University of Reading

The effects of climate change on the reproductive development of *Theobroma cacao* L.

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School of Agriculture, Policy and Development

Thesis by Liam Robert Handley

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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.

Liam Robert Handley

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Abstract

Cacao (*Theobroma cacao L.*) is widely farmed throughout the humid tropics where it is an important crop to smallholder farmers. Climate change projections suggest increased temperatures and altered rainfall patterns in these regions. The effects of two key climate change variables; elevated carbon dioxide (CO_2) concentrations and water deficit were investigated on the reproductive development of six genotypes of cacao (CL19/10, ICS 1, IMC 47, Pound 7/B, SCA 6, and SPEC 54/1). Genotypes showed variability in their response to treatments; however general observations were also made. At elevated CO_2 (700ppm) the mean length of pollen tubes decreased and the degree of fluctuation in flowering intensity increased over time without any overall reduction in flower production. Final pod size and maximum rate of growth increased in response to elevated CO_2 in the second year of study. Additionally in the second year there was an increase in husk weight and thickness, and individual bean weight. There was no increase in bean number or shell percentage. Total cocoa butter content and percentage unsaturated fat was lower under elevated CO_2 along with reductions in stearic, oleic and linoleic fatty acid content; however these responses varied between genotypes and sampling period. Final woody biomass increased under elevated CO_2 whereas leaf biomass was unaffected. Water deficit stress had no observed effect on pollen performance, flowering behaviour, or cocoa butter content and composition. Final pod size and rate of growth was reduced under water stress in the first year of study only. The reduced pod size was reflected in reduced husk and bean weights. Final wood and leaf biomass were both reduced in response to the water stress treatment. Across all parameters measured, no evidence of interaction between elevated CO_2 and water deficit stress was observed. Overall, these results demonstrate the potential for yield improvement in cacao through breeding in preparation for future climate scenarios.

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Chapter 1. General Introduction

1.1 *Theobroma cacao* L.

1.1.1 Introduction

Theobroma cacao is the tree species from which cocoa beans are produced and is the species most widely farmed from the genus (Wood, 1985b). *T. cacao* is native to tropical South America and is traditionally divided into 3 genetic groups (Wood, 1985b). The first is called Criollo. This was the first to be farmed and is used today for fine flavour and luxury chocolates despite its susceptibility to disease and pests (Argout et al., 2011). The second variety is called Forastero. These trees generally show increased vigour in growth, disease and pest resistance. Due to this they are also the most widely cultivated variety. Finally, the third variety is called Trinitario. This form is a hybrid of Criollo and Forastero and was selected for its combination of Criollo's taste and the resilience of Forastero (Argout et al., 2011). Further cultivars called Nacional and Amelonado have also been identified (Motamayor et al., 2003). The classifications above were largely grouped in reference to physical descriptions, or to the time of introduction and cultivation (Zhang and Motilal, 2016), and thus each category may contain significant genetic diversity. A more recent genetic analysis of cacao samples taken from Central and South America has involved genotyping using microsatellites. Analysis of the findings has resulted in the proposal of a new classification of cacao germplasm into 10 genetic clusters which better reflects the diversity available for breeding (Motamayor et al., 2008). Currently, field genebanks for cacao are estimated to contain between 15 and 44% misidentified material, and molecular analysis is identified as the tool required to ensure reliable identification (Motilal and Butler, 2003).

Cacao was first mentioned by Hernando Cortés in the early 16th century upon his invasion of Mayan territory. The Aztecs used the beans as a currency and to make a thick beverage referred to as Xocoatl. Upon Spanish occupancy of parts of South and Central America the name was modified to Chocolatl and later Chocolate as the beans became popular in Europe (Dillinger et al., 2000). The process of removing the cocoa butter from the beans and leaving cocoa solids was a large step in the development of chocolate in the 19th century. Conrad Van Houten developed a press to produce these cocoa solids (Minifie, 1989) and later the process of creating a chocolate 'bar' became possible (Morgan, 1994). Now cacao is of great economic importance as a crop and is farmed widely throughout the tropics. The cocoa beans are harvested and fermented then later processed to go on to be used in a wide variety of products such as chocolate, cosmetics and pharmaceuticals (Liendo et al., 1997). Around 3.9 million tonnes are produced annually, of which 71% is from Central and West Africa, most notably in Cameroon, Côte d'Ivoire and Ghana. Central and South America produce around 16% of the world cocoa output, mainly from Brazil and Ecuador, and finally Asia and Oceania produce around 13%, largely in Indonesia (ICCO, 2013). In Southern Cameroon cocoa forms the main source of household income demonstrating the economic importance cacao farming holds (Sonwa et al., 2008).

1.1.2 The botany of cacao

The classification of the genus Theobroma is somewhat undecided. Theobroma has been classified into both the Sterculiaceae and the Malvaceae family (kew.org, 2013; plants.usda.gov, 2013). Phylogenetic studies have proposed that Sterculiaceae should be reclassified under Malvaceae (Silva and Figueira, 2005). However, until this point the classification of *T. cacao* can be found as identified under both family groups. *Theobroma*

cacao is one of 22 species within the genus Theobroma and is an understory tree which grows in tropical forests (Toxopeus, 1985). Such forests are beneficial for cacao growth as they can provide relatively constant humidity and temperatures all year round (Kricher, 2011b).

The fruit/pods take 6 months to develop fully (Munoz and Beer, 2001) and contain the cacao seeds (often termed beans) which are surrounded by a white/pink/brownish, aromatic pulp. The fresh pulp acts as a germination inhibitor and prevents the beans from germinating until the pulp has decomposed (Young, 1994a). Upon bean germination the growth is vertical up to the height of around 1-2 metres with the leaves growing in a spiral arrangement around the central stem. The growth of cacao is dimorphic and when the vertical (orthotropic) stage of growth is complete the plant changes to develop 3-5 plagiotropic branches with a different alternate arrangement of leaves. This branch formation is known as a jorquette. New leaves are pale green or various shades of red/pink depending on the variety. Upon maturity the leaves turn dark green due to the synthesis of chlorophyll increasing upon the full expansion of a new leaf (Baker and Hardwick, 1973). The growth of *T. cacao* is slow and after several years a vertical stem called a chupon develops and grows above the level of the jorquette. This growth then develops its own jorquettes. This pattern of growth can continue until the tree reaches heights of between 4 and 10+ metres (Toxopeus, 1985). The stems and leaves grow in 'flushes' in which new shoots and leaves develop. These periods of growth also coincide with the senescence of old leaves. It is theorised that a balance between abscisic acid (ABA) and root cytokinins is involved in the stimulation of bud break in cacao (Alvim, 1977). As the leaf area increases, the more growth stimulating cytokinin is diverted away from growing tips and into the leaves. Equally, greater leaf area also increases the amount of ABA reaching the growing buds and inducing dormancy. The arrival of water deficit further increases ABA production which stimulates the formation of a leaf abscission layer. This

layer diverts cytokinins away from the leaves and prevents the translocation of ABA out of the leaves to the buds. Consequentially, bud dormancy is lost and flushing occurs. The return of rain or irrigation also acts to rupture the abscission layers resulting in leaf drop of older leaves (Alvim, 1977). The extent to which leaves are lost can also be an indication of plant health and nutrient availability (Toxopeus, 1985). As discussed, environmental stimuli such as water deficit can play a role in the triggering of bud break, however flushing also occurs in constant environmental conditions suggesting a degree of endogenous control also. Carbohydrate availability for new leaf development can influence flush occurrence as new leaves represent a large carbon sink. Assimilate consumption has been observed to exceed the photosynthetic production, thus requiring carbohydrates from storage within the plant. A limitation in carbohydrate availability is suggested to play a role in the ceasing of new leaf expansion resulting in the end of a flush. The regeneration of carbohydrate storage will then take place in between flushing events (Machado and Hardwick, 1988). Hormonal control of flushing is also identified by Taylor and Hadley (1988). High concentrations of free indole-3-acetic acid (IAA) were observed in cacao apical buds and leaves but concentrations decreased as leaves expanded. The IAA is suggested as an attractor of assimilates to the growing region, in addition to inhibiting the expansion of other leaf primordia. Removal of new leaves stimulated the next leaf primordia to develop. Continuous leaf removal continued the stimulation of subsequent primordia until all initiated buds (usually 10-15) were used. Evidence is presented to suggest the IAA may be synthesised in the mature leaves and transported to the growing tips where it accumulates prior to bud break (Taylor and Hadley, 1988).

Cacao flowers develop on the trunk and branches of the tree at the leaf axil. The flowers are small and measure between 1 and 2 centimetres in diameter. Each flower consists of 5 sepals, 5 petals, 10 stamens (5 fertile stamens and 5 non-fertile staminodes) and an ovary with 5

carpels. The petals curve up to cover the anthers which project outwards from the centre of the flower (Glendinning, 1972). The flower takes approximately 30 days to develop before it begins to open (Toxopeus, 1985). The sepals split late afternoon and the flower is fully open in the early morning the following day. Pollination is most successful within the first 12 hours, after which the viability of the pollen decreases (Aneja et al., 1992). If a flower is not pollinated then it will abscise 24-36 hours after it has opened (Almeida and Valle, 2007). *T. cacao* produces a surplus number of flowers when flower number is compared with pod number. In addition, some varieties produce more flowers than others. The level of pollination an individual tree requires for maximum yield is varied and would depend on several factors. Using manual pollination, raising pollination from 10% to 40% produced the greatest increases in yield. Beyond 40% no significant yield gains were seen (Groeneveld et al., 2010). It is further suggested that subsequent to pollination the tree's carrying capacity of pods is dictated by the nutrient resources available to the tree (Groeneveld et al., 2010).

T. cacao can be either self-compatible or self-incompatible. All cacao flowers are hermaphrodite and contain both male and female flower components (Almeida and Valle, 2007). In self-compatible flowers, the pollen from the stamen is capable of fertilizing the egg in the ovary of the same flower. Self-incompatible flowers are unable to self-fertilize and therefore depend on a pollinator to transfer pollen between itself and a different tree. Consequentially, a high proportion of self-incompatibility can reduce yields (Lockwood, 1977). Self-incompatibility involves the failure of syngamy (fusion of nuclei) between ovary and pollen tube (Cope, 1962). However, later research has also suggested that incompatibility may take place in two stages, and that the earlier stage, acting within the style, can be overcome with exposure to high concentrations of CO_2 (Aneja et al., 1994). This study reported that self-incompatible pollination of the genotype IMC 30 resulted in no pollen grain germination and no fruit set; however when pollinated flowers were enclosed to allow an

accumulation of 8% CO_2, the germination rate increased to 95% and the fruit set increased to 45%. It was concluded that the first stage of incompatibility was overcome by the CO_2, but the later stages were still in effect and causing a partial block to fruit set (Aneja et al., 1994). It is thought that self-incompatibility is linked to auxin levels and genetic control (Hasenstein and Zavada, 2001). Currently research into the use of molecular markers is in progress to speed up the identification of self-incompatibility in trees, to help in the selection of self-compatible trees for breeding and consequently to increase yields in the field (Da Silva et al., 2016). Pollination of flowers in the field is largely reliant on natural pollinator activity. There are several small insects considered to be possible pollinators of cacao such as phorid flies, gall midges (Cecidomyiidae), microdpotera, thrips, and solitary bees; however these are largely unconfirmed as such (Young, 1994b; Klein et al., 2008). The primary confirmed pollinators of cacao are the biting midges of *Forcipomyia* sp. (Diptera: Ceratopogonidae) (Young, 1994b; Klein et al., 2008), the females of which have been observed in the process of cross-pollination of cacao flowers (Glendinning, 1972).

Pod development is slow and growth reaches a maximum rate at around 80-120 days. During development, a large proportion of pods can be lost in a process called 'cherelle wilt'. Accepted as a method of reducing the number of fruits on a tree, the pods shrivel and blacken whilst still attached to the branch (Nichols, 1964). Cherelle wilt allows the tree to adjust its fruit carrying capacity dependent on its current condition and nutrient availability (Mckelvie, 1956). This theory is further supported by accounts of higher incidence of wilt during periods of leaf flushing, representing internal resource competition (Hurd and Cunningham, 1961). Mckelvie (1956) also identified periods of high incidence of cherelle wilt at 2 points in the 6 month development period of the pod. At around 50 and 70 days into pod development the incidence of cherelle wilt showed a clear peak. After the latter peak the incidence decreased to 0 at 95-100 days. This study used Trinitario, Amelonado and Upper Amazon varieties of

cacao and showed evidence of variation in results between each variety. In particular, the Upper Amazon variety showed a reduced incidence of cherelle wilt at the 70 day point. The two defined peaks in wilt have been later brought into question as more recent research identified only one peak in wilt followed by a gradual decline in incidence (Daymond, 2000). The peaks in wilt coincide with the exponential growth phase of the pods, a period which involves cell division and cell expansion. Beyond the point of wilting only cell expansion is occurring (Valle et al., 1990). In the later, linear growth phase, the pods represent a large investment from the tree, therefore the younger pods will be wilted preferentially to minimise investment loss (Valle et al., 1990).

A fully developed pod and its beans vary greatly in their appearance and quality. The shape of pod is divided into four categories (Ciferri and Ciferri, 1957):

1. Angoleta – ridged and warty, squared base and a rounded or pointed apex.
2. Cundeamor – narrowed base giving a bottle-neck shape and a pointed apex.
3. Amelonado – Diameter of the pod greater than one half of the length. Ovate.
4. Calabacillo – spherical in shape (Toxopeus, 1985).

The pod husks show variation in thickness and can be either rough, warty or smooth. The surface can also be ridged. The husk colour begins green, light green or red depending on the variety and often a colour change indicates maturation. The beans can be flat or rounded and range from white and brown to purple. A typical bean is around 2cm in diameter (Are and Gwynne Jones, 1974) and bean weight can hold importance for fat content and shell percentage (Wood, 1985c). Bean weights below 1g are associated with a higher percentage of shell and a reduced fat content, therefore market value becomes reduced (Wood, 1985c).

1.2 Environmental requirements of cacao

Cacao is native to tropical South America; specifically the upper Amazon rain forest (Zhang and Motilal, 2016). This region is classified as the humid tropics and has an mean annual temperature of over 24°C and high humidity levels (Kricher, 2011b). In contribution to the conditions of high humidity, the rainfall ranges between 1500mm and 3000mm annually (Salati and Vose, 1984). Overall this habitat is warm and wet. There is little variation in temperature throughout the year; however there is fluctuation in the rainfall resulting in a wet and a dry season. These conditions best describe cacao's place of origin; however the cultivation of cacao has spread across the tropics with the growth of the cocoa industry. Around 75% of the world's cocoa production comes from within an 8° band of the equator, and this is largely attributable to the high volume of rainfall found within these regions (Wood, 1985a). Cacao cultivation can also be found at greater extremes of latitude, most notably in Bahia, Brazil between 13° and 18° south, in the Dominican Republic between 18° and 19° north, and Malagasy, Africa at 14° south (Wood, 1985a). Naturally with increasing distance from the equator and spread across the tropics, cacao has come to be farmed in areas with altered climates to that of its origin. For example, the subtropical location of Bahia, Brazil has a period of reduced temperatures from June to September. In Ghana, lower temperatures also occur at this time due to increased rainfall (Daymond and Hadley, 2004). The tropical regions of Asia such as the cacao growing areas in Malaysia experience a more stable climate with smaller seasonal fluctuations in temperature and precipitation (Wood, 1985a). However, the dry seasons in some Indonesian cacao growing regions are more pronounced than others such as East Java (Boer and Subbiah, 2005). Furthermore, drought is also found in this region in association with El Niño Southern Oscillation (ENSO) (Schwendenmann et al., 2010).

1.2.1 Temperature

Daymond and Hadley (2004) stated that temperature is the main factor which restricts the locations in which cacao can be grown and that it also explains some seasonal variation in bean yield. They reported a base growing temperature between 18.6 and 20.8°C for cacao. This was based on the measurements of early vegetative growth. These results were higher than previous temperatures found by Alvim (1977) however it is pointed out that this may be due to the lower light levels used by Daymond and Hadley. This would suggest that under a non-shaded condition, lower temperatures may be tolerated by cacao. In a study by Joly and Hahn (1991) it was reported that cacao seedlings were exposed to temperatures down to 4.7°C. Severe photosynthetic inhibition was noted at temperatures lower than 10°C. Despite such inhibition it is reported that all plants made a full recovery of photosynthetic rate over a period of 7 days. This study involved chilling at night with a recovery of normal temperature the following morning. Overall it becomes apparent that the temperature tolerances of cacao are also dependent on other factors such as light levels and duration of temperature exposure. Daymond and Hadley (2004) further mentioned that there was a degree of variation between the tolerances of low temperatures between varieties studied. The genetic variation observed may hold importance to breeding new varieties of cacao which are tailored to their farming locations, especially if the climate of current growing regions is expected to change. The optimal temperatures for growth of cacao have been observed to vary for different plant organs (Sena Gomes and Kozlowski, 1987). For example, leaves had a higher optimal growth temperature than stems and roots in a series of growth chamber studies at various temperatures. Leaves were also found to act as a stronger sink for dry matter over stems and roots. Carbohydrate partitioning changed in response to rising temperatures with roots receiving less dry matter at higher temperatures. The author comments on the detriment that higher temperatures may cause to drought tolerance in cacao as the development of root

systems may be reduced, thus reducing the likelihood of the water uptake meeting transpirational demands (Sena Gomes and Kozlowski, 1987).

Aneja et al. (1992) investigated the effect of temperature and carbon dioxide levels on pollen germination in *T. cacao*. It was concluded that an optimum temperature range for germination was between 25°C and 30°C. At 15°C little germination was seen. Furthermore if pollen was stored at 15°C and then transferred to 25°C a lack of germination was still observed *in vitro*. It is speculated that a lack of enzymatic activity at low temperatures was the detrimental factor. In contrast at 35°C the cytoplasm of the pollen grains was reported as 'shrunken' suggesting the limits of the pollen grain were being reached. These results suggest agreement with Daymond and Hadley (2004) who proposed a base temperature for cacao overall is around 20°C.

In further studies, Daymond and Hadley (2008) saw a decrease in the time taken for a pod to mature with an increase in temperature. However, this faster rate of development was associated with a reduction in bean dry weight for the clones Amelonado and SCA 6. The study identified the different responses between varieties to different temperature, again emphasising the diversity available for future breeding. Incidence of cherelle wilt has been observed to increase under warmer growing conditions (max 33°C, min 23°C) compared to a cooler treatment (max 25, min 16°C) in a greenhouse study conducted by End (1990). Additional findings of the study identified increased saturated fatty acids and higher melting points in the cocoa butter of beans which developed in the hot conditions (End, 1990), providing insight into the diversity in cocoa butter characteristics across different growing regions (Wood, 1985c). In addition to pod development; flower production is also affected by temperature. In a study by Sale (1969), trees of the genotype ICS 95 were grown in growth rooms and maintained at day temperatures of either 23.3°C, 26.7°C or 30°C, and night

temperatures of either 23.3°C, 26.7°C or 30°C. Flowering was significantly greater in the two highest day temperature treatments whereas a night temperature of 26.7°C resulted in the largest amount of flowers regardless of day temperature treatments.

1.2.2 Water availability

The tropics experience a high level of rainfall per year, between 1500mm and 3000mm annually (Salati and Vose, 1984). Despite this relatively high volume of rainfall, the distribution is uneven throughout the year, resulting in wet and dry seasons (Kricher, 2011b). Cacao is considered drought sensitive (Moser et al., 2010b) and rainfall pattern is observed to be the main factor which dictates the main cropping season (Alvim, 1977). Annual rainfall of above 2500mm can lead to waterlogging, increased incidence of disease such as black pod (*Phytophthora palmivora*), and reductions in yield, whereas annual rainfall below 1200mm is likely to require irrigation if yields are to be maintained (Alvim, 1977; Wood, 1985a). The seasonal patterns of rainfall are reflected in the cropping patterns observed in the cacao of that region. For example the presence of 2 dry seasons in parts of West Africa results in the two cropping seasons in that region. Additionally, more uniform precipitation patterns in parts of South East Asia result in more uniform cropping throughout the year (Wood, 1985a).

1.2.3 Irradiance

The shaded origins of cacao have led to the development of shaded cultivation. Cacao trees are often grown alongside larger trees either remaining from forest land prior to cacao cultivation, or trees specifically planted for shade such as coconut, oil palm and rubber (Beer et al., 1997). However, although shade is recommended for young cacao tree establishment

(Wessel, 1985), the removal of shade from mature trees in Ghana has been observed to result in significant increases in yield, increased further by additional fertiliser applications (Ahenkorah et al., 1987). The removal of shade has been found to increase the intensity of flowering and thus the number of flowers setting pods, yet the percentage of flowers setting pods did not increase in comparison to shaded trees (Hurd and Cunningham, 1961). The resultant increases in yield and reproductive capacity was associated with the increase in leaf flush size, leaf number and leaf area which was suggested to have increased carbohydrate production in the unshaded trees (Hurd and Cunningham, 1961). However, the farming of high yielding, unshaded trees, requires greater fertiliser investment and is suggested to reduce the economic life span of a tree and a limit of 12 years intensive farming is suggested (Ahenkorah et al., 1987).

1.3 Climate change

Increases in economic growth and population size since the pre-industrial era have had a significant effect on the global climate. The creation of an Intergovernmental Panel on Climate Change (IPCC) was created to evaluate historic climate changes, their main driving forces, and predictions of the future. The findings of the latest IPCC report (IPCC, 2014) are discussed below.

Through the monitoring of ice cores it is possible to measure the gas levels in the atmosphere over thousands of years. Since pre-industrial times, greenhouse gasses have been emitted into the atmosphere at an increasing rate. The 3 main greenhouse gasses are carbon dioxide (CO_2) (largely emitted as a product of fossil fuel use), methane (CH_4) (largely emitted as a product of agriculture and fossil fuel use) and nitrous oxide (N_2O) (largely emitted as a product of agriculture). CO_2 concentrations have increased by 40% since 1750, and the rate of increase

between the years 2002 – 2011 is the fastest observed decadal increase (IPCC, 2014). Global CO_2 concentrations are currently averaging around 400ppm (NOAA/ESRL, 2016). This concentration is predicted to continue increasing, however the degree of growth is dependent on many anthropogenic factors including land use and greenhouse gas emissions.

CO_2 is the largest contributor to radiative forcing and is therefore the largest driver of increases in global temperatures. Combined global land and ocean temperatures have increased by 0.85°C between 1880 and 2012 in response to the anthropogenic gas emissions (IPCC, 2014). The increases in temperature have resulted in warming oceans, smaller polar ice caps, and higher sea levels. Changes in ocean salinity in combination with global temperature increases have influenced the global water cycle and altered precipitation patterns and intensity. Furthermore, extreme temperature and precipitation events have increased in intensity and frequency (IPCC, 2014). Prediction of future global climate patterns depends heavily on the rate at which greenhouse gas emissions continue. To address this, the IPCC generated a range of outcomes based on a range of emission scenarios (SRES) (Nakićenović et al., 2000). Four main groups of scenarios were created, each of which predicted a range of climate outcomes based on human social and economic development. For example: scenario A1 assumed rapid economic growth and fast advances in technology, whereas A2 described a larger population but with slow economic growth and slow technological advances. However, the latest IPCC report has modified SRES to become Representative Concentration pathways (RCPs) (IPCC, 2014). The updated RCPs now represent a wider range of climate outcomes as they take into account implemented climate policy. The pathways include an outcome in the event of strong preventative measures against climate change (RCP 2.6), two intermediate pathways (RCP 4.5 and 6.0), and a pathway with greatly increased gas emissions (RCP 8.5) (IPCC, 2014).

