Duch.) (Sun *et al.*, 2012) and tomato (Peet and Nair, 2003), in this study, there was an overall alleviation of the negative effect of warming under CO_2 enrichment on the majority of the reproductive parameters measured (pollen viability, fertilisation success, pod set), pod growth and bean yield. CCN 51 was more responsive to elevated [CO_2] than SCA 6 in this respect. Despite the small number of genotypes used for the evaluation of reproductive and yield parameters, this research produced preliminary evidence to suggest that high temperature stress can be alleviated under elevated [CO_2] conditions. Although temperature has been demonstrated to have an effect on the bean lipid content which is also determined by genotype (Daymond and Hadley, 2008), the impacts on bean quality have not yet been reported in combination with elevated [CO_2]. Therefore, there is a need to expand the research to

examine a broader range of genotypes and to determine if these climate conditions will also affect cacao bean quality in the context of predicted climate change.

It has been predicted that water deficit and high temperature events will simultaneously occur under climate change, impacting plant growth and productivity (Fahad *et al.*, 2017). Significant warming may reduce the beneficial impact of elevated CO_2 in some crops (Yu *et al.*, 2012), and may be exacerbated further by water deficit (Xu *et al.*, 2013). The examination of these three environmental factors was carried out in young seedlings in the short-term experiment described in Chapter 4. Under water-limited conditions, the compensatory effect of elevated [CO_2] across the temperature range was still observed but at a lower magnitude compared to the well-watered treatment. In young plants, the adverse effect of water deficit on growth and photosynthesis was mitigated in those grown at elevated [CO_2] compared to ambient [CO_2]-grown seedlings under a non-stress temperature regime (Lahive *et al.*, 2018). Subsequently, Hebbar *et al.* (2020), working with seedlings of one cacao genotype grown under OTCs in India, reported that the negative impact of high temperatures (3°C above ambient temperature) on photosynthesis, Ψ_{leaf}, and biomass accumulation was more severe under water deficit conditions. The authors also suggested that elevated [CO_2] could improve photosynthesis resulting in higher biomass under water deficit combined with high temperatures. Using a broader range of temperatures, the present study partially confirms that assumption. The potential mitigation of water deficit by elevated [CO_2] is dependent on the temperature regime (Chapter 4) with little beneficial impact of CO_2 above 36/27°C (day/night). This could have implications in field locations where very high temperature events occur, which potentially may exacerbate the impact of water deficit on cacao physiology and growth. Thus, this study provides evidence that the physiological responses of cocoa to the combined effects of elevated [CO_2] and temperature can be modulated by other environmental factors such as water availability, which might be accounted for in future climatic models. Further investigation should elucidate to what extent water deficit severity and recovery from such events will influence this interaction.

Although this research has provided key information in terms of genotypic variation and traits which can be incorporated into breeding/selection cacao programmes, farm management practices will also need to be adapted in order to face future climate change scenarios. For example, the likely increase in water demand with rising temperatures due to increased transpiration can be approached through improvements in WUE. Where irrigation is not feasible, several cultural practices such as mulching (Liu *et al.*, 2014; Q. Li *et al.*, 2018), crop arrangement (Barbieri *et al.*, 2012), and cropping systems that use shade (Hatfield and Dold, 2019) may be suitable. On the other hand, water and nutrient limitations define plant responses to climatic drivers such as rising temperatures and [CO_2] (Dusenge *et al.*, 2019). Therefore, the beneficial effect of elevated [CO_2] may only be effective where there are not significant

limitations to growth. Baligar *et al.* (2005), demonstrated that nutrient uptake and nutrient use efficiency were improved in cacao plants grown under elevated [CO_2]. Soils in cacao regions are often deficient in nutrients and acidic which may lead to nutrient deficiencies in plants (Van Vliet and Giller, 2017). This might imply that more nutrient inputs would be needed to see the potential benefits of elevated [CO_2], thereby increasing cost in areas with poor soils.

Future research is also required to better understand to what extent air humidity may limit or enhance the beneficial effect of elevated [CO_2] on cacao physiology under warming and water limitations. In addition, the development of phenotyping platforms for physiological traits and the inclusion of a wide range of cacao genotypes would enable the identification of resilient materials suitable for growth under climate change scenarios. As cacao has a long cropping lifespan, long-term studies are needed to begin to understand the extent to which acclimation to various environmental conditions occurs or for how long mature trees can tolerate such stressful conditions. Working in *Citrus aurantium* trees grown for 13 years under elevated CO_2, Idso and Kimball, (2001) observed interannual changes as trees grew from seedlings to middle-age reproductive maturity in terms of biomass and fruit production. However, the authors also reported acclimation following the prolonged period of exposure. This long-term examination of cacao trees would also need large, field-scale experiments, which may be achieved using Free Air Carbon dioxide Enrichment (FACE) facilities. Thus, moving from glasshouse simulated conditions to FACE would complement current findings and provide a better understanding of how cacao trees may respond to these combined climatic factors under more representative field conditions. However, because CO_2 may oscillate in FACE, and because recent experiments have shown reduced photosynthesis, growth, and yield under fluctuating CO_2 (Allen *et al.*, 2020), plants in FACE might be predicted to underestimate the benefits of consistently rising CO_2 and this needs to be taken into the account in future research.

7.6 Conclusions

The research presented in this thesis has gathered additional evidence about the impacts of the climate change variables such as elevated [CO_2], high temperature, and water deficit on the photosynthetic, vegetative and reproductive responses of various genotypes of cacao. The results of the experiments have revealed that climate factors interact, and a single effect can be modulated by others or expressed according to the plant development stage. Overall, the enhancement of photosynthetic and growth parameters due to CO_2 elevation was more significant in juvenile plants than in mature trees. Under non-limiting water conditions, elevated [CO_2] mitigates the negative effect of increases in temperature on photosynthesis, growth and reproductive components. However, the compensatory effect of elevated [CO_2] diminished above a certain optimal temperature. Genotypic variation in response to the

combined effects of elevated $[CO_2]$ and temperature also modulates this alleviation suggesting a potential for breeding programmes for selecting resilient genotypes for future climatic projections. Under water-limiting conditions, the positive effect of elevated $[CO_2]$ on photosynthesis and growth under warming treatments was lowered. However, above the optimum temperature, this compensatory effect declines, exacerbating the negative effect of water deficit. More research is required to improve our understanding of how the cacao crop is likely to respond to different climate scenarios by incorporating a wide range of genotypes, corroborating findings under long-term field-trials, as well as developing agronomic practices to help to mitigate the effects of climate change.

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