Between the intermediate RCP 6.0 and the high emission RCP 8.5, atmospheric CO_2 concentrations are expected to reach between 720ppm and upwards of 1000ppm (IPCC, 2014). Global temperature increase is predicted to exceed 1.5°C by the year 2100 for RCP4.5, 6.0, and 8.5. Hot and cold temperature extremes are predicted to increase in frequency and are expected to occur for longer periods of time. The global water cycle offers a more varied response with patterns of wetting and drying occurring across different global regions. Finally, extreme events of precipitation are expected to increase along with the intensity and coverage of monsoon systems and El Niño-Southern Oscillation (ENSO). Overall these changes will be in effect globally but the impacts will be greater for poorer disadvantaged communities (IPCC, 2014). Increases in temperature and altered precipitation patterns pose a threat to yields of staple foods including wheat, rice and maize, ultimately posing risk to food security. Water availability is threatened along with a risk to livelihoods and infrastructure. Changes in agricultural practices are expected with recommendations to develop new crop varieties which will be suitable under conditions of elevated CO_2, drought and higher temperatures. Small-holder farms are identified as high-risk under climate change, and their safe-guarding through financial credit and investment is also recommended (IPCC, 2014).

1.4 Climate change in cocoa producing regions

Theobroma cacao is grown widely across the tropics. Around its origins, cacao is grown in northern South America, parts of the Caribbean including Trinidad, and in North East Brazil. The south-east of the state of Bahia in Brazil produces around 90% of Brazil's cocoa and experiences a moderate dry season with temperatures which can show large fluctuations due to the distance of this state from the equator (Wood, 1985a). Since the success of cacao as a commercial crop, Africa is now the world's largest exporter of cocoa and the dominant cacao

farming region is in West Africa. West Africa shows similar climate patterns to the Amazon but has more pronounced dry seasons. There is a wide range of variation between the cacao growing countries in Africa. Some have dry seasons which last up to four months and some areas such as east Nigeria and west Cameroon have a more sustained wet season (Wood, 1985a). There are small levels of cocoa production in India which displays more pronounced seasons with high temperatures in the dry season (Wood, 1985a). Finally, cacao is also grown in tropical South East Asia and Indonesia which has an overall more uniform warm and wet climate (Wood, 1985a), with some exceptions such as the cacao growing region of East Java which has a more pronounced dry season (Boer and Subbiah, 2005). There is a large degree of variation in the predictions of climate change across South East Asia.

Looking at individual regions, Africa has experienced significant warming of around 0.5°C to 0.8°C between 1970 and 2010, and an increase in the number of warm days and nights between 1961 and 2000 (IPCC, 2014). Predictions suggest that the warming in West Africa could continue with increases of between 2°C and 6°C in the next 100 years (Hulme et al., 2001; IPCC, 2014). Warming in Africa is expected to occur at a faster rate than the global average. Additionally, the narrow climate range of tropical West Africa means the effects of warming will be felt 1 to 2 decades earlier than other more variable regions (IPCC, 2014). This warming trend dominates the continent; however, cooling is also suggested in the cacao growing region of Cameroon and other areas of western Africa (Hulme et al., 2001). Cooler conditions may reduce plant transpirational water loss and thus plants may become less likely to suffer from water stress. However, parts of northern tropical Africa have experienced a decline in precipitation with strong drying trends in northern sub-Saharan Africa extending between the Sahel and the rainforest regions of West Africa and north Congo (Malhi and Wright, 2004). Dry season index/intensity (DSI) represents a measure of the relative variation in dry season length and severity over space and time, and has increased significantly in West

African rainforest regions (Malhi and Wright, 2004). Due to the rainforest locations on the outer edges of tropical forest areas, these regions experience the largest seasonal changes. Overall, a distinct drying trend has been observed in Africa with exceptions in equatorial areas which show a multi-decadal variation in rainfall pattern. There appears to be a mixture of drying and wetting trends across a relatively small area surrounding tropical Africa. The western Sahel regions have seen drying of up to 25% in the last century, whereas a wetting zone across the equator has shown precipitation increases of up to 10% (Hulme et al., 2001). The fluctuation in annual rainfall across the Central Africa region ranges between -10 and +39mm yr^{-1} per decade and further serves to demonstrate the variability in precipitation in the area (Maidment et al., 2015). Despite strong suggestion of future warming patterns, rainfall predictions are less confident and show a range of predictions in rainfall over west Africa (Hulme et al., 2001; Cook and Vizy, 2006). A lack of comprehensive historical precipitation data in the region has limited the reliability of future predictions and as a result there is a great degree of uncertainty surrounding rainfall patterns (IPCC, 2014). Overall, Africa is deemed as a region with the highest vulnerability to climate change and also a region with the least ability to adapt. Food production in Africa is deemed the most vulnerable in the world due to a heavy reliance on rainfed agriculture. It is suggested that the areas suitable for growing economically important perennial crops such as cacao are expected to diminish, especially in areas of low altitude (IPCC, 2014).

Temperatures in south-east Asia have been increasing between 0.14°C to 0.2°C per decade since 1960 and the frequency of warmer days has increased, as well as a reduction in cooler days (IPCC, 2014). Generally, Southeast Asia experiences a weak dry season, with patterns of a strong dry season occurring once per decade. As a consequence Asia is one of the least water stressed regions across the tropics. Southeast Asia has experienced an increase in wetting of around 22mm per decade; however the wetting is seasonal and is reflected in the

increase in ratio of rainfall in the wet season compared to the dry season (IPCC, 2014). Predicted mean annual temperatures are expected to increase between 3℃ and 6℃ under RCP8.5 predictions, tropical cyclone precipitation intensity is expected to increase, and monsoon related precipitation extremes are also predicted to increase. Changes in the occurrence of drought are expected to interfere with the phenology and growth of plants in lowland rainforest regions. Southeast Asia is identified as one of the most poorly performing regions for economic and human development. Climate change is expected to have large impacts on water resources, agriculture and resource dependent livelihoods (IPCC, 2014).

In the South American tropics due to the vast size of the Amazon rainforest, precipitation is generated from the forest itself. If large scale deforestation is to continue in these areas along with other climate changes, the precipitation will reduce in the region and the surrounding areas. As a result, the dry seasons will become elongated and droughts will become more severe (Kricher, 2011a). For example, a drought occurred in the Amazon in 2005 and was considered the worst in a century. Its cause is linked to an increase in Atlantic sea temperatures causing higher air temperature and reduced precipitation in the southern regions of the Amazon. A project entitled RAINFOR monitored several plots across the Amazon forest. Before the drought of 2005, the plots consistently gained biomass and showed the forest acting as a carbon sink. Furthermore, biomass was also gained in years of ENSO events. However, the drought in 2005 resulted in no net gain of biomass and mortality rates increased in plots most severely affected by the water shortage (Peacock et al., 2009; Kricher, 2011a). It is predicted that in extreme cases of persistent drought, areas of tropical rainforest could be converted to savannah as the area will no longer be suitable to sustain tropical plant and animal species (Kricher, 2011a). The observed trends in climate change vary significantly across the tropical regions of South and Central America. Temperatures in the Andes have increased by around 0.1°C per decade over the last 60 years and precipitation has

shown small increases in the inner tropics but decreased in the outer regions. Conditions within the Amazon basin show no consistent trends and patterns of wetting and drying have been linked to El Niño-Southern Oscillation (ENSO) or natural climate variability (IPCC, 2014). Future projections of precipitation are diverse and include wetting in southeast South America, northwest Peru and Ecuador, and drying in northern South America, eastern Amazonia, central and northeast Brazil. Projections also show a large degree of variation, suggesting uncertainty in future trends (IPCC, 2014). Deforestation to make way for agricultural land is a key factor in precipitation change and damage to fragile ecosystems, and is expected to continue. Crop productivity is expected to decrease in areas of increased temperature and reduced rainfall. Crop management with an emphasis on crop genetic improvements is a recommended adaptation (IPCC, 2014).

The greatest threat to small islands such as Trinidad and the Dominican Republic is sea level rise. Many small islands are at very low elevation and are exposed to tropical storms which are expected to increase in frequency. The diversity in location of small islands and variation in physical and human attributes makes climate data and predictions highly unique to individual nations. Further concerns include the availability of fresh water and increasing cases of invasive non-native species establishing on islands and out competing other native organisms. International support is emphasised as an appropriate way to aid the ability of small islands to adapt to and mitigate climate change (IPCC, 2014).

In summary, an increase in temperature is largely the trend predicted to effect most tropical regions. Predicted precipitation trends are highly varied between locations and poor quality or quantity of historic data, particularly in Africa, generates a high degree of uncertainty in the precipitation projections over the next 100 years. Wetting is a common theme in the tropical regions however shifts in the arrival and duration of dry seasons is also expected to change, naturally having an effect on local agriculture. In almost all regions, emphasis on the

development of new crop varieties is mentioned as a pathway for future adaptation to climate change. It is likely that elevated CO_2, flooding, salinity, water deficit and high temperatures are all factors which will impact agriculture (IPCC, 2014).

1.5 Plants and climate change

1.5.1 Plants and elevated CO_2

Increased concentrations of atmospheric CO_2 have a direct effect on the rate of photosynthesis in terrestrial C_3 plants. The maximum velocity of the carboxylation reaction, performed by the enzyme Rubisco, is achieved at roughly double the current atmospheric concentrations of CO_2. Therefore an increase in CO_2 concentrations increases the velocity of the carboxylation reaction, thus increasing photosynthetic rate. Photorespiration is also performed by Rubisco and can sequester ATP and NADPH away from photosynthetic assimilation. An increase in CO_2 concentrations out-competes the reaction between oxygen and Rubisco (photorespiration) and thus improves the efficiency of CO_2 uptake (Long et al., 2004). In consequence to the elevated photosynthetic rate, assimilate availability for plant development is increased. Free-air CO_2 enrichment experiments (FACE) have identified yield increases of between 7-15% in rice when grown at 200ppm above the atmospheric concentrations (KIM et al., 2003). Similarly FACE experiments at an elevated CO_2 of 550ppm have demonstrated yield increases of 8% for wheat (Kimball, 1995) and 15% for soybean (Ort et al., 2006). Furthermore, 20% increases of pollen production and increases in pollen tube lengths were observed when soybean was grown in growth chambers at 720ppm CO_2 (Koti et al., 2005). Significant variation in treatment response was also present between the different soybean genotypes used (Koti et al., 2005).

The effects of elevated CO_2 on crops such as rice, wheat and soybean are often of primary focus due to their importance as staple foods. Perennial crops have substantial physiological differences to the main annual staple foods. Despite the often cash-crop nature of perennials, they still hold significant economic importance and their responses to climate change are equally as important. The trans-location of perennials is often more difficult due to re-establishment times, in addition to other factors such as accessibility to processing facilities and labour (Glenn et al., 2013). Overall, it is suggested that increased CO_2 concentrations may have greater and longer-lasting effects on perennials due to the increase in life-span (Glenn et al., 2013). A seventeen year study of four Sour Orange trees (*Citrus aurantium* L.) resulted in a substantial 70% increase in final total biomass in trees grown at 300ppm above atmospheric concentrations (approximately 700ppm). Initially, as the trees were young, biomass enhancement due to increased CO_2 was heavily invested into woody growth; however after year 5 of the experiment the biomass investment in fruit was greater than that of wood. In the later years of the study, biomass enhancement levelled-off and fruit enhancement stabilised at around 70%. Increases in fruit biomass were attributed to an increase in fruit number with no detriment to fruit size or quality (Kimball et al., 2007). Generally, elevated CO_2 studies of other perennial crops are limited, potentially due to the complications added with longer life cycles and larger organisms. FACE studies, in addition to providing experimental conditions closer to that of the field, can remove the size limitations imposed by using growth chamber CO_2 studies (Long et al., 2004). FACE may provide viable research options for large perennial species, although costs can be large with this type of study.

1.5.2 Cacao and elevated CO_2

Studies have been conducted to observe the effect of elevated CO_2 on the photosynthetic and vegetative growth responses of cacao seedlings. Baligar et al. (2005) grew cacao seedlings for 57 days in climate controlled greenhouses. Elevated CO_2 (700ppm) increased both shoot and root growth, and nutrient uptake; however under high photon flux density of 1050 µmol m^{-2} s^{-1}, growth and nutrient uptake was hindered regardless of CO_2 concentrations. Furthermore the detrimental effects of high light were more severe under ambient CO_2 suggesting increased CO_2 provided some alleviation. The study concluded that elevated CO_2 conditions are favourable for the growth of cacao but that nutrient demands may increase and soils may degrade as a result. An atmosphere with higher CO_2 levels may increase the need for fertilizer use and increase the cost of pod production. Later work by Baligar et al. (2008) identified significant increases in photosynthetic rates of cacao seedlings with increasing concentrations of CO_2 (up to 850ppm). Leaves were measured in measurement chambers and exposed to instantaneous increases in CO_2. Additional observations included decreases in stomatal conductance and transpiration rates. Controlled greenhouse experiments by Lahive (2015) also identified significant increases in photosynthetic rates of cacao seedlings and also six genotypes of adult cacao trees either when exposed instantaneously or grown under conditions of elevated CO_2 (700ppm). However, in contrast to findings by Baligar, no changes in stomatal conductance were observed.

The elevated CO_2 studies in cacao have largely used young cacao seedlings/trees. Consequentially there is little evidence demonstrating CO_2 effects on reproductive growth. However, studies have been conducted as to the effect of CO_2 on cacao pollen germination. It has been observed that incubating cacao flowers at high concentrations of CO_2 increased pollination success and fruit set (Aneja et al., 1992). Prior to pollen use, the flowers were sealed in vials for 6 hours. The concentration of CO_2 within the vials increased to 8500ppm

through respiration. This resulted in increased germination of pollen grains and as a result increased fruit set. Leading on from this research Aneja et al. (1994) noted that the initial stages of self-incompatibility were overcome by similar treatment of pollinated flowers. When flowers were enclosed to allow CO_2 levels to increase, germination was 95% with a fruit set of 45%. It was identified that self-incompatibility has two levels. Stage one is the germination stage which is overcome by increasing CO_2 levels. The second stage is the gamete fusion stage which is not affected by CO_2 and is attributed as the reason why fruit set is only 45%. Overall, the exposure of pollen to high concentrations of CO_2 appeared to stimulate pollen performance; however the application of this research to climate change research is limited as the concentrations of CO_2 which were achieved are much larger than projected atmospheric concentrations.

1.5.3 Plants and water deficit

Analysis of various grain crops, including rice and maize, under drought conditions demonstrated clear inhibition of reproductive development and yield (Saini and Westgate, 1999). Floral induction and development is often inhibited during drought along with reduced grain filling. Meiosis was identified as one of the major processes that are highly sensitive to water stress, with detrimental impacts almost exclusively effecting male gamete development (Saini, 1997; Saini and Westgate, 1999). Soybean has demonstrated decreases in photosynthetic rate and water potentials in leaves, flowers, and pods when plants were exposed to drought around anthesis (Liu et al., 2004). The author suggests that the drought exposed pods had an inability to utilize sugars in addition to a reduced sugar flow from leaves, thus resulting in pod abortion.

In tropical regions, a pattern of wet and dry periods is the main contributor to seasonality. Having annual periods of dry weather is suggested to play a regulatory role in the reproductive development of plants. Guava is reported to enter a state of inactivity during drought conditions which is broken upon the return of the rains (Mercado-Silva et al., 1998). Similarly, rains following a dry season are referred to as 'cafeteleras' in native Costa Rica as it triggers a sudden mass-flowering of the coffee crops (Opler et al., 1976). Such plant behaviour has led to various studies on the reproductive responses of tropical plants to times of drought and water stress. With reference to coffee, when the plants are supplied with regular and substantial water irrigation, they develop fewer fruits and flowers (Alvim, 1960). Whereas a period of drought followed by a period of irrigation stimulated flowering and thus increased fruiting. Flowering occurred 10-11 days post-irrigation after a period of no water. Here the need for a drought treatment was likened to the chilling requirement seen in some plant species of temperate climates (Alvim, 1960). Additionally, the use of irrigation to synchronise fruiting is a suggested method of reducing labour requirements in coffee harvests (Masarirambi et al., 2009). Borchert (1983) has suggested that flower initiation and flower emergence/anthesis were separately controlled and that flower initiation was controlled largely by endogenous factors, not through environmental control. This is contradicted by the increase of floral initiation in coffee when exposed to dry conditions (Alvim, 1960). The synchronisation of flowering through rainfall is suggested as an evolutionary advantage to maximise the likelihood of cross-pollination with other individuals which are flowering in response to the same stimulus (Opler et al., 1976).

1.5.4 Cacao and water deficit

Sale (1970b) reported on the growth, flowering and fruiting responses seen in cacao when the trees were exposed to varied soil moisture levels. When the trees were continuously maintained at 85% of the soil field capacity (wet treatment), the flushing frequency decreased but the total dry weight, leaf area, leaf longevity, and number mature pods was greater compared to trees exposed to continuous soil moisture conditions of around 15% field capacity (dry treatment). Trees in the dry treatment underwent a significant leaf flush when re-watered; however the new growth showed poor leaf expansion and early abscission. The water use efficiency was considered the worst when the trees were maintained in a continuously dry period, suggesting little/no acclimation to the dryer conditions. Overall it was suggested that an absence of a dry period was preferred by cacao and that the dry conditions 'seriously impaired growth'. The flowering declined in the trees maintained under the dry conditions; however if a tree was transferred to a wetter treatment flowering returned in great volumes indicating flowering initiation had been stimulated by the dry period. This research was carried out over 2 years and a reduction in flowering in the continuously wet conditions suggests a dry period may play an important role in the stimulation of flowering. Alternatively, the reduction in flowering in the wet treatment may be a consequence of reduced light levels caused by increased self-shading in the wet treatment through the stimulation of vegetative growth. This theory is in line with observations of increased flowering with the removal of shading on cacao farms (Hurd and Cunningham, 1961).

A pronounced dry season can create drought conditions for the cacao trees in the field. Bae et al. (2008) monitored the stress response of cacao when watering was withheld. Using the presence of polyamines as a measure of stress response, the speed at which water shortage is detected was visible. After 7 days without water there was a drop in photosynthesis rates and 50% drop in stomatal conductance. Severe water stress was suggested after 10 days through

polyamine biosynthesis. The author suggests that an increase in the genes involved in the biosynthesis of polyamines could be involved in the changing of the structure of the root system in response to water stress, as seen in other crops. Using field conditions and natural dry and wet weather conditions, Araque et al. (2012) studied the effects of dry weather when establishing young cacao plants. Osmotic adjustment was observed and associated with higher potassium concentrations as seen in high yielding cacao clones. Stomatal closure was noted in dry seasons, causing 73% lower assimilation and transpiration rates. Higher water use efficiency was also observed in response to drought. The author concludes that osmotic adjustment in the first 5 years of growth has a large importance when looking at plant survival and production.

Humidity is another aspect considered when water availability is studied. Cacao has been observed to have smaller leaves and a greater weight per unit area indicating thicker leaves when grown at high relative humidity (Sale, 1970a). In addition, cacao showed high sensitivity to low humidity with stomatal closure and a low net photosynthesis (Sena Gomes et al., 1987). Experimental treatments of high humidity alternating with low humidity resulted in a greater dry weight gain and higher levels of flowering (Sale, 1970a). This could suggest that a dry and wet season, as seen in the field, could play an important role in the growth stimulation of cacao. The author comments on the restrictions in application to the field due to the analysis of humidity in the absence soil water stress.

1.5.5 Interaction between water deficit and elevated CO_2

A common response of plants to elevated CO_2, in addition to increased rates of photosynthesis, is a reduction in stomatal conductance (Long et al., 2004; Drake et al., 1997). This reduces transpirational water loss and thus improves water use efficiency. This can

result in a degree of alleviation from water deficit stress. However, a decrease in stomatal conductance does not always occur in response to increased CO_2 therefore alleviation may not always occur, as was the case for Beech (*Fagus sylvatica* L.) and Birch (*Betula pubescens Ehrh.*) (Beerling et al., 1996). Stomatal conductance in cacao under elevated CO_2 has shown varied results with significant reductions observed by Baligar et al. (2008), yet no response was observed in studies of six different genotypes by Lahive (2015). Improvements of water use efficiency were observed by Lahive (2015); however these were largely attributed to the increases in photosynthetic rate.

1.6 Justification of this research

It is likely that the growing regions of cacao will be exposed to increased concentrations of atmospheric carbon dioxide and increased variability in water availability. Although precipitation patterns are largely expected to increase in the humid tropics, cacao is also grown on the boundaries of these regions and a mixture of wetting and drying is expected depending on the specific location (IPCC, 2014). The high incidence of smallholder farming of cacao places a large section of cocoa production under high risk from climate change. To preserve and improve yields, a deeper understanding of the responses of cacao to climate variables will be essential in safeguarding supply and farmer's livelihoods. Furthermore, exploration of the genetic diversity amongst cacao genotypes in response to climate change will be crucial in breeding new varieties for farms.

This body of work aims to examine the response of six cacao genotypes to elevated CO_2 and water deficit stress, and to identify any interaction between these two treatments. There is an emphasis on investigating the reproductive responses to treatment, to compliment the associated body of work examining the photosynthetic and vegetative responses of cacao

conducted by Lahive (2015). Investigation into the effect of treatment on the development of pollen grains are analysed through observation of pollen performance and viability when pollen is germinated *in vitro*. Flower emergence and flowering intensity are monitored throughout long-term exposure to experimental conditions. Pod development is recorded from manual pollinations through to fruit ripening and harvest. Partitioning of resources within the pod is compared between treatments in combination with the attainment of lipid and fatty acid profiles from the beans. Finally, a destructive harvest was carried out to build a picture of overall tree performance and canopy size. An estimation of canopy productivity is also calculated.

Chapter 2. General materials and methods

2.1 Experimental Design

For the purpose of this study, six genotypes of *Theobroma cacao* L. were grown in either elevated or ambient levels of carbon dioxide (CO_2), and with either surplus water or under water-stressed conditions. Trees were grown in their allocated treatments within a controled environment glasshouse facility (Figure 2.1) for two years. Temperature, lighting, and humidty were kept constant between the experimental treatments.

Figure 2.1 Glasshouse Facility
Horticultural grounds, Whiteknights Campus, University of Reading, UK.

2.2 Glasshouse Facilities and Set-Points

To enable research on the effects of environmental parameters on cocoa, a purpose-built glasshouse facility was utilised in the University's horticultural grounds. This facility consists of one large glasshouse building divided into 6 houses by glass partition walls. (From this point onwards the term 'house' will be used to describe a singular division within the whole

glasshouse building. The whole complex will be referred to as the 'glasshouse facility'). For each house; the temperature, CO_2, and lighting were controlled using a TomTech T200 computer system (*Tomtech*, Lincolnshire, UK). For the purposes of this experiment, only houses 2, 3, 5 and 6 were used (see Figure 2.2).

House 4 House not in experiment	**House 5** Ambient CO_2 WW and WS treatment	**House 6** Elevated CO_2 Set point: 700ppm WW and WS treatment
House 1 House not in experiment	**House 2** Elevated CO_2 Set point: 700ppm WW and WS treatment	**House 3** Ambient CO_2 WW and WS treatment

Figure 2.2 Glasshouse facility layout
The layout and numbering of the glasshouse facility identifying houses with either an ambient or elevated CO_2 concentration. Achieved CO_2 concentrations can be found in Table 2.1. WW – well watered, WS – water stressed.

Houses 2 and 6 were maintained at a target CO_2 concentration of 700ppm and houses 3 and 5 remained at ambient CO_2 concentrations. Each house had a well-watered (WW) and a water-

stressed (WS) irrigation treatment (see Section 2.3). Temperature, lighting and humidity parameters remained the same across all houses.

2.2.1 Temperature

The temperature within each house was maintained by a Benson PV100-1 gas heater (*AMBIRAD Ltd*, West Midlandsm UK. Output: 29.4 kW.h.) The temperatures were maintained between 20°C and 30°C minimum by means of a combination of heating and venting. The temperature set-points followed a sine-curve from the lowest temperature at 06:00 hr, ramping up to 30°C at 14:00 hr, and then ramping back down to lower temperatures throughout the night. This is to simulate a natural daily temperature curve typical of that in West Africa growing regions (Wood, 1985a). During the summer months internal temperatures increased under high levels of sunlight. When temperatures increased above the set-points, the venting system in the roof opened. Details of greenhouse temperatures can be found in Table 2.1.

Additional 50% shade screening was hung on all walls which were exposed to direct sunlight during the day. This included the short external walls of houses 5 and 6, and the long external walls of houses 3 and 6 (Figure 2.2). The shade screening was to reduce the impact of sunlight on the internal temperatures of the glasshouses, and to restrict the direct sunlight reaching the cocoa trees which are commonly grown in shaded conditions.

Table 2.1 Achieved greenhouse conditions
Greenhouse conditions across the 2 year study period. Instances of heater failure and anomalous recordings were not included in the calculation of maximum and minimum values. Mean calculations are inclusive of all data. Ambient CO_2 houses (house 3 and 5) also show a mean CO_2 value for between 6am and 6pm. This is to demonstrate the achieved CO_2 levels during the photosynthetically active periods of the day. (NB: CO_2 levels were higher in the evenings due to CO_2 release from plant respiration in the enclosed greenhouses).

		Temperature (°C)	Relative Humidity (%)	Carbon dioxide (ppm)
House 2	Mean	26.2	70.2	727
	Max	41.3	100	981
	Min	19.2	28.3	603
House 3	Mean	26.3	64.8	470 (446 6am-6pm)
	Max	43.3	100	600
	Min	19.2	18	306
House 5	Mean	26.5	78	457 (420 6am—6pm)
	Max	42.1	100	639
	Min	19.5	36.5	267
House 6	Mean	26.5	67	714
	Max	43.8	100	966
	Min	19.2	27.4	579

2.2.2 Lighting

A 12 hour day/night cycle was maintained during the experiment using the glasshouse supplementary lighting. Six 400w sodium lamps were used per house between 06:00 and 18:00 to extend the day length when external light levels fell below 8 kilolux (148 µmol m^{-2} s^{-1}). During the summer months, day length was longer than 12 hours due to natural UK day length. Shade screening fitted on tracks across the length of the house ceiling were opened and closed to manage excess light levels within the greenhouse (XLS Firebreak shade

screening, *AB Ludvig Svensson*, Kinna, Sweden). Cocoa originated in the under storey of evergreen rainforests (Toxopeus, 1985) and as a result photosynthesis saturates under low light levels. High light levels can be detrimental to cocoa growth (Baligar et al., 2005; Raja Harun and Hardwick, 1988) therefore the screens were closed above 35 kilolux (648 µmol m^{-2} s^{-1}). To reduce heat loss the screens were also closed at night.

2.2.3 Humidity

Humidity was recorded but not controlled using feedback sensors. Water misters were programmed to switch on at fixed times using a HERON MCI-96 timer system (see Irrigation 2.3) and switched on initially for 15 minutes at 05:30, 06:30, 08:30, 10:30, 12:30, 14:30, 16:30, 18:30 and 20:30 each day. Roof venting for temperature control had a large effect on humidity. During the winter months, humidity was well maintained as the roof vents rarely opened due to low external temperatures and light levels. However, in the summer months when external light levels increased, roof vents opened more frequently to reduce internal temperatures thus allowing humidity to escape. To reduce the impact of venting, misting was increased from 15 to 30 minutes of activation during such periods. Additionally, capillary matting was used to retain ground surface water. Water entering the mister system was pre-treated using an ultra violet disinfecting system (model SE2, lamp 550 T5C) to meet health and safety regulations. The mister pipework was also cleaned periodically by external contractors (*SMS Environmental Ltd*, Theale, Reading, UK). Details of humidity levels within each house can be seen in Table 2.1.

2.2.4 Carbon dioxide

Carbon dioxide (CO_2) levels were monitored using a wall-mounted infra-red gas analyser (IRGA) (MYCO_2 Gascard II, *Edinburgh Sensors*, Livingstone, UK) which fed data back to the T200 computer. In the elevated CO_2 houses, the exhaust gas from the greenhouse heater was diverted via an in-line fan through perforated plastic tubing running at ground level along the side walls of the house. The CO_2 fan activated after initial heater ignition to ensure only clean combustion gases were used. To improve the level of control over the CO_2 conditions, circulation fans (Thermotecnica Pericoli ACF 18, *Thermotecnica Pericoli SRL*, Albenga, Italy) were installed into all houses to promote movement of air and thus more accurate monitoring of CO_2 levels. As the heaters naturally produced heat during CO_2 elevation, a venting system was installed on the heat outlet of the boilers. When temperatures exceeded the desired set-points the vents directed heat out of the greenhouse. Expandable foam was used on the glasshouse facility partition walls to ensure the isolation of CO_2 and prevent leaking into ambient CO_2 houses.

CO_2 levels remained ambient within houses 3 and 5 (see Figure 2.2) however, as the greenhouses created an enclosed growing environment; plant respiration increased the levels of CO_2 during the night. Across the whole experimental period the average CO_2 levels for the ambient houses was 470 and 457ppm for house 3 and 5 respectively. However if this average is calculated between the hours of 06:00 and 18:00 only (day time only), these averages are lower at 446 for house 3 and 420ppm for house 5. CO_2 levels within house 2 and 6 were elevated to a set-point of 700ppm with a hysteresis value of 50ppm (when the CO_2 levels fell below 650ppm the CO_2 system would switch on until 750ppm was reached). As the trees became larger, CO_2 distribution became slower and the variation around the set point of 700ppm became greater. To tighten the level of control over the CO_2 levels the hysteresis was

reduced to 10ppm in February 2015 to counteract the slower feedback loop. Details of the CO_2 levels in each house can be seen in Table 2.1.

2.3 Irrigation

The irrigation system consisted of a 227L nitrate stock solution; 227L sulphate stock solution, an 80L acid stock solution, and a 227L mixer tank (see Table 2.2). The mixer tank was filled with water and maintained at capacity with a ball-cock valve. Conditions within the mixer tank were monitored using a pH controller and sensor (HANNAH pH controller, model HI8710 and HANNAH pH electrode, model HI1332P), and a conductivity meter and sensor (HANNAH meter, model HI943500 and HANNAH conductivity sensor, model HI7638). The solution was agitated by a water pump (Hozelock cascade 700 fountain and waterfall pump, *Hozelock Ltd*, Birmingham, UK) to ensure even solution mixing. Conductivity was maintained at 2.00ms and pH was maintained at pH5.7. When sensors detected a fall in conductivity or a rise in pH, dosing pumps (HANNAH dosing pumps, model: BL15) were activated to draw solutions from the relevant stock tanks into the mixing tank.

From the mixer tank, the diluted nutrient solution was delivered to the trees via a water pump (Jet 40 pump, *Stuart Turner Ltd*, Oxfordshire, UK) through solenoid valves (*Bermad UK Ltd*, Berkshire, UK), which were controlled by a HERON MCI-96 timer system (*Heron Electric*, West Sussex, UK). The irrigation pipework was comprised of 16mm PVC piping and compatible sized joints and junctions. At each tree, three lengths of 5mm tubing were connected to the larger pipework using 5mm insert nipple connectors. Netafim pressure compensated dripper valves (*Netafim Ltd*, Tel Aviv, Israel) were added to the 5mm tubing

and secured above the potting substrate with 5mm pipe clamp stakes. This resulted in three drippers supplied to each tree.

Table 2.2 Nutrient stock solution recipes
A Long Ashton nutrient solution which has been modified for use with cacao (End, 1990). Nitrate and sulphate solutions were made up to 227L with water. Acid solution was made up to 80L with water.

Nitrate solution:	
Potassium Nitrate (KNO_3)	6048 g
Ammonium Nitrate ($NH4NO_3$)	5.5 L
Sulphate solution:	
Potassium Sulphate (K_2SO_4)	1680 g
Magnesium Sulphate ($MgSO_4$)	3312 g
Potassium dihydrogen phosphate (KH_2PO_4)	2112 g
EDTA	480 g
Nitric acid	500 ml
Boric acid (H_3BO_3)	120 g
Manganous sulphate ($MnSO_4$)	68 g
Zinc sulphate	324 g
Ammonium molybdate	10.4 g
Copper sulphate	9.6 g
Acid solution	
Nitric acid	2.5 L
Orthophosphoric acid	1.25 L

There were two watering treatments; well-watered (WW) and water-stressed (WS). Each treatment had a separate circuit of pipework and valves connecting it to the irrigation system. The WW treatment provided trees with water for 7 minutes at 8:00, 10:00, 12:00, 14:00, 16:00 and 18:00 each day. The set-point of 7 minutes was used as this volume of water was sufficient to obtain run-off from the base of the pots. This prevented the build-up of nutrient in the pot substrate (Savvas et al., 2007). The irrigation duration was later increased to 12 minutes as the trees became larger and their water usage increased.

The ‘WS’ treatment maintained the trees in a state of water-stress without causing wilt. The percentage moisture level within the pot was determined by a profile moisture probe (AT

Delta-T Devices profile probe, model PR2, *Delta-T Devices Ltd*, Cambridge, UK). An initial method was devised to maintain water stress conditions (see water stress method 1) however a more practical method was devised (see water stress method 2). As the trees grew larger and their water usage increased, method 2 became impractical and a further method was devised (see water stress method 3) and used for the remainder of the experiment.

2.3.1 Water stress method 1: (02.09.13 – 12.09.13)

For this method, pot weight was used as a measure of water loss. To reduce the time needed to maintain the water treatment, a representative sample of trees was selected to be weighed every other day to monitor water loss. First, a representative sample of four trees per house (2 "small" and 2 "large") was selected (see 2.4 Cocoa Genotypes for tree sizing). Every other day the sample trees were weighed and the soil moisture recorded using digital scales (KERN scales, model DE150K20D *KERN & SOHN*, Balingen, Germany), and the PR2 moisture probe. The new weight was subtracted from the previous weight for each tree and the water usage was calculated (1g of weight loss = 1ml of water usage). The water usage for "small" trees was averaged. The same was calculated for all of the "large" trees. The difference in water usage between "small" and "large" trees was added back into all "large" trees. This theoretically raised the moisture level of the large trees up to that of the "small" trees and allowed all the trees to dehydrate at the same rate. This continued until an average probe reading of 15% pot moisture had been reached. From this point, the average water usage for small trees was added back to all small trees to maintain 15% moisture content. The same method was applied to all large trees. It soon became apparent that there was large variation in tree water use within tree size groups. Use of a representative sample was not able to maintain all trees at a specific moisture level. Trees with a higher water use rate began to wilt

and lose leaves. The water treatment was stopped and irrigation was set to ‘WW’ for all trees to allow for recovery. Weighing every tree for individual water use measurements was not practical; therefore a new method for the water stress treatment was devised.

2.3.2 Water stress method 2: (17.09.13 – 08.07.14)

From the data which had already been collected, the average water loss per day and the average drop in moisture % per day could be correlated. From this correlation, it was calculated that a drop of 1% in soil moisture equates to approximately 500ml of water loss (Figure 2.3). Using this result as a model, a new method of maintaining a 15% pot moisture level was devised.

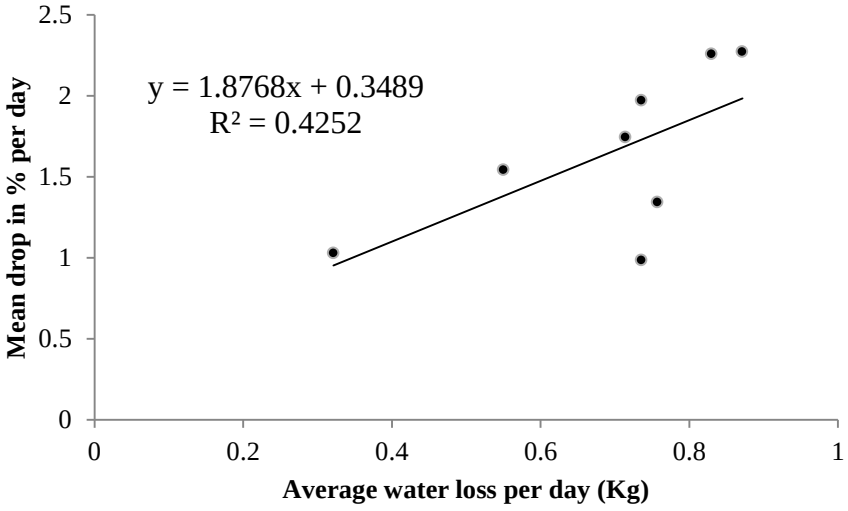

Figure 2.3 Correlation of reduction in soil moisture percentage with reduction in weight (1g = 1ml water)
Using the data collected in water stress method 1 a linear regression analysis was calculated. It was then calculated that a reduction in 1% equates to 533ml. This was rounded to 500ml for the purposes of the study. Figure and data also appears in (Lahive, 2015).

All of the ‘WS’ trees were monitored with the moisture probe every other day. The values from all ‘WS’ trees in all houses were used to calculate a mean, a minimum and a maximum. Any tree with a moisture level below the average value was watered precisely to bring its

moisture level up to the average point (see Figure 2.4). From the previous data collected it was known that 500ml approximately raised the pot moisture by 1%. Therefore to calculate a precise re-watering value, the difference in percentage from the mean value (ΔSM) was multiplied by 500 to give a volume in millilitres to be added back to the pot. This water volume was weighed on electronic scales (Adam equipment scales, model CBK 32, *Adam Equipment Inc*, CT, USA), and added to each pot. As this was calculated on a pot-by-pot basis the chance of a tree reaching wilting point was greatly reduced. The desired end point was a moisture level between 10-15%. Once the average moisture across all 'WS' trees dropped below 15%, the average percentage value (used above) was substituted for a fixed percentage of 15% when calculating ΔSM. Once the 15% average had been reached the trees were maintained at this level of dehydration for 2 weeks. After this time they were re-watered and saturated. This cycle can be seen in Figure 2.5. Upon saturation, run-off from the base of the pots was collected and the conductivity was measured using the conductivity sensor from the irrigation mixer tank. It was assessed whether nutrient build up had occurred in the pots which can happen when the irrigation frequency is low (Savvas et al., 2007). If the conductivity readings were considerably higher than 2.0ms after pot saturation, a larger volume of water was applied to create a high volume of run-through to remove the excess nutrients in the potting mix. After this point the dehydration process was resumed again.

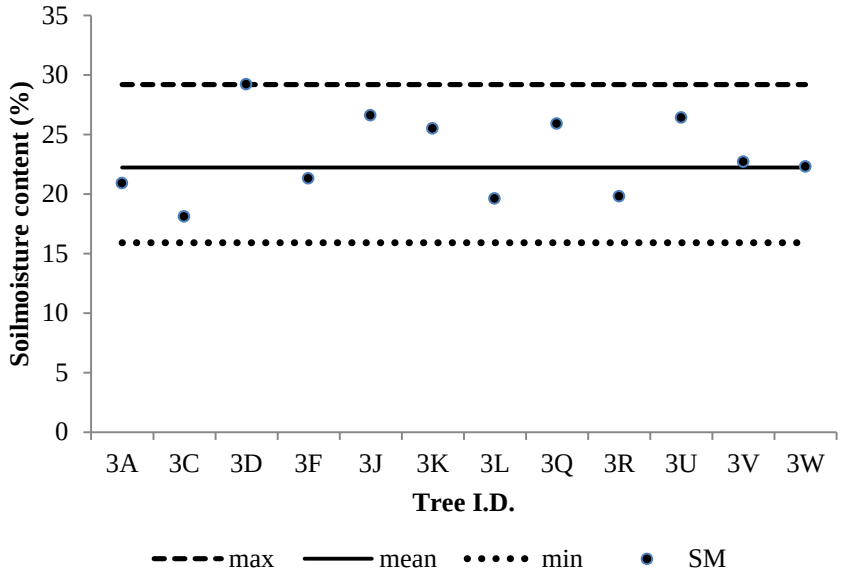

Figure 2.4 Soil moisture levels for water-stressed trees in house 3 on 13.11.13
The percentage soil moisture level for an individual tree (sm) was plotted with the mean, minimum and maximum values across all WS trees in all houses. Note only trees within house 3 are shown here.

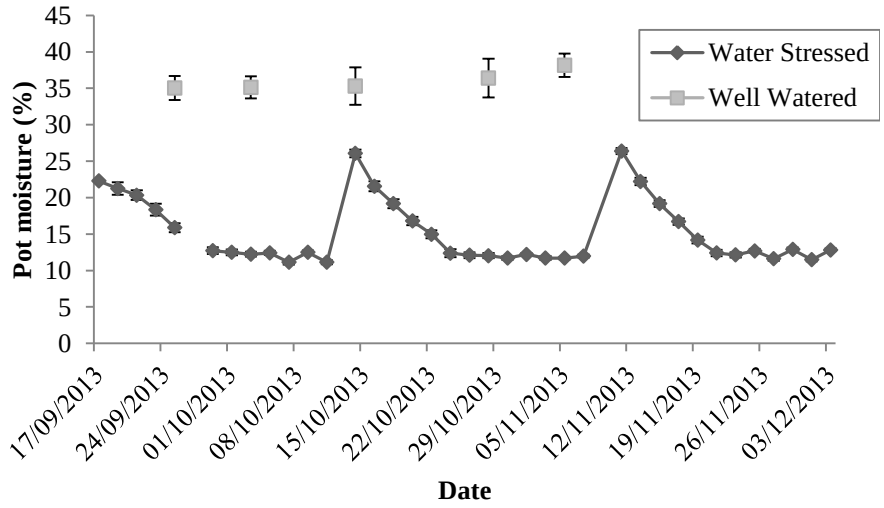

Figure 2.5 Mean water percentage for all trees between 17.09.13 and 03.12.13
Well-watered trees remain at a high moisture level (average of 36.3% across the study). Water stressed trees follow a cycle of dehydration, maintained water content, and re-water.

As the trees became larger and their water usage increased, soil moisture content began to fall below 10% moisture levels within the 48 hour period between watering. In an attempt to reduce risk of leaf loss and minimise the time required to maintain the water-stress, the upper limit of the desired dehydration percentage was increased from 15% to 17%. As a result, all

trees were watered to 17%, thus reducing the risk of the percentage falling below 10%. Water usage continued to increase as trees grew larger and the light levels and temperatures increased in the summer months. The time required to weigh the volumes of water for each tree became impractical and a final revision of a water-stress method was created. On the 9th July 2014 method 3 began.

2.3.3 Water stress method 3: (09.07.14 – 18.07.15)

Each tree had access to three drippers connected to the irrigation system. Watering method 3 used the drippers to supply a small dose of water regularly throughout the day. Following a re-water, all drippers were removed from each pot until that tree had reached soil moisture of between 10-17%. From this point onwards the drippers were added back to the pot to maintain the water content within this bracket. The number of drippers in the pot was then altered every 48 hours to meet the rate of water use for that tree. A benefit of this method was that the percentage measured was a true representation of the moisture level of the pot rather than a measure of the lowest level of moisture. The pot moisture level became much more stable as the water supply was not added in one large quantity every 48 hours as in other water-stress methods. As this method continued it became possible to predict water usage of trees and their dripper requirements based on previous records.

2.4 Cocoa Genotypes

Six genotypes of *Theobroma cacao* L. were selected on the basis that they display contrasting physiological features (Table 2.3) (Daymond et al., 2011).

Table 2.3 Cocoa genotypes
Genetic material selected for use in this experiment. Pod index indicates the number of pods required to obtain 1kg of dried beans. All information sourced from (Turnbull and Hadley, 2015)

Genotype	Origin	Compatibility	Notes
CL19/10	Ecuador	Inconclusive	Shows moderate resistance to frosty pod rot. Pod index: 35
ICS 1	Trinidad	Inconclusive	Varied resistance/susceptibility to disease. Used as parent for breeding. Pod index: 19-24
IMC 47	Peru	Self-incompatible	Resistance shown to witches broom. Pod index: 22-27
POUND 7/B	Peru	Inconclusive	Pod index: 19 Often used as a parent
SCA 6	Peru	Self-incompatible	Largely resistant to witches broom. Susceptible to frosty pod rot. Used as parent for breeding. Pod index 35-52
SPEC 54/1	Colombia	Self-compatible	

Where possible there were four replicates of each genotype in each house (two well-watered (WW) and two water-stressed (WS)). However, due to some limitation on tree availability, in some instances there were only 3 replicates of a genotype in a given house (see Table 2.4). The trees were grafted between July 2009 and October 2012. For the purpose of treatment allocation trees were categorised as either “small” or “large” in size initially by visual estimation. This was then followed up by quantifying side stems and categorising based on “small” trees having 0-20 side stems and “large” trees having greater than 20 side stems. Most of these calculations matched the visual estimate; however in the instance of the visual

estimation not matching the size based on branch number, a final decision was made based on an additional visual estimation of overall tree size. From here it was ensured that relatively equal quantities of "large" and "small" trees were allocated to each treatment to give representative samples. The trees were given a unique ID code and their location was distributed evenly in the houses. Note: as the experiment progressed all trees developed into "large" trees and the sizing became irrelevant.

2.5 Tree Husbandry

Trees were provided for the project from the International Cocoa Quarantine Centre, University of Reading, UK. Using genetic material from the quarantine centre, patch budding was carried out onto seedlings of genetically distinct 'GU' clones (originating in French Guiana; (Lachenaud et al., 2007)) To eliminate root variations, all trees were grafted onto the same root stock. All grafting was carried out between July 2009 and October 2012. The trees were grown in a mixture of sand, gravel and vermiculite (1:2:2) and watered using a Long Ashton nutrient solution which has been modified for use with cacao (End, 1990). The potting mixture contained no nutritional value. All trees were potted into 50ltr pots in June and July 2013 (see Figure 2.6).

Initially trees were not pruned as to minimise any interference with tree growth and experimental effects. However, as trees became larger pruning was required to reduce glasshouse overcrowding. As a general rule, each tree was permitted up to 3 main plagiotropic branches. Lateral growth was removed around the base of the tree allowing access to the lower level woody material (required for monitoring of flowering and pod growth). Additionally, chupon development from the base of the tree was also removed. Lateral growth higher in the tree canopy was supported using bamboo canes and twine. Trees

were permitted to reach a height of 3m (the height at which the trees would reach the shade screening). Above this height the branches were pruned to prevent further vertical growth. Weekly checks for pests, diseases and or signs of nutrient deficiency were conducted. Pests and nutrient deficiencies and the respective applied treatments are documented in Table 2.5. Any pesticide applications were applied using a fogger (DYNA-FOG Tornado, model 2897. Curtis Dyna-FOG Ltd, Westfield, Indiana, USA).

Figure 2.6 Trees in situ

Table 2.4 Genotype availability

Figure displays the total number of replicates available for each genotype. Note: houses 2 and 6 share the same experimental conditions and houses 3 and 5 share the same conditions.

House 2

Genotype	WW	WS	Total
CL19/10	2	2	4
ICS 1	2	2	4
IMC 47	2	2	4
POUND 7/B	2	2	4
SPEC 54/1	2	2	4
SCA 6	2	2	4
Total	**12**	**12**	**24**

House 3

Genotype	WW	WS	Total
CL19/10	2	2	4
ICS 1	2	2	4
IMC 47	2	2	4
POUND 7/B	2	2	4
SPEC 54/1	1	2	3
SCA 6	2	2	4
Total	**11**	**12**	**23**

House 5

Genotype	WW	WS	Total
CL19/10	1	2	3
ICS 1	2	2	4
IMC 47	2	2	4
POUND 7/B	2	2	4
SPEC 54/1	2	2	4
SCA 6	2	2	4
Total	**11**	**12**	**23**

House 6

Genotype	WW	WS	Total
CL19/10	2	1	3
ICS 1	2	2	4
IMC 47	2	2	4
POUND 7/B	2	2	4
SPEC 54/1	2	2	4
SCA 6	2	2	4
Total	**12**	**11**	**23**

Table 2.5 Pest and deficiency management
Pest and deficiencies encountered during this study and the methods used as treatment.

Pest/Deficiency	Treatment	Frequency
Red spider mite	Abamectin (Dynamec) - 5ml in 10ltr water dilution	Application every 1-2 months.
Aphid	Thiacloprid (Calypso) – 7.5ml in 10ltr water dilution	Two applications in January 2015 only.
Iron deficiency/chlorosis	2250g EDTA diluted in 227ltr acidified water (see section 2.3 for pH) added as a pot-rinse. Standing time of 3-4 hours followed by a rinse with water acidified to pH 5.7 with the acid solution described in Table 2.2.	Only two applications throughout the experiment on 30/01/2015 and 22/05/2015.

Chapter 3. The effects of elevated CO_2 and water stress on aspects of cacao pollen viability

3.1 Introduction

With rainfall patterns expected to change and atmospheric carbon dioxide (CO_2) levels increasing (IPCC, 2014), it is important to understand the effects such changes can have on crops of commercial and economical importance such as cocoa. Water stress during pollen development, specifically during meiosis, has been demonstrated to result in pollen sterility in wheat (Saini and Aspinall, 1981). A later review by Saini (1997) discussed observations by Morgan (1980), in that the water potential of the reproductive organs was preserved at a high level even when leaf turgor was reduced under water stress. This paper suggested that pollen sterility observed from water stress was induced by signalling from the leaves, potentially as a result of reduced sugar transport from lower rates of photosynthesis. Koonjul et al. (2005) suggested that reductions in sugar flux to the reproductive organs may be the signal for observed down regulation of anther invertase after a period of water stress during meiosis. Anther invertase is responsible for the accumulation of starch in the developing pollen grains. Starch stores are essential to support pollen germination and tube growth. Therefore, a reduction in expression of the invertase leads to the reduction of pollen viability.

The effect of increased levels of CO_2 on the rates of photosynthesis in C_3 plants is well documented (Ainsworth and Long, 2005). Generally, growth and biomass increases as a result of increases in photosynthetic rate. As an example, woody material, root and vegetative growth were all seen to increase in Sour Orange in response to elevated CO_2 conditions (Kimball et al., 2007). Pollen has been shown to be effected by elevated CO_2 both in development and post germination. For example, during pollen development, heat stress has been shown to result in reduced germination of pollen grains in bell pepper (Aloni et al.,

2001). However, Aloni et al. also demonstrated that elevated levels of CO_2 (800µmol mol^{-1} air) alleviated the impacts of high temperatures and restored germination levels to those observed under normal temperature conditions. The authors suggest that elevated CO_2 increases assimilate availability and as a result counter-act the detrimental effect that high temperatures have on sucrose and starch metabolism. It should be noted, however, that similar results were not found for kidney bean when exposed to high temperatures and elevated CO_2 (Vara Prasad et al., 2002) suggesting contrasting responses depending on the species involved. Effects of elevated CO_2 have also been seen on the quantity of pollen produced in ragweed (Rogers et al., 2006). The study aimed to identify the effects of growing season length and elevated CO_2 on allergenic pollen production. Increases in pollen quantity of 32% and 55% were observed in mid and late season crops respectively when grown at 700ppm CO_2. The ragweed which was grown at elevated CO_2 had increased biomass, inflorescence weight and number. The larger biomass in combination with a reduced effect of growing season length is suggestive of increased growth and assimilation rates, all of which appeared to contribute to an increased flower and pollen production.

Additional effects of CO_2 have also been observed on pollen performance. During pollen germination it is suggested that CO_2 concentration within the style may be used to regulate germination and tube growth (Sfakiotakis et al., 1972). Lily pollen was germinated *in vitro* in CO_2 concentrations between 0.03% (atmospheric) and 5%. Germination increased up to around 1.3% CO_2, beyond which point no further increase in germination was achieved. Furthermore, CO_2 levels within the style were found to be 1.59% which corresponded to the optimum levels observed for *in vitro* germination. In *T. cacao*, high levels of CO_2 has been observed to increase the percentage of fruit set from *in vivo* pollen germination (Aneja et al., 1992). If flowers were stored in vials, and CO_2 levels allowed to increase (final concentration of 85,000ppm), pollen from the flowers resulted in 100% fruit set when used in manual

pollinations of cacao flowers. Furthermore, pollen did not germinate *in vitro* unless the flowers were also stored in vials under increased CO_2. Later work by Aneja et al. (1994) also observed that the same pre-treatment of pollen prior to pollination can also overcome the early stages of self-incompatibility in cacao.

It has been suggested by some authors that pollination may be a limiting factor in the yield of cocoa (Groeneveld et al., 2010). Reduced pollen viability through water stress during pollen development, may reduce pollination success and have detrimental effects on yields. However, there is the potential that conditions of elevated atmospheric CO_2 may help alleviate the negative impacts induced by water stress by increasing the levels of assimilates available for pollen grain development. Furthermore, water use efficiency (WUE) is known to increase under elevated CO_2 (Lahive, 2015). Improved WUE could reduce the impacts of water stress on the tree, in-turn reducing the potential negative impacts of water stress on pollen viability.

The aim of this study was to evaluate the effects of water stress and elevated CO_2 on the pollen viability of six cocoa genotypes. The effects of water deficit stress and elevated CO_2 concentrations were recorded on the germination rates of pollen *in vitro*, and the lengths of developed pollen tubes.

3.2 Materials and methods

As previously outlined in Chapter 2, trees of six cocoa genotypes (CL19/10, ICS 1, IMC 47, Pound 7/B, SCA 6, SPEC 54/1) were grown under ambient (averaging 437ppm) and elevated (700ppm) CO_2 for 23 months in two replicated glasshouse compartments per treatment. Each compartment also contained a well-watered (WW) and a water-stressed (WS) treatment. Therefore, in total there were 4 treatments:

- Ambient CO_2 – 'WW'
- Ambient CO_2 – 'WS'
- High CO_2 – 'WW'
- High CO_2 – 'WS'

Prior to the start of the main pollen study, a 10% sucrose germination medium (Shivanna and Rangaswamy, 1992) was made and autoclaved at 121°C to sterilise the medium (small amounts of fungal contamination had been observed in prior experiments).

It should be noted that the treatment period was during the development of the pollen grains. Germination and pollen tube growth occurred *in vitro* under standard laboratory conditions at ambient levels of CO_2. The first replicate of the study began on 13th October 2013 and lasted for 7 days. The following protocol was used:

- First day: Old, and open flowers were removed from all trees from the first genotype.
- Second day: Newly opened flowers were collected from the first genotype and pollen was sampled in the morning. Prepared samples were placed in an incubator (model: WE52B5 – 959, Sanyo Electric Co. Ltd., Moriguchi, Japan) at 30°C and 85% RH for 24 hours. In the afternoon, old flowers from the second genotype were removed.
- Third day: Newly opened flowers from trees of the second genotype were collected, pollen was sampled and then placed in the incubator. The previous days pollen samples from the first genotype were then removed from the incubator (after 24 hours) and analysed under an 'Axioskop 2 MAT' microscope (Carl Zeiss, Light Microscopy, Göttingen, Germany). Old flowers from trees of the third genotype were then removed.

The cycle continued until all genotypes had been sampled and all pollen images had been analysed under the microscope. Genotypes were sampled in alphabetical order. The study

was repeated 3 times. Firstly starting on 13th October 2013, then on the 14th and 20th of January 2014.

3.2.1 Flower sampling

As new cocoa flowers open each day, and pollen only remains viable for up to 24 hours (Toxopeus, 1985), it was essential that only fresh flowers were sampled. Old, open flowers were removed from the trees on the day prior to pollen sampling to ensure that only newly opened flowers were collected. There was a maximum of 4 trees per genotype, per treatment across 2 houses. This equated to two trees of the same genotype and treatment in each house. Five flowers from each tree were collected and pooled with the flowers from the tree of the same treatment and genotype in that house. If five flowers were not available from one tree, an additional five were sampled from the equivalent tree in that house.

3.2.2 Pollen preparation and incubation

Once flowers had been collected they were transported in petri dishes in an insulated box to protect the pollen from temperatures external to the glasshouse. Pollen extraction and slide preparation was carried out in a laminar flow cabinet which had been cleaned with 70% ethanol. All tools were also cleaned with 70% ethanol and placed in a hot bead steriliser (Steri 250, Simon Keller AG, Burgdorf, Switzerland). Pollen was brushed from the flower onto a glass microscope slide using a horse-hair paintbrush. Five flowers were brushed onto 1 slide. Pollen was brushed to the centre of the slide and 30µl of the germination medium was added. Using a needle/pointed tool the drop was stirred to incorporate the pollen. The slide was placed on top of two additional glass slides and placed into a labelled petri dish lined

with moist filter paper. The petri dish was sealed with parafilm. Assuming sufficient flower numbers, this method resulted in 2 slides of pollen for each genotype, of each treatment in each house.

All prepared pollen slides were placed in a Sanyo environmental test chamber (model: WE52B5 – 959, Sanyo Electric Co. Ltd., Moriguchi, Japan). Conditions in the chamber were set to 30°C and humidity was set to 85% RH. Lighting was continuous and set to give an mean of 1134µmol s^{-1} m^{-2}. The dishes were left in the chamber for 24 hours after which pollen development was stopped by adding 20µl 70% ethanol.

3.2.3 Microscope and image analysis

The slides were viewed using an 'Axioskop 2 MAT' microscope (Carl Zeiss, Light Microscopy, Göttingen, Germany) at 20x magnification. Three representative locations on the slide were selected for image capture. A scale bar was added for later measurement of pollen tube length.

The microscope images were analysed using 'Image J' software (Schneider et al., 2012). The scale bar was used to calibrate the measurement tool. Germinated and ungerminated pollen grains were counted. Pollen was considered germinated if the pollen tube length was greater than the diameter of the pollen grain. The line drawing tool in Image J was used to trace along the length of all visible pollen grains and the calibrated length was recorded. It should be noted that whilst it was possible to count all germinated pollen grains, not all pollen tubes were clear enough to measure. Number of pollen tubes measured on a single slide image ranged from 1 to 28 depending on number of pollen tubes present and number of pollen tubes clear enough for measurement. For the calculation of percentage germination, the data from

each slide image was combined for each genotype in each repeat. The percentage value was then averaged for each treatment to give a combined percentage across all genotypes.

3.2.4 Statistical analysis

Pollen tube length and germination percentage was analysed using GenStat 15th edition statistical software (GenStat, VSN International Ltd., Hemel Hempstead, UK) using an unbalanced design analysis of variance (ANOVA). The effect of treatments (CO_2 and water stress) were analysed using all data across all genotypes. Where the effect of genotype significantly interacted with treatment, genotypes were then analysed individually using a further unbalanced design ANOVA. Two block designs were created to test for any spatial effect between greenhouses.

3.3 Results

3.3.1 Percentage germination

There were no significant effects of either treatment ($P > 0.05$), and no significant effect of genotype ($P > 0.05$) on percentage germination. The mean percentage across all genotypes for each treatment is shown in Figure 3.1. There were no significant interactions between treatments and/or treatment and genotype. There was also no significant block effect of greenhouse location.

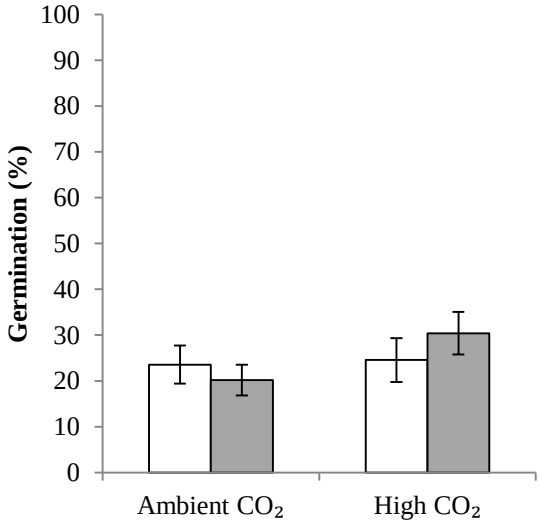

Figure 3.1 Percentage pollen germination from trees grown at two concentrations of CO_2 and two watering treatments. Data are combined across six genotypes.
□ - Well-watered; ▒ - Water-stressed. Error bars show standard error of the mean.

3.3.2 Pollen tube length

Pollen tube length data was initially averaged across all genotypes per treatment (see Figure 3.2). Tube length was significantly reduced under the high CO_2 treatment ($P < 0.01$), however, there was no significant effect of water treatment ($P > 0.05$). There was a significant effect of genotype ($P < 0.001$) and a significant interaction between water treatment and genotype ($P < 0.01$). The trend for a reduced tube length under high CO_2 was significant for genotypes CL19/10 ($P < 0.01$), ICS 1 ($P < 0.05$), and SCA 6 ($P < 0.01$)

(Figure 3.3). The water stress treatment significantly increased tube length for the genotypes IMC 47 ($P < 0.05$) and Pound 7/B ($P < 0.01$), however the effect of water stress was not significant for the remaining genotypes. There were no significant interactions between water treatment and CO_2 treatment. The genotype SPEC 54/1 did not provide sufficient numbers of flowers for pollen analysis and was therefore not included in this analysis. Overall, the genotype Pound 7/B had the longest mean pollen tubes at 359.15µm, whilst SCA 6 had the shortest mean of 140.23µm. There was also no significant block effect of greenhouse location.

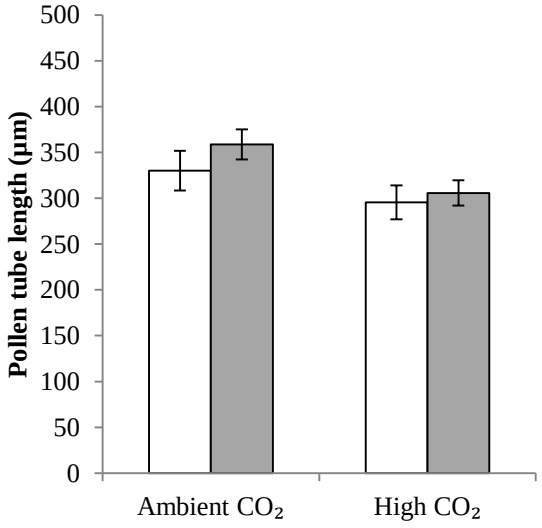

Figure 3.2 Mean pollen tube length of pollen from trees grown at two concentrations of CO_2 and two watering treatments. Data are combined across six genotypes.
□ - Well-watered; ▒ - Water-stressed. Error bars show standard error of the mean.

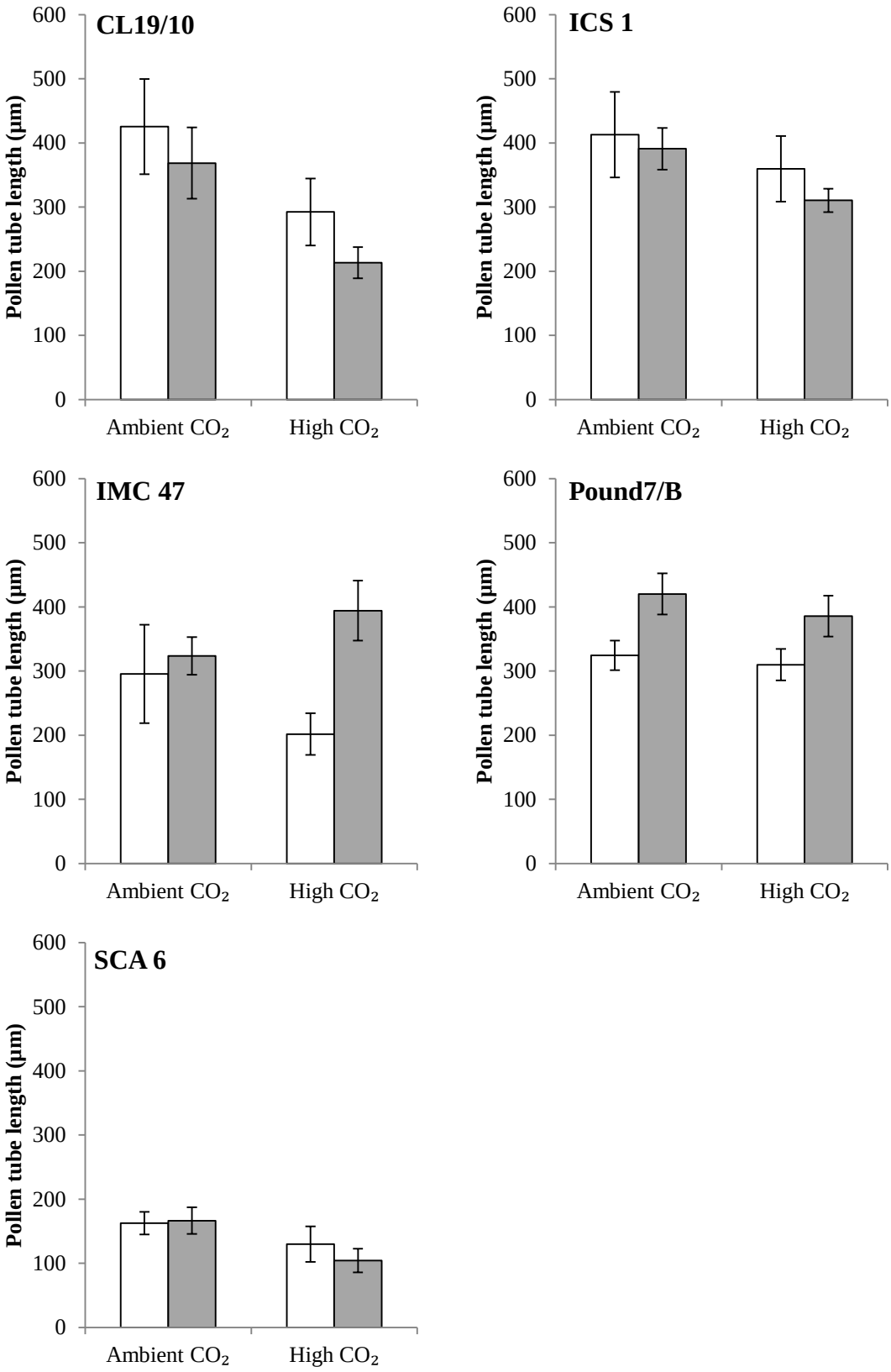

Figure 3.3 Mean pollen tube length of pollen from six cacao genotypes grown at two concentrations of CO_2 and two watering treatments.
□ - Well-watered; ▒ - Water-stressed. Error bars show standard error of the mean.

3.4 Discussion

The results presented here demonstrate no significant response of germination percentage to the treatments imposed. As discussed by Saini (1997), the sugar availability to anthers in pollen development is likely to have an important role in invertase and sucrose synthase gene expression. Invertase and sucrose synthase expression can be increased or decreased in response to sucrose levels (Xu et al., 1996). Under high CO_2, sucrose levels may be higher due an increase in photosynthetic rate. Improved sucrose metabolism in the anthers could improve pollen development and reduce the risk of pollen sterility. This was not the case in this study, suggesting the increased assimilate availability did not affect pollen development. With regards to the effects of water stress, the meiosis developmental stage of a pollen grain is identified as the most sensitive process to water stress resulting in pollen grain sterility (Saini, 1997; Salter and Goode, 1967). No effect of water stress was observed on germination in this study. The pollen of *T. cacao* takes approximately 30 days to develop (Toxopeus, 1985), with meiosis occurring in the early stages of development. The water stress treatment in this study operated on a cycle involving a re-water every 2 weeks. To more accurately study the effects of water stress on pollen development, the mitotic developmental stage of cacao would need to be precisely identified within the 30 day developmental period. The water stress treatment could then be coordinated with this developmental stage to study the effects.

Pollen tube length was monitored in this study as a measure of pollen grain health. This is with the understanding that a longer pollen tube is more likely to be successful in reaching the ovule resulting in fertilisation. Data across all genotypes in this study showed a significant decrease in pollen tube length from pollen grains which had developed under the high CO_2 treatment. Higher rates of photosynthesis in high CO_2 conditions (Lahive, 2015; Baligar et al., 2008) would have increased the photosynthate production within the trees. It

appears here that the increased photosynthate did not provide any developmental advantage to the developing pollen grains. The decrease in tube lengths imply a reduction in pollen grain health, suggesting a decrease in resources partitioned to pollen development. The trees which were kept under the high CO_2 treatment developed a significantly larger tree biomass, attributable to increased woody development (Chapter 7). The increased vegetative growth may have competed with the floral/pollen development for resources. The priority of vegetative growth over reproductive growth is also demonstrated in cacao through the increased incidence of cherelle wilt during periods of leaf flushing (Alvim, 1954). The significant trend for reduced pollen tube length under high CO_2 is present in the genotypes CL19/10, ICS 1, and SCA 6. However, the remaining genotypes IMC 47 and Pound 7/B demonstrate longer tube lengths under the water stress treatment. As discussed previously, water stress is documented as causing pollen sterility in many crop species (Saini, 1997; Salter and Goode, 1967). Furthermore, through a reduction in stored starch in the pollen grain, it is expected that pollen tube growth would be reduced by water stress (Koonjul et al., 2005). Therefore the observed positive effect seen here in pollen tube length contradicts what may be expected under water stress conditions. The trees maintained in the water stress treatment demonstrated significant reductions in overall tree biomass, including leaf weight, leaf area and woody growth (Chapter 7). With these significant reductions in vegetative growth, the resource competition may have been greatly reduced. Although overall photosynthesis and thus assimilate production was also reduced under water stress (Lahive, 2015), the reduced vegetative sink competition may have provided a developmental advantage to pollen grain development, resulting in the increases in pollen tube length seen here.

Although germination rates were unaffected here, the pollen tube data showed significant genetic variation in response to the two treatments. It is suggested that pollination success

may be a strong limiting factor on yield of cocoa (Groeneveld et al., 2010). The genetic variability observed here holds potential for breeding cacao varieties which will maintain and improve yields of cacao through the improvement of pollen performance under climate conditions of elevated CO_2 and water stress.

Future research into the effects of climate change on the development of cacao pollen may need to assess the merits of handling the pollen in a sterile, but dry and cool laminar flow environment, versus the warm, humid and potentially contaminating environment of the greenhouses. Additionally, previous research has reported that cacao pollen does not germinate well in liquid medium (Cheesman, 1927) despite this being the method of choice here. As an alternative, cacao pollen is suggested to germinate best on 1.5 to 2% agar with 5% cane sugar (Cheesman, 1927). A limitation of the liquid medium is the visualisation of the pollen under the microscope, as the tubes may project in and out of focus. It is possible that agar may present clearer images for analysis as the surface is flat.

3.5 Conclusions

Overall, pollen germination was unaffected, however pollen tube growth varied significantly in response to treatment. The combined data revealed a significant reduction in tube length in response to elevated CO_2, potentially due to CO_2-stimulated woody growth diverting resources away from pollen development. The effect of CO_2 was observed in some genotypes but not others. Although no effect of water stress was observed overall, two genotypes demonstrated significantly increased pollen tube growth in response to this treatment. This may be an effect of a reduced vegetative sink under water stressed conditions, freeing assimilates for pollen development.

Chapter 4. The effects of elevated CO_2 and water stress on the flowering intensity of cacao

4.1 Introductions

Theobroma cacao is cauliflorous, in that its flowers develop from meristematic tissues located on the mature trunk and woody branches of the tree. Flowering occurs at the site of abscised leaves and over time the point of flowering thickens and is commonly referred to as a flower 'cushion' (Toxopeus, 1985; Carr and Lockwood, 2011). Cocoa flowers are small (approximately 15mm diameter) and numerous on the tree. As many as 120,000 flowers can be produced in a year on one tree with only a very small proportion (0.5 - 5%) being successfully pollinated and reaching pod maturity (Carr and Lockwood, 2011). In a detailed study of the floral development of *T. cacao*, Swanson et al. (2008) studied the development of 65 flowers from 6 different flower clusters from genetically identical trees. Flower development was observed using a combination of time-lapse photography, light and electron microscopy from initial meristem development (day 1) to flower abscission (day 31). All flowers began to open around 6pm on the 29th day of development and were fully open by around 7am the following day. Additional findings in Swanson et al. (2008) revealed that flower opening is highly synchronised amongst other flowers with 7 out of 8 flowers observed on different flower clusters beginning to open in the same hour (6pm-7pm). The speed of the flower opening varied during the 12 hour opening period. Swanson et al. suggest that this demonstrates mechanisms within the plant which control the rate of flower opening. The flower cushion, once formed, produces flowers throughout the tree's lifetime (Carr and Lockwood, 2011) and flower production fluctuates across the year depending on the genotype and the local climatic conditions (Toxopeus, 1985).

Borchert (1983) distinguishes between the processes of floral initiation and floral anthesis/emergence. In many herbaceous plant species, floral initiation is controlled endogenously and development is a continuous process from initiation to anthesis (sylleptic flowering). However, in many tree species, floral initiation occurs first and is endogenously controlled, followed by anthesis which is triggered by environmental cues. Initiation and anthesis can also be separated by a dormancy period (proleptic flowering). For many tropical trees, and also in the case of cocoa, flowering has been associated with leaf fall and dry seasons (Alvim and Alvim, 1977), however the endogenous trigger for floral induction is unknown (Borchert, 1983). Swanson et al. (2008) observed that cocoa flowers open consistently after 29 days of development, suggesting there is no delay between initiation and anthesis in cocoa. As flowering in cocoa is seen to fluctuate with seasonal climate, floral initiation may be responding to environmental conditions. For clarity, the term 'flowering' will be used in this study to describe the opening and anthesis of flowers unless stated otherwise.

Control of flowering is often attributed to photoperiod or temperature as seen in many temperate, herbaceous plants and crops (Borchert, 1983). Temperature varies across different cocoa growing regions, for example, in Brazil average minimum monthly temperature varies across the season whereas temperature in regions such as Malaysia are fairly constant (Wood, 1985a). However, day length in the humid tropics is more stable across the year. As a result, the seasons are divided into wet and dry to reflect rainfall patterns (Kricher, 2011b). The relationship between flowering and water availability in the tropics has previously been investigated. Irrigation experiments by Alvim (1960) demonstrate that water stress is required by coffee plants before flower buds can emerge. Continuous irrigation resulted in no flowering at all, however heavy flowering was observed on irrigated trees which had previously been water stressed. Further research by Masarirambi et al. (2009) has suggested

that using irrigation to synchronise the flowering of coffee could have beneficial applications in reducing the labour required to harvest the crop and maximising crop quality. An increase in the flowering and yield of litchi in Israel was also observed when the trees were subjected to water stress (Stern et al., 1993).

In cocoa, changes in flowering intensity have been associated with temperature (Sale, 1969), fruit and flower assimilate competition (Alvim, 1977), humidity (Sale, 1970a) and predominantly water availability and rainfall patterns (Alvim, 1977; Alvim and Alvim, 1977; Sale, 1970b). Regarding temperature, cocoa showed reduced flowering when maintained at a day temperature of 23.3°C as opposed to trees which were exposed to temperatures of 26.7°C or 30°C which showed larger flower numbers, more flower cushions and more flowers per cushion (Sale, 1969). Periods of minimum levels of flowering coincide with periods of maximum pod load (Glendinning, 1972). It has been suggested that this is due to a sink competition between flowers and fruit for photosynthates (Alvim, 1977). This theory was further extended with the observation that older trees develop much larger numbers of flowers in periods of heavy flowering than younger trees. Also, when flower emergence is low, the rate of flower emergence is much lower in older trees. Older trees show greater variation in flower emergence. This may be due to younger plants receiving greater amounts of solar energy per unit area or older plants having a greater proportion of non-photosynthetic material such as trunk and roots which acts as a sink for photosynthates, reducing the resources available for floral development in times of abiotic stress. Alvim (1977) states that competition with fruits for assimilates are a major factor affecting floral inhibition. However, environmental factors can have an influence but only when pod load is minimal. In addition, Sale (1970a) observed profuse flowering when trees grown in growth rooms were moved from conditions of low (50-60%) to medium or high (70-80% or 90-95%) relative humidities.

The environmental factor which is accepted as having the greatest control over flowering in cocoa is soil water availability. Alvim and Alvim (1977) observed that trees in the field flowered more when rainfall followed a period of drought. Sale (1970b) conducted a glasshouse study and observed decreased flowering of trees under a dry soil treatment and 'profuse' flowering when trees were transferred from dry soil moisture to a wet soil moisture treatment. He argued that floral initiation is stimulated by water stress, however a continuation of flowering was observed in the wettest soil treatment suggesting that water stress is not a pre-requisite for flowering in cocoa as it is for coffee (Alvim, 1960).

The effect of elevated CO_2 on plant reproduction varies widely depending on the species. Elevated CO_2 glasshouse experiments identified changes in flowering time for *Phlox drummondii*, *Datura stramonium* and *Abutilon theophrasti* (Garbutt and Bazzaz, 1984). Both *P. drummundi* and *D. stramonium* flowered earlier under high CO_2. No changes in flowering time were observed for *A. theophrasti*. Hesketh and Hellmers (1973) also report delayed flowering in sorghum, cotton, corn and sunflower under elevated CO_2. However, flower number was found to be consistently higher in a meta-analysis of 79 plant species under conditions of elevated CO_2 (500-800ppm) (Jablonski et al., 2002).

As discussed in Bazzaz (1990), increased levels of CO_2 increases rates of photosynthesis, reduce stomatal conductance and as a result increase water use efficiency (WUE) in many plant species. Under elevated CO_2 , cocoa also shows increased photosynthetic rate, although stomatal conductance and stomatal density are unaffected (Lahive, 2015). Despite the lack of effect on stomata, instantaneous WUE increases as a result of increased photosynthesis. With improved WUE in conditions of elevated CO_2, it is possible that the reducing effects of water stress on flowering in cocoa (Sale, 1970b) will be less pronounced, potentially maintaining flowering rates under conditions of drought. Additionally, competition for photosynthates is suggested as a reason for reduced floral emergence during the 'main crop' season for cocoa

(Alvim, 1977). It is therefore possible that under elevated CO_2 greater assimilate availability within the tree as a result of increased photosynthesis reduces sink competition under high pod load.

In this study six cocoa genotypes were maintained for 22 months in controlled environment glasshouses. Trees were grown in either ambient (mean 437ppm 6am-6pm, September 2013 to July 2015) or high (mean 724ppm September 2013 to July 2015) CO_2 and maintained under either a well-watered or water-stressed treatment (Chapter 2). Here the aim was to identify the effects that water stress and elevated CO_2 on the rate of floral emergence in cocoa, and any interactions which may occur between CO_2 treatment, water availability, and genotype.

4.2 Materials and methods

As described in Chapter 2, six cocoa genotypes were maintained under either ambient (mean 437 ppm) or elevated (mean 724 ppm) CO_2; and either well-watered or water-stressed conditions. The trees were grown under these conditions for 22 months. Data for this study were collected weekly between months 6 to 22 (February 2014 to July 2015).

There were between 3 and 4 trees per treatment from each genotype. An area on the central trunk and three smaller lateral branches were identified on each tree. To increase accessibility, branches were selected in the lower half of the tree. The lower branches were also older and were producing flowers. The sample area from which flowers were counted was between either the trunk base or where the branch met the trunk, and a tag which identified 40cm of branch length and marked the ‘top’ or the outer most point of the sample area.

Flower number was counted weekly. On the day prior to flower counting, all open flowers were removed from the identified sample areas. Care was taken not to disturb unopened buds. Flower removal was carried out in the morning as flowers for the following day begin to open late afternoon and may be confused with old flowers (Toxopeus, 1985). On the following day, all fully open flowers were counted.

The diameter (at base and position of tag) and length of the sample branches was measured in July 2014, February 2015 and July 2015. The surface area of the sample branch was calculated using **Equation 4.1**. Based on the dates that surface area was calculated the study was divided into three periods:

- Period 1 – Feb 2014 to July 2014
- Period 2 – July 2014 to Feb 2015
- Period 3 – Feb 2015 to July 2015

The surface area was not measured at the start of the study. For Period 1, surface area was assumed to be that calculated in July 2014. For period 2 and 3 respectively, the surface area was calculated as the average of the size at the beginning and the end of the period. Flower number was expressed as flowers.cm^{-2} each week. The value for flowers.cm^{-2} was accumulated across the 16 month period to give a total value per tree.

Equation 4.1 Branch surface area calculation
'Diameter1' and 'Diameter2' represent the diameter of branch measured at the base and top of the sample area.

$$\left(\frac{(\pi\ Diameter1) + (\pi\ Diameter2)}{2}\right) \times length$$

4.2.1 Statistical analysis

Statistical analysis was carried out using GenStat 15th edition statistical software (GenStat, VSN International Ltd., Hemel Hempstead, UK). The effects of treatments (CO_2, water stress and genotypes) were compared using an unbalanced design analysis of variance (ANOVA). Treatment effects on the number of flowers over time were compared using repeated measures ANOVA. Variation between time periods was tested for using a one-way analysis of variance (ANOVA) with a Tukey HSD post hoc test. The coefficient of variation was also calculated to assess the degree of variation within a treatment. Effect of greenhouse was tested for using two block designs.

4.3 Results

4.3.1 Flowering over time

The total number of flowers recorded each week was plotted to show the weekly fluctuation in flower number. Means across all genotypes are shown in Figure 4.1. Repeated measures ANOVA revealed a significant effect of date ($P < 0.001$) with mean flower numbers ranging between 0.036 and 0.002 per cm^2. There was a significant interaction between water treatment and date ($P < 0.05$) representing an effect of water treatment on some dates but not on others. For example, on the 17th June 2014, well-watered trees averaged 0.028 (ambient CO_2) and 0.029 (high CO_2) flowers per cm^2, whilst water stressed trees averaged relatively low values of 0.016 (ambient CO_2) and 0.005 (high CO_2) flowers per cm^2. However, on the 9th September 2014 all treatments averaged relatively similar flower numbers at 0.011 (ambient CO_2, WW), 0.008 (ambient CO_2, WS), 0.008 (high CO_2, WW), and 0.009 (high CO_2, WS) flowers per cm^2. There were no significant effects of water treatment or CO_2 treatment. There were no interactions between water treatment and CO_2 treatment. There was

a significant effect of genotype on flowering over time ($P < 0.001$) with mean flower numbers ranging between 0.019 flowers per cm^2 for genotype ICS 1, and 0.003 flowers per cm^2 for genotype SPEC 54/1. Means for individual genotypes are shown in Figure 4.2 (CL19/10), Figure 4.3 (ICS 1), Figure 4.4 (IMC 47), Figure 4.5 (Pound 7/B), Figure 4.6 (SCA 6), and Figure 4.7 (SPEC 54/1).Flower number fluctuated significantly over time in all genotypes: Cl19/10 ($P < 0.01$), ICS 1 ($P < 0.01$), IMC 47 ($P < 0.001$), Pound 7/B ($P < 0.01$), SCA 6 ($P < 0.001$), and SPEC 54/1 ($P < 0.05$). A period of generally high flowering occurred throughout period 1 for genotypes CL19/10, ICS 1, Pound 7/B, and SCA 6. This was to a lesser extent in genotype IMC 47 and not at all in SPEC 54/1 which shows little variation in flowering over time across the study. Flowering is generally reduced during period 2, notably in genotypes IMC 47 and Pound 7/B. Finally, during period 3, flowering showed a general increase for genotypes ICS 1, IMC 47 and SCA 6. There were no significant effects of water treatment or CO_2 treatment and no interactions between treatments.

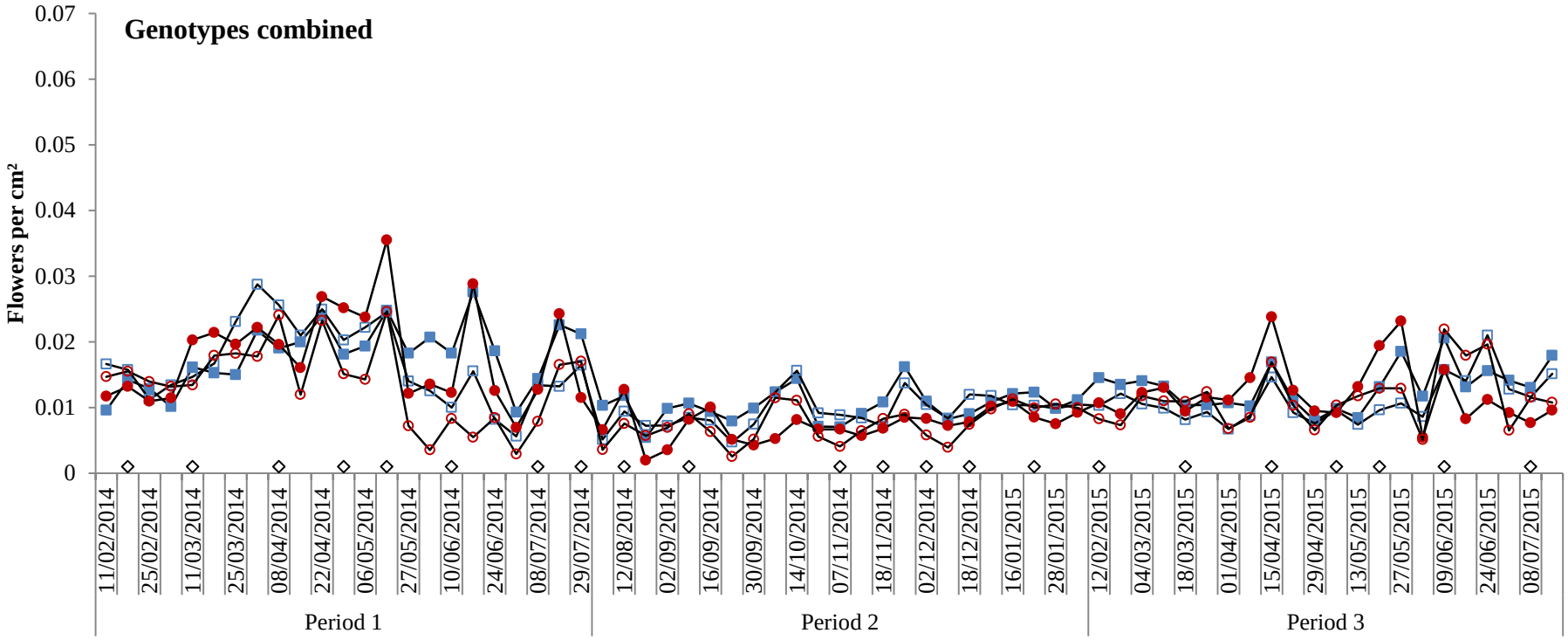

Figure 4.1 Mean flower number over time from trees grown at two concentrations of CO_2 and two watering treatments. Data are combined across six genotypes.

The mean flower number per cm^2 of sample branch across all genotypes per treatment.

■ - Ambient CO_2 WW, □ - Ambient CO_2 WS, ● - High CO_2 WW, ○ - High CO_2 WS, ◇ - Re-water event.

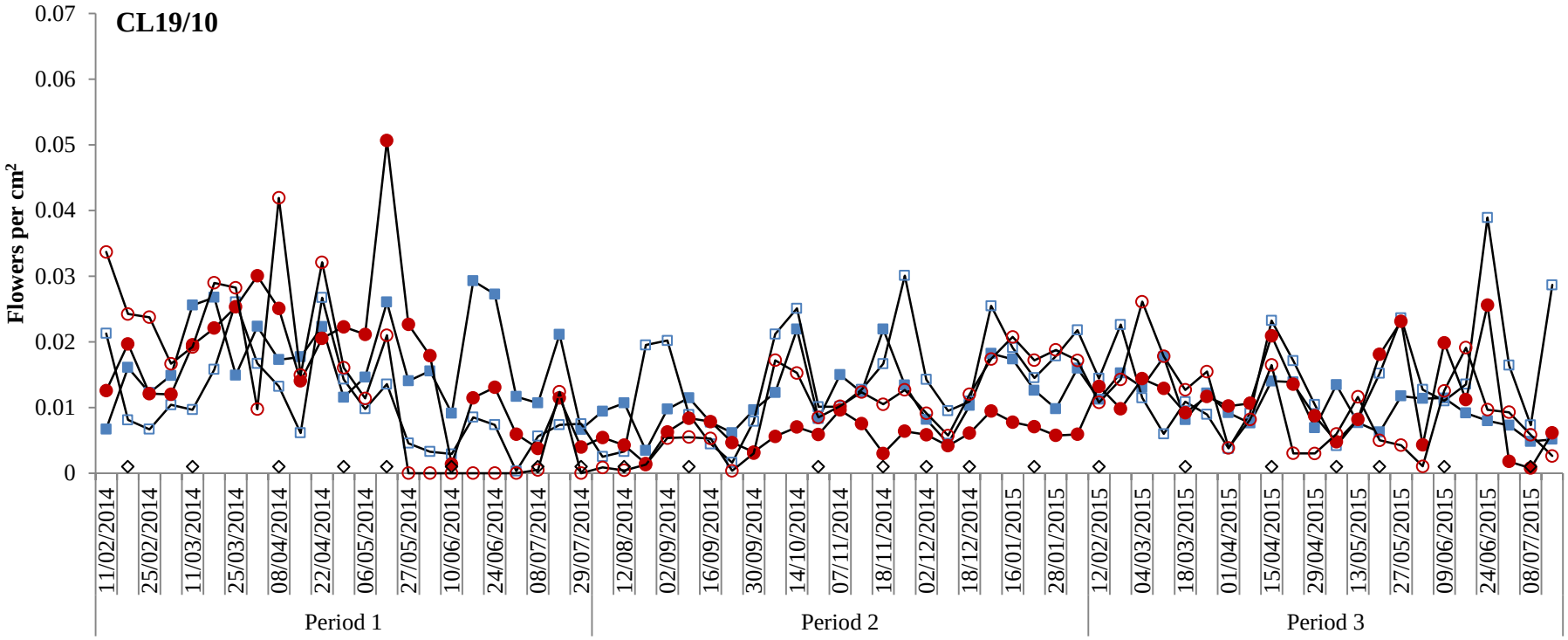

Figure 4.2 Mean flower number over time from trees grown at two concentrations of CO_2 and two watering treatments. Genotype CL19/10.
The mean flower number per cm^2 of sample branch per treatment for genotype CL19/10.
■- Ambient CO_2 WW, □ - Ambient CO_2 WS, ● - High CO_2 WW, ○ - High CO_2 WS, ◇ - Re-water event.

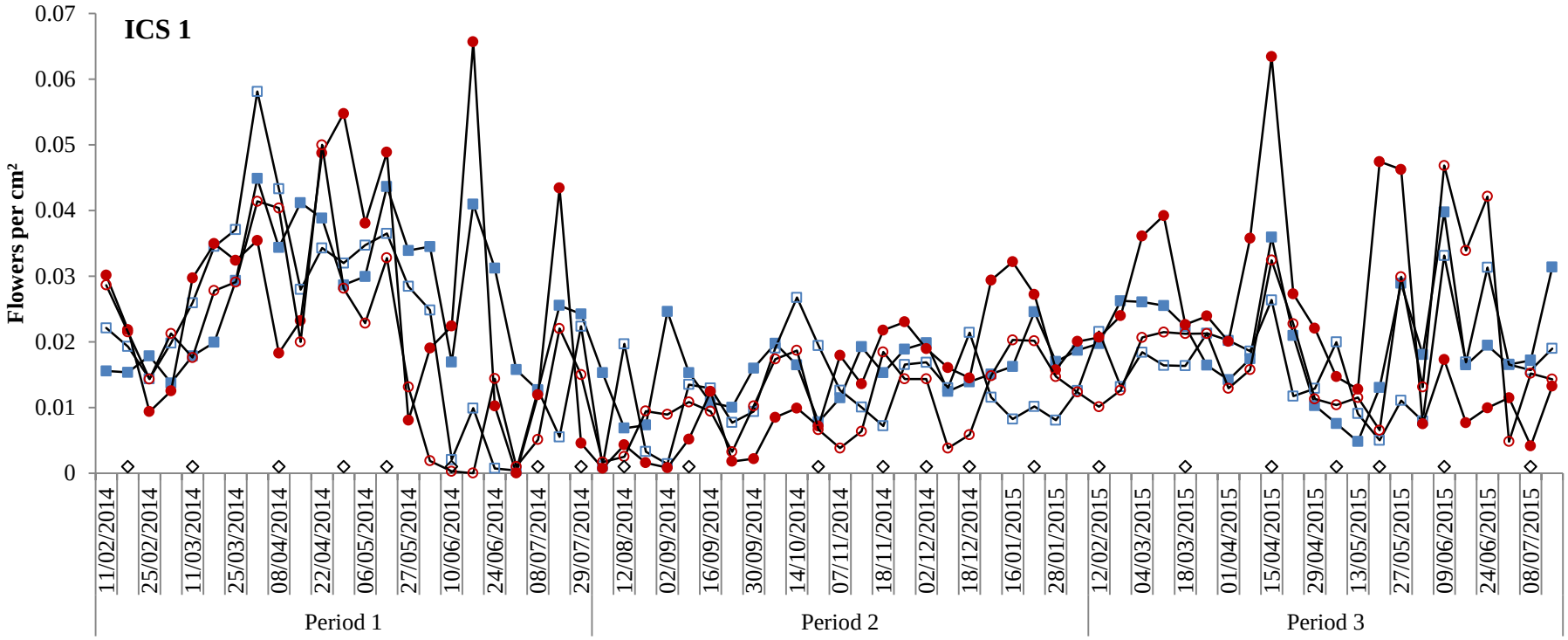

Figure 4.3 Mean flower number over time from trees grown at two concentrations of CO_2 and two watering treatments. Genotype ICS 1
The mean flower number per cm^2 of sample branch per treatment for genotype ICS 1.
■- Ambient CO_2 WW, □ - Ambient CO_2 WS, ● - High CO_2 WW, ○ - High CO_2 WS, ◇ - Re-water event.

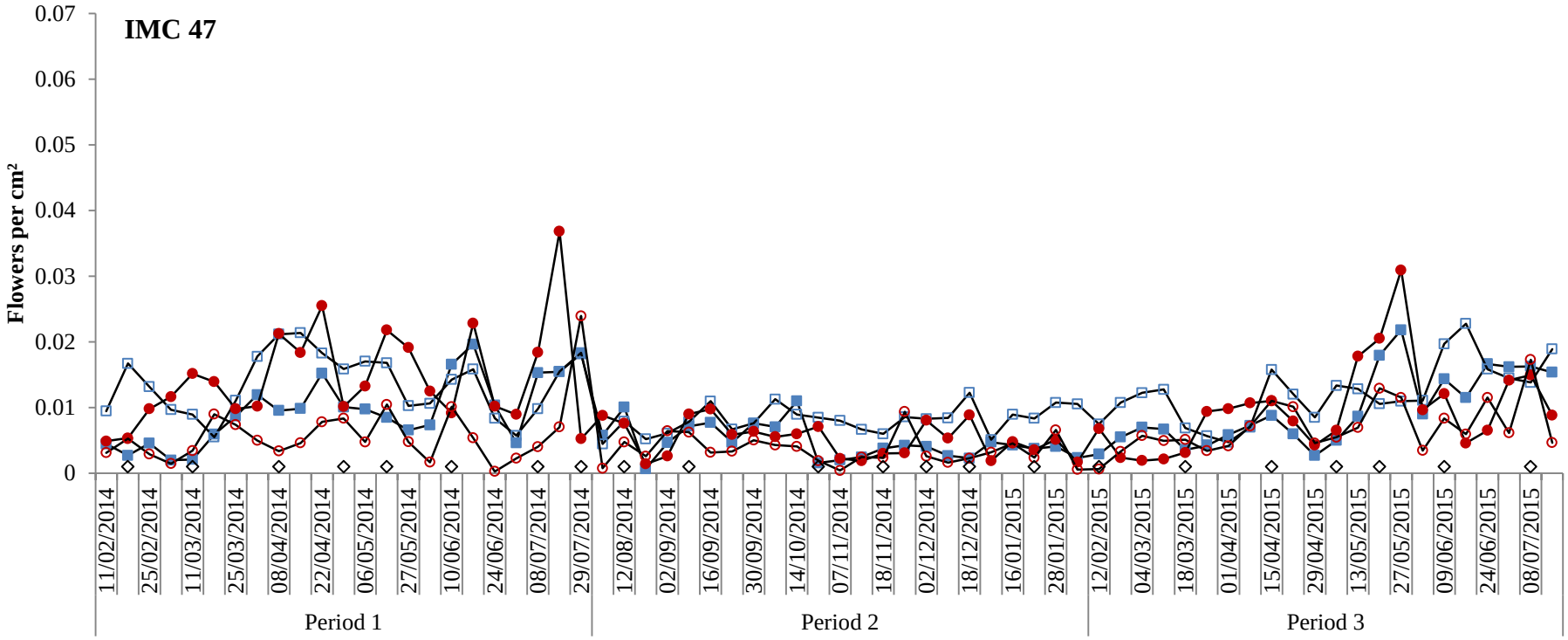

Figure 4.4 Mean flower number over time from trees grown at two concentrations of CO_2 and two watering treatments. Genotype IMC 47
The mean flower number per cm^2 of sample branch per treatment for genotype IMC 47.
■- Ambient CO_2 WW, □ - Ambient CO_2 WS, ● - High CO_2 WW, ○ - High CO_2 WS, ◇ - Re-water event.

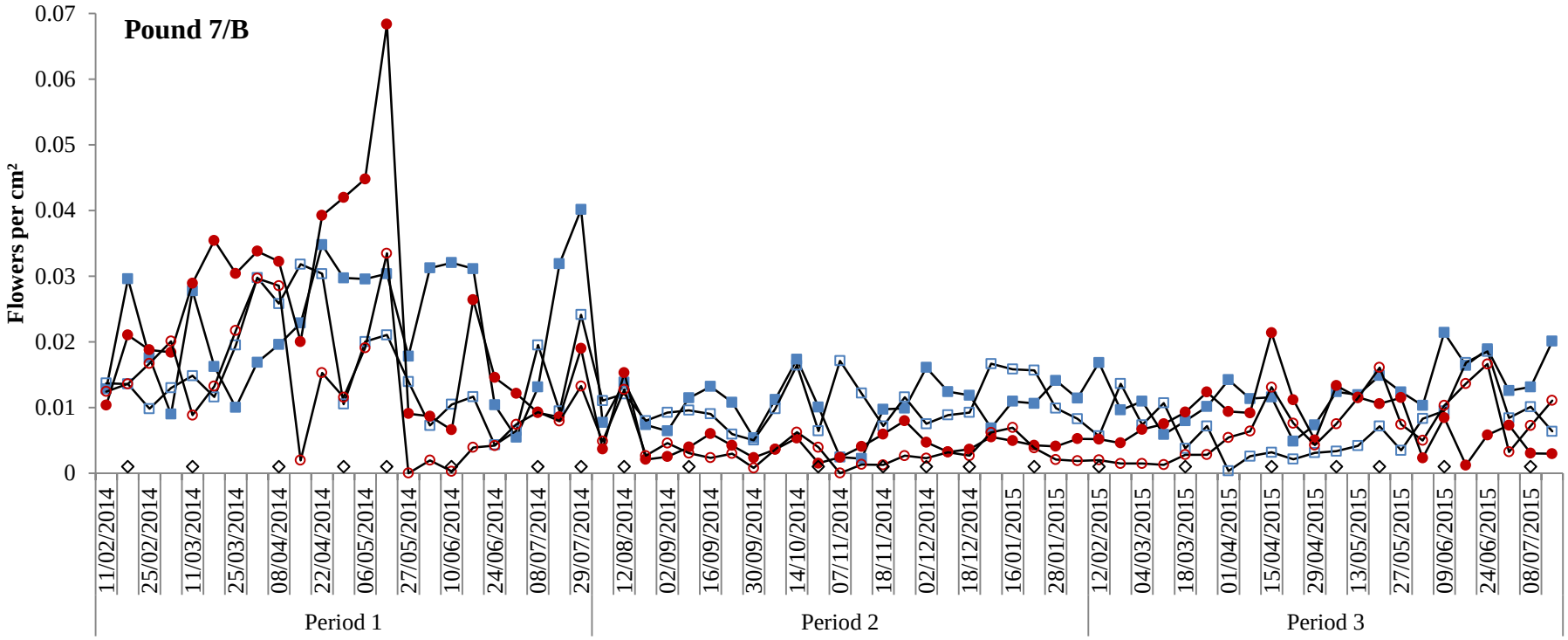

Figure 4.5 Mean flower number over time from trees grown at two concentrations of CO_2 and two watering treatments. Genotype Pound 7/B
The mean flower number per cm^2 of sample branch per treatment for genotype Pound 7/B.
■- Ambient CO_2 WW, □ - Ambient CO_2 WS, ● - High CO_2 WW, ○ - High CO_2 WS, ◇ - Re-water event.

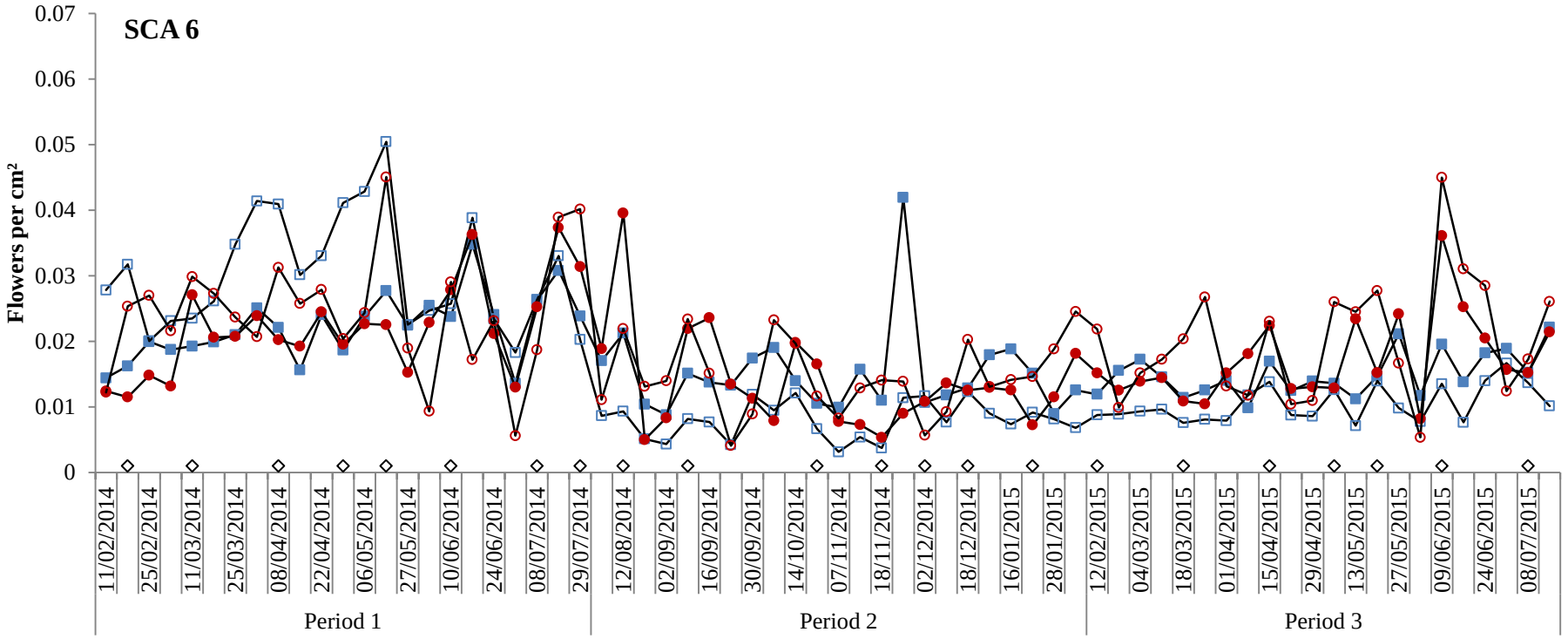

Figure 4.6 Mean flower number over time from trees grown at two concentrations of CO_2 and two watering treatments. Genotype SCA 6
The mean flower number per cm^2 of sample branch per treatment for genotype SCA 6.
■- Ambient CO_2 WW, □ - Ambient CO_2 WS, ● - High CO_2 WW, ○ - High CO_2 WS, ◇ - Re-water event.

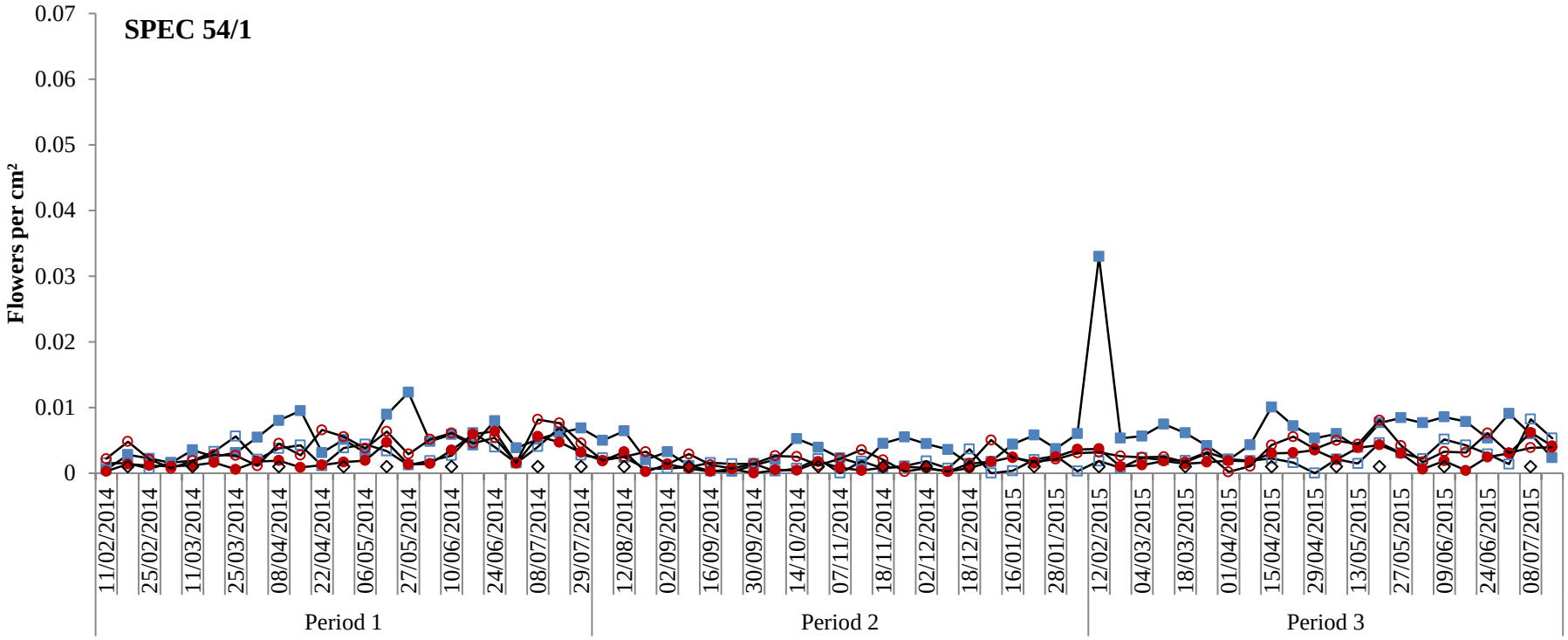

Figure 4.7 Mean flower number over time from trees grown at two concentrations of CO_2 and two watering treatments. Genotype SPEC 54/1
The mean flower number per cm^2 of sample branch per treatment for genotype SPEC 54/1.
■- Ambient CO_2 WW, □ - Ambient CO_2 WS, ● - High CO_2 WW, ○ - High CO_2 WS, ◇ - Re-water event.

4.3.2 Total number of flowers

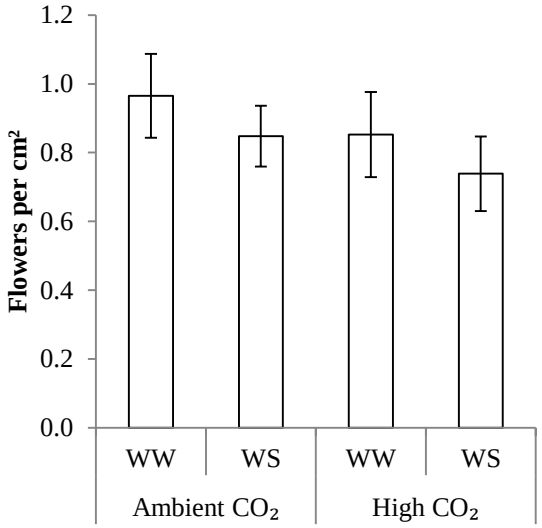

Figure 4.8 Mean total number of flowers per cm^2 from trees grown at two concentrations of CO_2 and two watering treatments. Data are combined across six genotypes.
The weekly number of flowers per cm^2 was combined to create a total per tree. This was averaged across all genotypes per treatment. Error bars display standard error of the mean.

Values for weekly flower number per cm^2 were combined for all genotypes and time periods to give the mean total flower number per cm^2 for each treatment (Figure 4.8). No significant effect of treatment was observed ($p > 0.05$). However, there were significant differences between genotypes ($p < 0.001$) (Figure 4.9). No significant effect of treatment was found for any of the genotypes ($p > 0.05$). ICS 1 had the highest mean total flower per cm^2 (1.31), followed by SCA 6 (1.21), CL19/10 (0.87), Pound 7/B (0.81), IMC 47 (0.59), and SPEC 54/1 with the lowest (0.21 flowers per cm^2). No effect of greenhouse was observed.

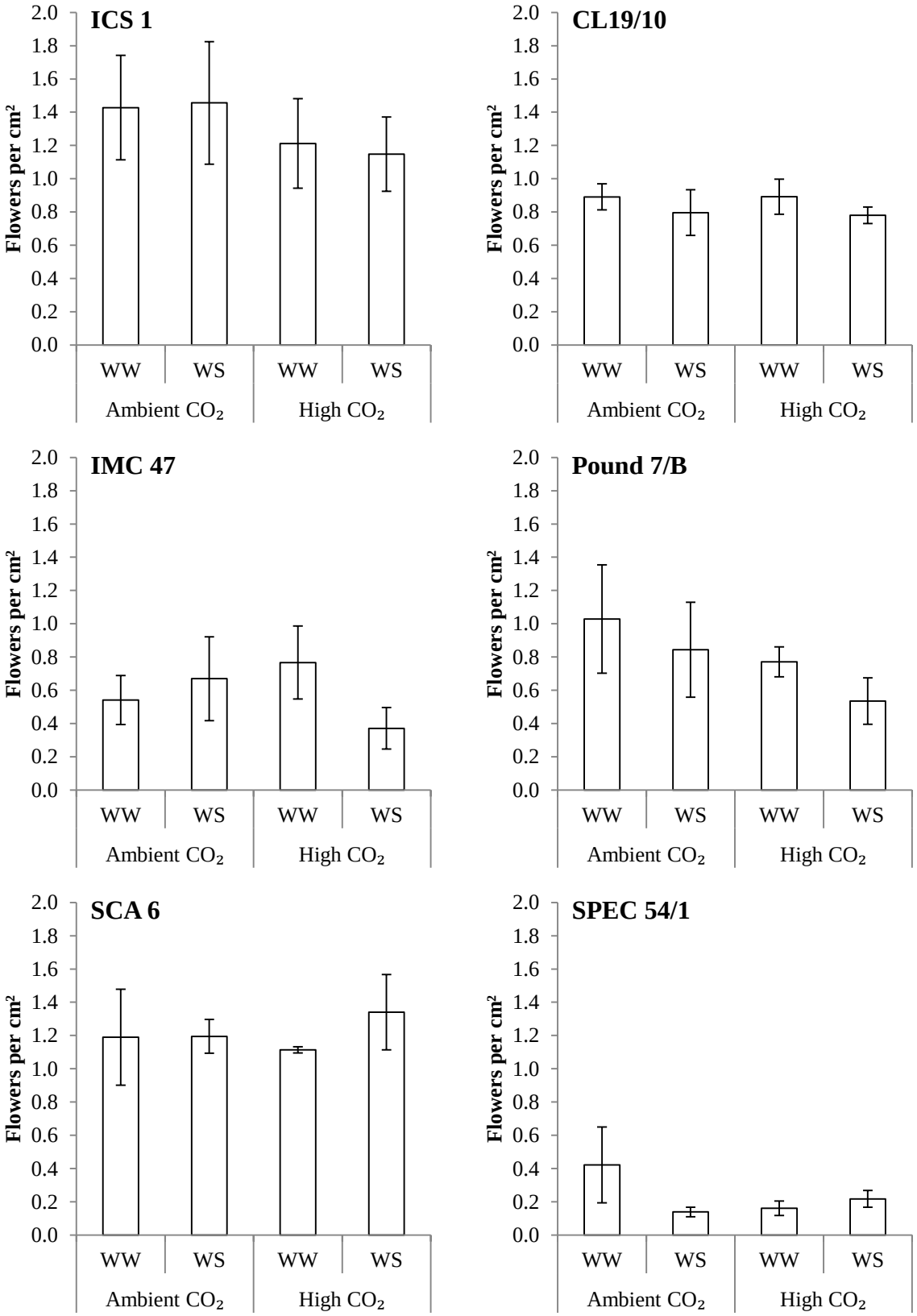

Figure 4.9 Mean total flowers per cm^2 of six cocoa genotypes grown at ambient and elevated CO_2 and under two watering treatments.
Weekly flowering per cm^2 values were combined for each tree and averaged per treatment across all time periods. Error bars display standard error of the mean.

Mean total flower number was also calculated with the time periods separated (see Figure 4.10) There were no significant effects of treatment found within time periods ($p > 0.05$), however period 1 was found to contain significantly greater flowering intensity than period 2 and 3 ($P < 0.001$).

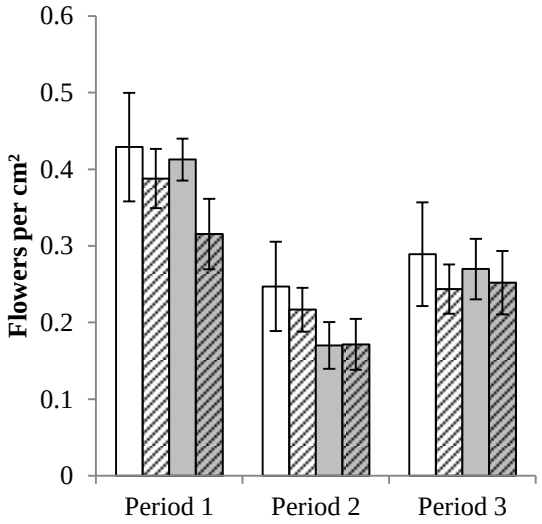

Figure 4.10 Mean total number of flowers per cm^2 across three time periods of trees grown in two concentrations of CO_2 and two watering treatments. Data combined across six genotypes.
The weekly number of flowers per cm^2 value was combined for each time period for each tree. This was averaged across all genotypes per treatment. Period 1 – Feb-14 to Jul-14. Period 2 – Aug-14 to Jan-15. Period 3 – Feb-15 to Jul-15. □ - Ambient CO_2 WW, ▨ - Ambient CO_2 WS, ▩ - High CO_2 WW, ▨ - High CO_2 WS. Error bars display standard error of the mean.

4.3.3 Coefficient of variance

In order to quantify temporal variation under each of the different treatments, a coefficient of variation was calculated for each tree across all time points (Figure 4.11). There was a significant increase in variation for elevated CO_2 compared to ambient conditions ($p < 0.01$) and a significant effect of genotype ($p < 0.001$). The mean coefficient of variance ranged between 1.14 for the genotype IMC 47, and 0.72 for the genotype SCA 6. There was no significant effect of water treatment. When genotypes were studied individually (Figure 4.12), Pound 7/B was the only genotype to show a significant increase in variation under high CO_2

treatments ($p < 0.05$). There were no significant effects of water treatment on individual genotypes. No greenhouse effect was observed.

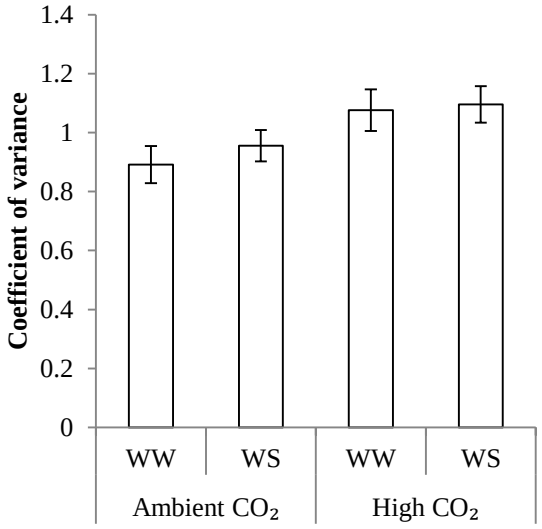

Figure 4.11 Mean coefficient of variation of flower number per cm^2 throughout the duration of the study of trees grown at two concentrations of CO_2 and under two watering treatments. Data is combined across six genotypes.
Coefficient of variation was calculated for each tree and averaged for each treatment, combining all genotypes and time periods. Error bars display standard error of the mean.

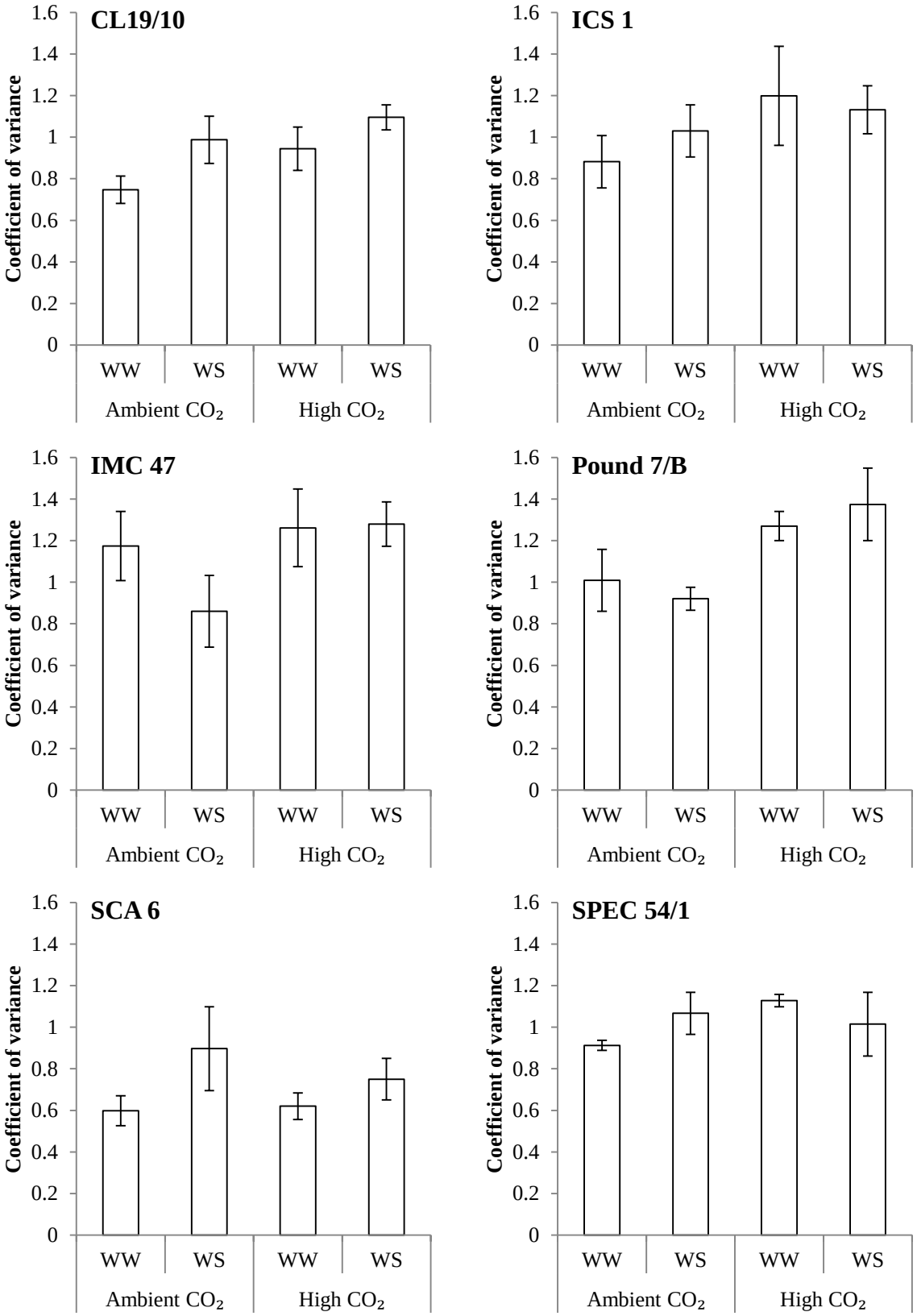

Figure 4.12 Mean coefficient of variation of flower number per cm^2 throughout the duration of the study of trees grown at two concentrations of CO_2 and two watering treatments. Data is combined across six genotypes.
Coefficient of variation was calculated for each tree and averaged for each treatment, combining all time periods. Error bars display standard error of the mean.

4.4 Discussion

Elevated CO_2 has been shown to increase the number of flowers produced for a large number of plant species (Jablonski et al., 2002). However the effect of increased CO_2 on flowering in cocoa has not been studied previously. In this study, no effect of elevated CO_2 was observed on the overall pattern of floral emergence or the quantity of flowers produced over the entire 16 month recording period (Figure 4.1 and Figure 4.8). An increase in flowering may have been expected as elevated CO_2 increased photosynthetic rates of the trees in this study (Lahive, 2015), and thus assimilate availability would have increased. A relationship between flowering intensity and assimilate availability has already been suggested as increased fruit loads and thus competition for assimilates is seen to decrease flowering intensity (Valle et al., 1990; Alvim, 1977). Although the total number of flowers did not significantly change, the degree of variation in flowering intensity was greater for high CO_2 treatments when compared to trees grown in ambient conditions (Figure 4.11). In Figure 4.1, the largest periods of flowering variation, with large peaks and troughs, are during period 1 and again later in period 3. Both times of increased variation begin around March/April and represent a time of year when light integral begins to increase. This was also a time when there were pods developing on the trees. Light levels have been associated with flowering in cocoa with decreased light integral resulting in reduced flowering (Almeida and Valle, 2007; Hurd and Cunningham, 1961). The increasing light integral around March/April may have resulted in the peaks in flowering observed at this time; meanwhile the reductions may be due to assimilate competition with developing pods. Under high CO_2 the increased photosynthetic rates would further enhance the peaks in flowering through increased assimilate availability. Furthermore, pods developing in the high CO_2 treatments were significantly larger in size, further increasing the assimilate demand for pod growth. Overall, this may explain the increased variance in flowering patterns observed under high CO_2.

No significant effect of water stress was observed on the total number of flowers across the study. The patterns of floral emergence revealed an interaction between date and water treatment suggesting, despite a lack of overall effect of water stress, floral emergence was altered in response to water stress at certain time points. Water stress has been observed to supress flower emergence in cocoa (Alvim and Alvim, 1977; Sale, 1970b) and the return of rains or irrigation then triggers the emergence/opening of flowers. Sale (1970b) observed a heavy floral emergence when cacao trees were transferred from a dry treatment to a medium/wet irrigation treatment. In this study, no overall floral response to water stress was observed. Floral emergence did not appear to respond to the occurrence of re-water events in the water stress treatment. Additionally, the total number of flowers which were induced did not alter in response to water treatment. Prior studies into the effects of water stress on cocoa flowering have all included a clear shift from a long period of drought to a long period of watering. This study maintained trees under a water-stressed condition continuously, with small breaks in treatment for a re-water to remove nutrient build-up within the soil substrate (Savvas et al., 2007). The absence of a shift of treatment from 'dry' to 'wet' may have prevented long-term effects of the water stress treatment from emerging. Furthermore, Sale (1970b) stated that for the 'dry' treatment (15% field capacity) trees only required watering once every three weeks. This time period between watering is much greater than in this study. The trees in Sale's study were maintained in soil, compared to this study which maintained trees in sand, gravel and vermiculite mix, which was irrigated with nutrient solution. To maintain 10-17% moisture content in this experiment, watering was required every 48 hours in small quantities. It is possible that the longer periods without any water additions, as seen in Sale (1970b), is required to reduce the flowering of cocoa. In addition, the regular re-watering events in this study may have counteracted any long-term effects of water stress.

The total number of flowers produced varied significantly between genotypesin this study highlighting the natural variation in flower production between the six genotypes in question. This is in line with the natural variation in canopy architecture (Yapp and Hadley, 1994), photosynthetic rates (Daymond et al., 2002b; Lahive, 2015), and yield (Hadley and Yapp, 1992) which have already been identified in various genotypes of cocoa. No interaction between genotype and treatment response was observed suggesting all genotypes responded to treatment similarly. However, as little treatment response was observed here in general, further investigation would be required to identify any genotypic differences in response to elevated CO_2 and water stress.

4.5 Conclusions

Overall floral induction and emergence was unaffected by water stress, contradicting other research. This is likely to be due to the duration of induced water stress which was much shorter in this study due to the water retaining properties of soil substrate used. Elevated CO_2 increased the temporal fluctuation in flower emergence without altering the total number of flowers in the study period. This may be due to increased photosynthate production in elevated CO_2 trees, and additional assimilate competition from larger developing pods.

Chapter 5. The effects of elevated CO_2 and water stress on pod development of cacao

5.1 Introduction

There are many factors which have an effect on a plant's ability to produce fruit and seeds. Assuming a successful pollination with an adequate number of pollen grains, variation in environmental conditions, nutritional availability and internal resource allocation can all affect a plant's ability to grow fruits to maturity. An ability to control the fruit load on a plant can be essential in ensuring resource depletion is not too great and that the fruits which remain on the tree develop successfully and reach maturity. Fruit abortion or fruit 'thinning' is found across many types of fruit trees including apple, citrus and oak, and is generally accepted as a mechanism to reduce the number of developing fruit, to optimise investment depending on resource availability (Stephenson, 1981). The cocoa tree uses fruit abortion to reduce its pod load in a process referred to as 'cherelle wilt', the defining features of which involve the young pods (cherelles) aborting and remaining on the tree (Toxopeus, 1985). A pod takes between 5 and 6 months to mature (approximately 150-180 days) (Toxopeus, 1985). Mckelvie (1956) reported that there are two time periods at which cherelle wilt occurs, referred to as first and second wilt respectively. The first wilt occurs around 50 days after pollination and coincides with the formation of the cellular endosperm. The second peak occurs around 75 days after pollination and coincides with an accumulation of fat and starch within the pod. Later research into the wilting points of cocoa revealed a gradual decline in the incidence of wilt in the time after the 'first wilt period', as opposed to a second peak in wilt (Daymond, 2000).

Several factors can affect whether a given fruit will be aborted. The number of developing seeds within the fruit is one such factor. For example, fruit development in Trumpet Creeper

(*Campis radicans*, Bignoniaceae) was studied and mature fruits were rarely found with fewer than 100 seeds. It was noted in this study that at least 400 compatible pollen grains were required to meet a pollination threshold and avoid fruit abortion (Bertin, 1982). Furthermore, Stephenson (1981) reported that in apples the minimum seed number required by the tree, varied from year to year, suggesting adaptation to resource availability. In cocoa, links between fruit size and selection for wilt have been established as wilted cherelles were generally smaller and grew more slowly therefore acting as weaker sinks for assimilates (End, 1990). The distance between the fruit and the source of assimilates (photosynthesising structures such as leaves) can also have a bearing on fruit abortion. It has been observed in Milkweed (*Asclepias tuberosa*) that fruit set is greater in umbels located more centrally on a branch/stem. The terminal growth receives reduced resources and therefore fruit set is lowest here (Wyatt, 1980). Additionally, basal flowers in lupin show a higher rate of pod set over the terminal flowers, further suggesting a locational effect of fruit development. However, as the basal flowers develop earlier, it is also suggested that assimilate competition increases with fruit development. The younger fruits may be aborted over the older ones as the younger fruits represent a smaller investment by the plant (Herbert, 1979). Finally, the nature of the pollination may also play a role. For example, *Macadamia ternifolia* sets many more fruits from cross-pollinations than from self-pollinations (Urata, 1954). Self-pollinated fruits are aborted over cross-pollinated ones as cross-pollination provides a genetic advantage, especially when an environment is not predictable (Solbrig, 1976). This links back with reference to Stephenson (1981), where the ability to reduce fruit load and pod set provides an advantage to a plant when resource availability is unpredictable.

A link between assimilate availability and fruit drop has been established through a sucrose supplementation and defoliation experiment on Satsuma mandarins (*Citrus unshiu* (Mak.) Marc.) cv. Okitsu. Sucrose injections into stems resulted in a decrease in fruit abortion by 10-

15% and an overall increase in fruit yield of a tree, whilst partial defoliation increased fruitlet abortion from 25.7 to 42.8%. Furthermore, sucrose supplementation lessened the negative impacts on fruit abortion of partially defoliated trees, reducing the fruit drop by 15% (Iglesias et al., 2003). Fluctuations in the availability of assimilates is largely attributable to changes in environmental conditions. The resource availability within the plant from carbon storage or canopy size and photosynthetic rates, will be altered by the environment and will determine the plant's ability to support fruit growth. For example, temperature has a direct effect on the rate of photosynthesis, and natural temperature fluctuations throughout any given growing season will have an effect on the development of a plant. Increased temperature was found to speed up the floral development in Apricot when pre-blossom temperatures were high. However, the reduced development time had a detrimental effect on pistil development and as a result, floral viability and pod set were reduced (Rodrigo and Herrero, 2002). In cocoa, several studies have identified the effects of temperature on pod development. Trees which were grown in semi-controlled environment glasshouses showed an increase in cherelle wilt under high temperatures (Daymond and Hadley, 2008; Hadley et al., 1994). This increase in wilt is attributed to higher assimilate demands from an increased rate of respiration at higher temperatures, reducing the carrying capacity of the tree. Additionally, higher temperatures lead to a decrease in time to reach 95% maximum size, and overall final size was also reduced. Final pod size in the Amelonado variety was reduced as a result of faster growth rates, however in other varieties, final size seemed to be strongly influenced by bean number (Daymond and Hadley, 2008). Further evidence that assimilate availability plays a role in fruit size is also provided by Daymond and Hadley (2008) as increases in light levels also resulted in larger pods.

Variation in rainfall patterns has the potential to limit the water availability to the plant for photosynthesis. Water stress has been observed to reduce fruit numbers in citrus (*Citrus*

clementina) by around 58-86%, especially when stress occurred during flowering and fruit set. The reduction in fruit number was due to a heavy fruit drop. Water stress which occurred at a later developmental stage, did not alter fruit number, however fruits were smaller and of reduced quality (Ginestar and Castel, 1996). A higher volume of fruit wilt is also observed in cocoa during water stress as assimilate availability is reduced from a lower rate of photosynthesis (Sale, 1970b). Additionally, if the wilting periods for cocoa coincide with a flush of new leaf growth, the volume of wilt is increased further representing a sink competition within the trees (Alvim, 1954). In an experiment using controlled soil moisture conditions, the dry soil treatments reduced pod set in cocoa when compared to medium and wet conditions (Sale, 1970b). Furthermore, any pods which did set under the dry treatment subsequently wilted. No effect of water availability was found on the size of the wilted pods although the greatest pod weight was obtained from the wettest treatments. No reference was made to final pod size in the study (Sale, 1970b).

Atmospheric carbon dioxide (CO_2) levels have been increasing globally and are set to continue rising in the future (IPCC, 2014). As increasing CO_2 directly increases the rate of photosynthesis in C_3 plants (Long et al., 2004), it might be expected that assimilate availability and thus reproductive capacity may also increase in an environment of elevated CO_2. Up to 70% increases in fruit retention have been observed in Valencia orange (*Citrus sinensis* (L.) Osbeck) when trees were grown at elevated CO_2 (800 µbar). Furthermore, the fruits obtained were no smaller in fresh weight or size compared to control tree fruit. During the study, the photosynthetic rates were observed to increase by 23% more than the control trees at the end of flowering, to 77% higher rates during fruit development. Closer to fruit maturity, the increase in photosynthetic rate compared with the controls was reduced to 18% demonstrating a dynamic response to meet assimilate demand (Downton et al., 1987). Increase in fruit load was also observed in sour orange (*Citrus aurantium* L.) after 17 years

grown at 700ppm CO_2 (Kimball et al., 2007). However, not all plants respond so positively to the increases in CO_2 as identified in Garbutt and Bazzaz (1984). *Datura stramonium* and *Abutilon theophrasti* were grown at 300, 600 or 900ppm CO_2 in glass growth chambers. *D. stramonium* showed significant increases in final fruit weight as a result of a thicker fruit wall; however both *D. stramonium* and *A. theophrasti* displayed no changes to fruit abortion. It is worth noting that these two examples are both annual plants, and a lack of cumulative gain from several years of growth in high CO_2 conditions, may place an upper limit on the reproductive advantages CO_2 enrichment may have. In comparison, the citrus examples given above may show a stronger reproductive gain through CO_2 enrichment as perennial crops are able to slowly increase their reproductive capacity after prior investment in storage reserves. This was demonstrated in the sour orange which only dramatically increased fruit load after around 5 years of CO_2 enrichment (Kimball et al., 2007).

In addition to the effects on photosynthesis, CO_2 has also been observed to influence fruit set though other means. Fruit set of cocoa increased from around 50% in normal pollinations to almost 100% when pollinated flowers were sealed in vials to allow an accumulation in CO_2 up to levels of around 85ml $liter^{-1}$ (8500ppm) (Aneja et al., 1992). The increase in pod set was attributed to an enhancement of pollen performance under elevated CO_2. However, these experimental conditions generated a very high concentration of CO_2 compared to many studies which use predictive atmospheric simulations of between 500 and 700ppm (Kimball et al., 2007; Baligar et al., 2005; Ort et al., 2006; Oberbauer et al., 1985). It has yet to be demonstrated whether an increase in cocoa pod set is seen with smaller increases in CO_2. An additional effect of elevated CO_2 is an increase in water use efficiency (WUE) of plants due to a reduced stomatal conductance reducing transpirational water loss (Ainsworth and Rogers, 2007; Long et al., 2004). As a result, some alleviation from the effects of water stress may be observed when combined with elevated CO_2. However, previous studies with cocoa

have showed little to no response of stomatal conductance to elevated CO_2 (Lahive, 2015). Furthermore, reduced transpiration will also reduce the heat loss which transpiration facilitates. If water stress is combined with high temperatures, heat stress may play a stronger detrimental role in elevated CO_2 conditions.

This chapter aims to investigate the effects water stress and elevated CO_2 may have on the pod set, cherelle wilt and pod development of *T. cacao*. Both of the experimental treatments have the potential to alter the assimilate availability within a plant through influences on photosynthetic rate. However, these parameters may also interact to alter treatment responses.

5.2 Materials and methods

As described in Chapter 2, six genotypes of *Theobroma cacao* were grown in either elevated (700ppm) or ambient (averaging 437ppm) carbon dioxide and either well-watered (WW) or water-stressed (WS) conditions. The study of pod development took place over a two year period and consisted of two periods of pod growth (dates shown represent time from first pollination attempt to time of last pod harvest):

- Harvest 1 – 14.02.2014 to 07.11.2014
- Harvest 2 – 26.11.2014 to 18.07.2015

5.2.1 Pollinations and cherelle wilt

All pollinations were carried out by hand and used pollen from trees of the Amelonado variety which were housed in standard growing conditions in neighbouring glasshouses. Freshly opened flowers were collected at 09:00am and transported in petri dishes to the experimental glasshouse compartments. A newly opened flower was located on an

experimental tree and the staminodes were removed on one side to allow access to the pistil, taking care not to damage the peduncle and its connection to the tree. An Amelonado flower from the petri dish was prepared by removing all petals with tweezers, taking care to preserve the anther. The stamen was detached at the base of the filament and the anther was then rubbed along the length of the pistil on the pre-prepared experimental flower approximately 5 times. This process was repeated using two further stamens. A second flower was selected on the same tree and pollinated in the same way using the remaining two stamens from the Amelonado flower. The pollinated flowers were labelled with the date. The following week, flowers which had remained on the tree and had a swollen ovary, were measured and a unique pod I.D. was added to the label. The pod I.D. was formulated using the tree I.D. (e.g. 3G) and a successional number based on the number of successful pollinations which had occurred on that tree (e.g.5) to give an I.D. of 3G5. In the second year, a '2-' was inserted after the tree I.D. to identify the second harvest (e.g. 3G2-5).

Manual pollinations took place on a two week cycle. In week 1, pollinations took place on all trees in houses 2 and 3; in week 2, pollinations were conducted on all trees in houses 5 and 6. Unless sufficient flowers were not available, two flowers on each tree were pollinated in one cycle. Pollinations were carried out between 14.02.2014 and 4.06.2014 in year 1 and between 26.11.2014 and 16.01.2015 in year 2. For year 1, since the pollination technique was being developed, a record of pollination attempts was not initially taken. However, later in the process there was an 8 week period where the rate of pollination attempts was known and measurable. The number of developing pods which resulted from this period was used to calculate the percentage of pollination success. For harvest 2, the number of pollination attempts was recorded for each tree throughout the whole period. The percentage of pollination success was calculated using this information and the number of developing pods for that year.

Any pod which had been identified as a successful pollination and therefore had been measured at least once, was considered as being lost to 'cherelle wilt' if it blackened and ceased growing beyond this point. The number of matured pods was used with the total number of successful pollinations to calculate the percentage of cherelle wilt.

5.2.2 Pod growth

Once a pod had been allocated a pod I.D. (roughly one week after pollination), weekly length and width measurements were taken using digital callipers. Care was taken to ensure the widest and longest part of the pod was measured each week.

Using the weekly length and width values, pod volume was calculated using **Equation 5.1** from Jessop et al. (2010). As discussed in Ten Hoopen et al. (2012), a prolate spheroid shape best describes the shape of a cocoa pod and Jessop's equation best described pod volume.

Equation 5.1 Volume calculation for a prolate spheroid (Jessop et al., 2010)
Where V is volume (cm^3), a is the equatorial radius (cm) and b is the polar radius (cm).

$$V = \frac{4}{3}\pi a^2 b$$

Regression analysis was used to describe the increase in pod volume over time, using a four parameter logistic function (see Equation 5.2). Using the parameters provided from the regression analysis, the maximum pod volume was calculated by the sum of the A and C values. Additionally, using a day-by-day timeline, a smooth growth curve could be plotted for each pod (see Figure 5.1). From this timeline the maximum growth rate, day at which maximum growth rate occurred, and the time to reach 95% maximum pod size was calculated. Means using the data from multiple pods were calculated for each of these parameters to represent genotype and treatment response.

Equation 5.2 Regression formula for pod growth (GenStat, VSN International Ltd., Hemel Hempstead, UK)
Standard logistic regression analysis, where *A*, *C*, *B* and *M* are parameters provided by the regression analysis and *X* is the number of days after pollination.

$$\frac{A + C}{1 + EXP\ (-B(X - M))}$$

An average growth curve was derived by calculating a weighted mean for each formula parameter (A, C, B and M) for a given treatment. These means were then placed into Equation 5.2 and plotted using a day-by-day timeline. This allowed for a visual comparison of treatments in addition to the parameter averages.

5.2.3 Statistical analysis

The main effect of treatments on pollination success and cherelle wilt was tested for significance using a chi-squared test. A standard logistic regression analysis was carried out using the calculated volume and days after pollination for each pod. An unbalanced design analysis of variance (ANOVA) was used to test for treatment effects on the maximum growth rate, time to maximum growth rate, maximum pod size and time to 95% maximum size. Two block designs were created to test for any greenhouse spatial variability. Finally, regression analysis was used to assess the relationship between pod size and duration of pod growth. All statistical analyses were carried out using GenStat 15th edition statistical software (GenStat, VSN International Ltd., Hemel Hempstead, UK).

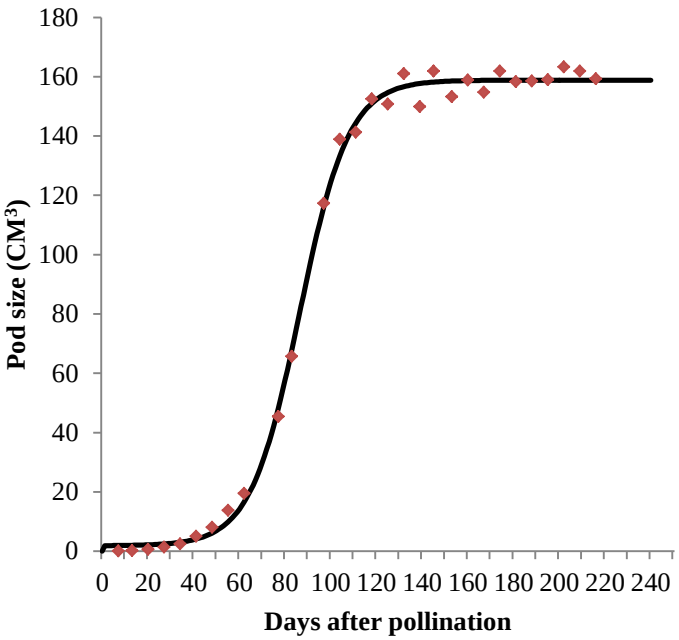

Figure 5.1 Example of a pod volume growth curve.
Raw data volume calculation (◆) plotted with the growth curve generated through standard logistic regression analysis (●). Data taken from pod 2E1.

5.3 Results

5.3.1 Pollination success

The success rate of manual pollinations across all genotypes is shown in Figure 5.2. These results were calculated using the total number of successful and unsuccessful pollination attempts across all genotypes. As a result, the data was not suitable for analysis using an ANOVA and instead a chi squared test was used. A significant increase ($P < 0.01$) in pollination success was seen under high CO_2 for harvest 1. No significant effect of water stress was seen ($P > 0.05$), however 'WS' treatments showed consistently lower pollination success compared to 'WW' treatments. The reverse pattern was seen in harvest 2 as high CO_2 consistently reduced the pollination success, although the result was not significant. A significant increase ($P < 0.01$) of pollination success was seen under 'WS' conditions, this being the reverse of the trend seen in harvest 1. A significant increase in pollination success

was seen under high CO_2 for the clones IMC 47 and Pound 7/B (both $P < 0.01$) in harvest 1 (Figure 5.3). No other clones showed a significant response in harvest 1. In harvest 2, a significant increase in pollination success was observed under water stress treatments for the clones CL19/10 and ICS 1 ($P < 0.01$ and $P < 0.05$ respectively). No other clones demonstrated a significant response to treatment.

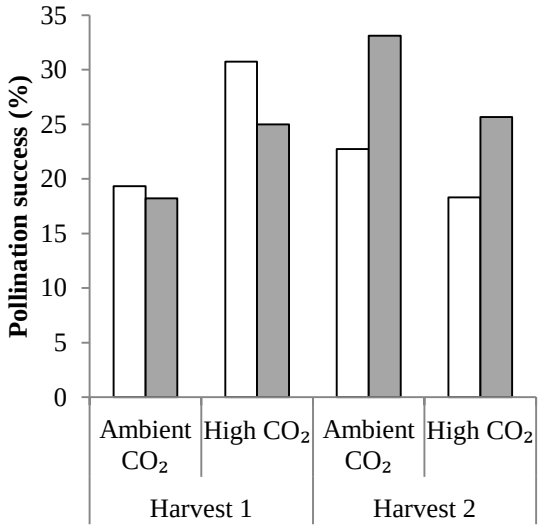

Figure 5.2 Success rate of manual pollinations of trees grown under two concentrations of CO_2 and two water treatments. Data combined across six genotypes.
Percentage of success was calculated using the number of pollination attempts and the number of developing pods from all genotypes in each treatment. Only data from a period of known pollination attempts was used for harvest 1 compared to harvest 2 which used all data. □ - Well-watered. ▩ - Water-stressed.

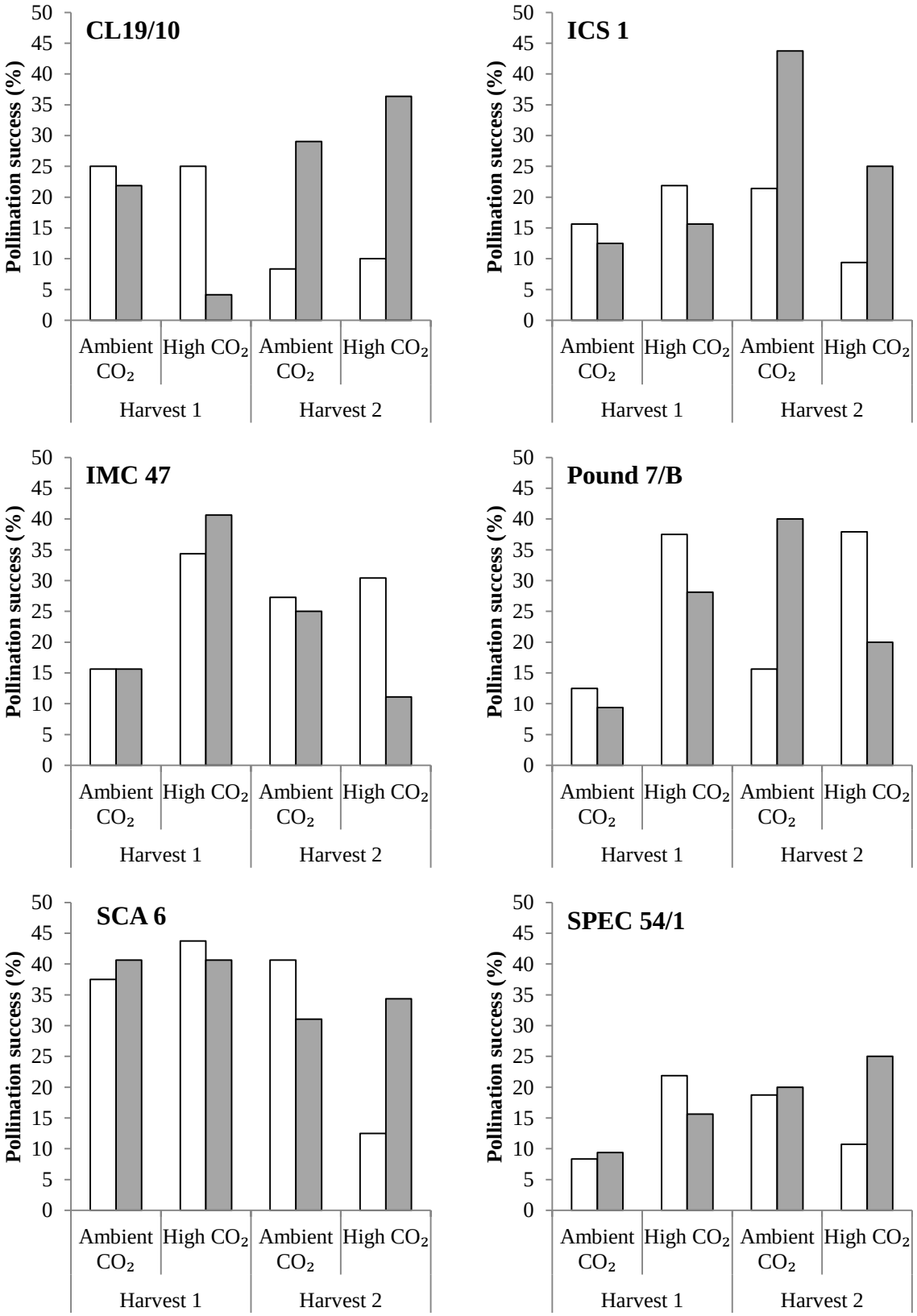

Figure 5.3 Success rate of manual pollinations of six cocoa genotypes grown at ambient and elevated CO_2 and under two watering treatments.
Calculated using the number of pollination attempts and the number of developing pods in each treatment. Only data from a period of known pollination attempts was used for harvest 1 compared to harvest 2 which used all data. □ - Well-watered. ▩ - Water-stressed.

5.3.2 Cherelle wilt

The percentage of developing pods which wilted before reaching maturity is shown in Figure 5.4. In both harvest 1 and 2 there were no significant effects of treatment on the percentage of cherelle wilt ($P > 0.05$). The wilt percentage was consistently lower under water stress and high CO_2. An additional chi squared analysis was conducted using only the data from the high CO_2 treatment to test for the effect of water stress; however no significant effect of water stress was observed ($P > 0.05$). Genotypes CL19/10, Pound 7/B, SCA 6 and SPEC 54/1 all showed no significant responses to treatments ($P > 0.05$) (Figure 5.5). For the clone ICS 1 a significant increase in cherelle wilt was observed under high CO_2 ($P < 0.05$) in harvest 1 but no significant differences were seen in harvest 2. A consistent reduction in cherelle wilt was observed for the clone IMC 47 under 'WS' treatments ($P < 0.01$ and $P < 0.05$ for harvest 1 and 2, respectively).

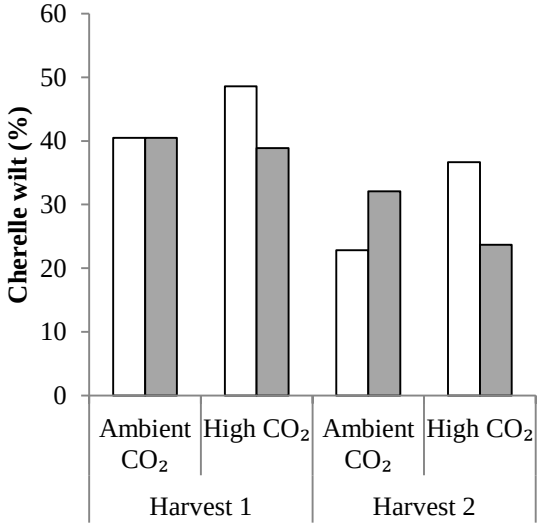

Figure 5.4 Cherelle wilt of trees grown under two concentrations of CO_2 and two water treatments. Data combined across six genotypes.
Percentage of cherelle wilt was calculated using the number of successful pollinations and the number of pods to reach maturity from all genotypes in each treatment. □ - Well-watered. ▩ - Water-stressed.

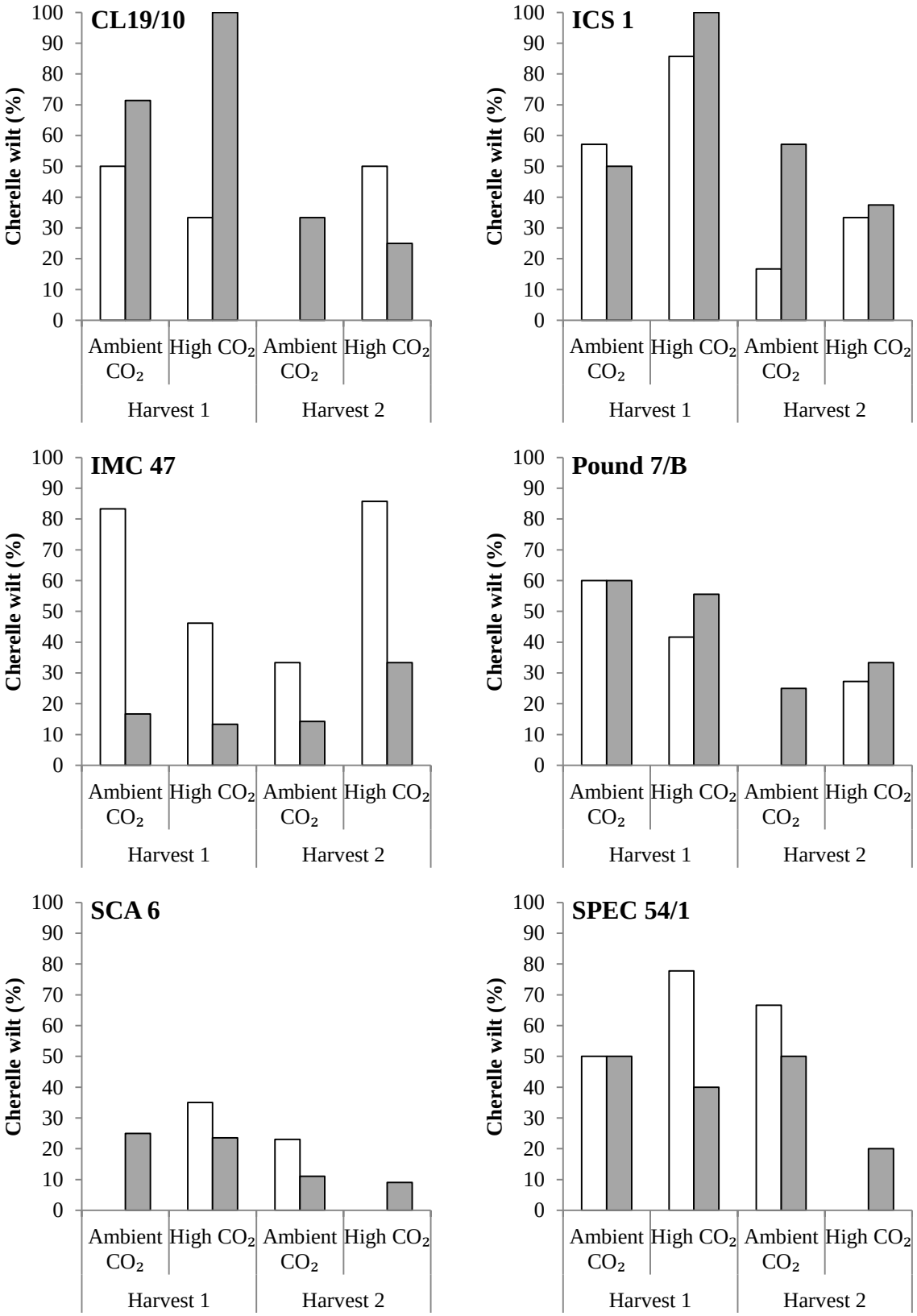

Figure 5.5 Cherelle wilt of six cocoa genotypes grown at ambient and elevated CO_2 and under two watering treatments.
Percentage of cherelle wilt was calculated using the number of successful pollinations and the number of pods to reach maturity from each genotype in each treatment. □ - Well-watered. ■ - Water-stressed.

The number of weeks of growth before a pod wilted is shown in Figure 5.6. There was a significant increase in the length of time before wilt under water stress in harvest 1 ($P < 0.05$) but no significant differences in harvest 2. There was no significant effect of CO_2 treatment in either harvest ($P > 0.05$). Additionally, an effect of genotype was also observed in harvest 1 ($P < 0.05$) but not in harvest 2 ($P > 0.05$). An increase in time to wilt was observed in harvest 1 for the clone CL19/10 ($P < 0.05$); however a decrease was also observed in IMC 47 ($P < 0.05$) (Figure 5.7). Contrasting results were also observed for the clone ICS 1 as a decrease in time to wilt was observed at elevated CO_2 at harvest 1 whereas an increase in time to wilting was observed at harvest 2 (both $P < 0.05$). A significant interaction between water and CO_2 treatments was observed in harvest 1 for SPEC 54/1. This represents an increase in time to wilt under water stress when in ambient CO_2, but a decrease under water stress when under high CO_2. In harvest 1 the shortest average time to wilt was observed for SPEC 54/1 at 3.33 weeks. Pound 7/B had the longest average time to wilt at 6.62 weeks.

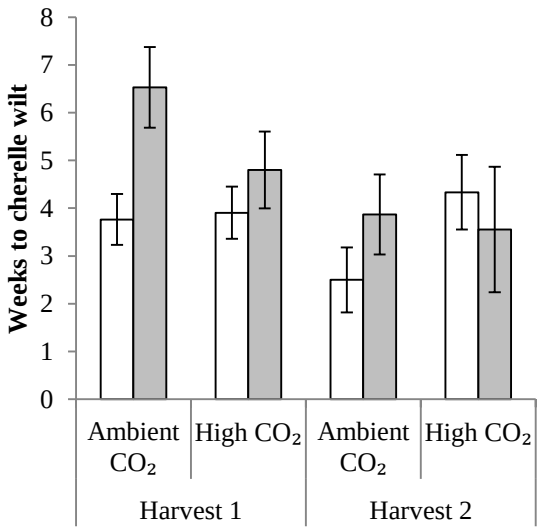

Figure 5.6 Time to cherelle wilt of trees grown under two concentrations of CO_2 and two water treatments. Data combined across six genotypes.
The number of weeks of recorded pod growth prior to pod wilt was averaged across all genotypes per treatment. □ - Well-watered. ▩ - Water-stressed. Error bars show standard error of the mean.

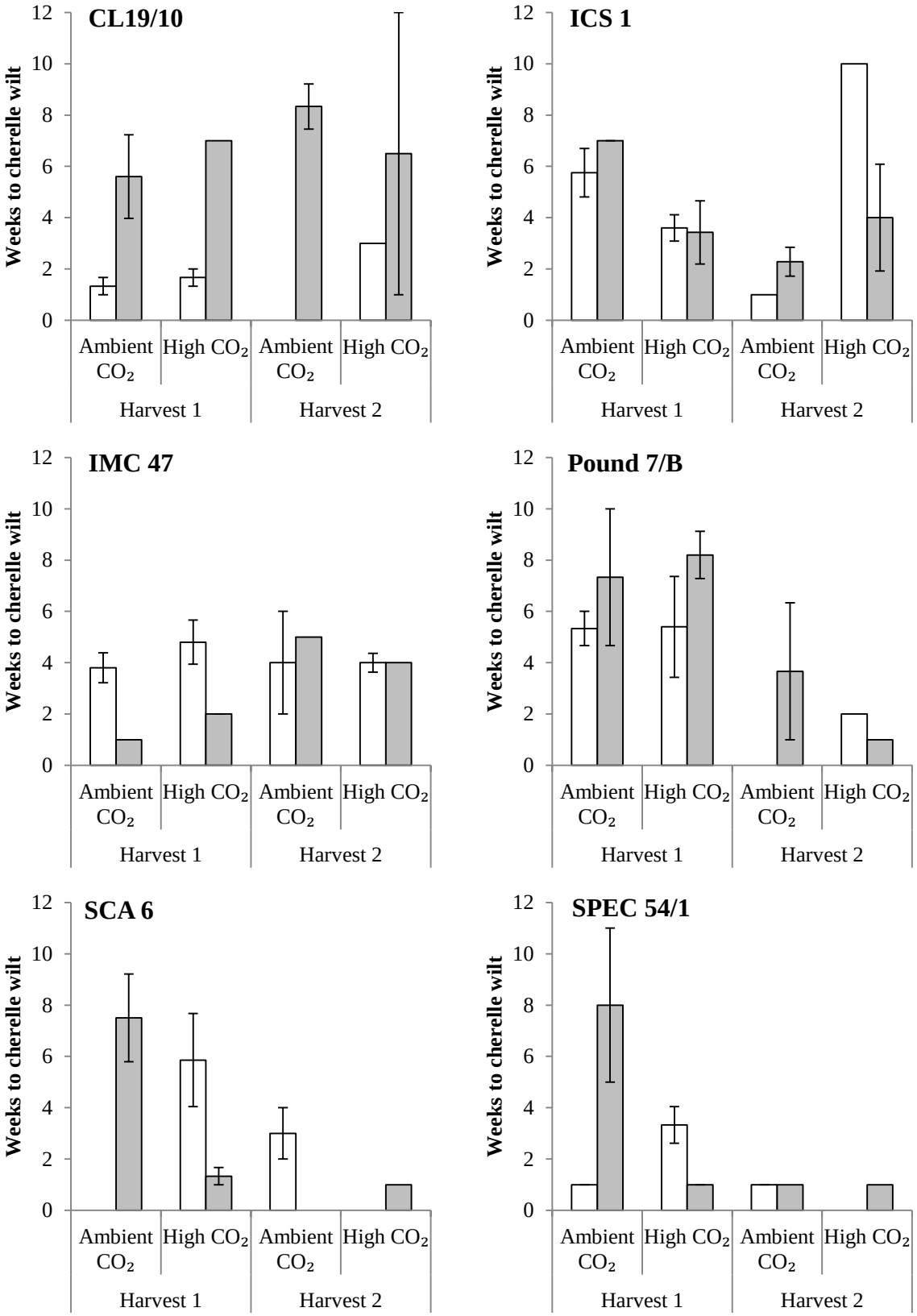

Figure 5.7 Time to cherelle wilt of six cocoa genotypes grown at ambient and elevated CO_2 and under two watering treatments.
The number of weeks of recorded pod growth prior to pod wilt was averaged for each genotype per treatment. □ - Well-watered. ▒ - Water-stressed. Error bars show standard error of the mean.

5.3.3 Maximum pod size

Maximum pod size (sum of regression parameters 'A' and 'C') was averaged for each treatment using data from all genotypes (Figure 5.8). The effect of treatment can also be observed in the average growth curves (Figure 5.9) A significant reduction of 22.6% in maximum pod size was observed under water stress in harvest 1 ($P < 0.001$), and a significant increase of 20.8% in maximum pod size was observed under high CO_2 in harvest 2 ($P < 0.001$). A significant effect of genotype was observed at both harvests ($P < 0.001$) as was a significant interaction between genotype and water stress ($P < 0.01$ and $P < 0.05$ for harvest 1 and 2 respectively). An additional interaction between genotype, water treatment, and CO_2 treatment was also observed in harvest 2 ($P < 0.01$). Maximum pod size was also studied for individual genotypes (Figure 5.10) and the effect of treatment can also be observed in the average growth curves (Figure 5.11). In harvest 1, a significant increase in maximum pod size was observed in response to elevated CO_2 for the clone ICS 1 ($P < 0.05$). A significant decrease in maximum pod size was observed in response to water stress for the clones ICS 1 ($P < 0.05$) and Pound 7/B ($P < 0.01$). This trend was also observed for SPEC 54/1 however this was not significant. No other treatment responses were observed in this harvest. In harvest 2, a significant increase in maximum pod size was observed in response to elevated CO_2 for the clones CL19/10 ($P < 0.01$), Pound 7/B ($P < 0.05$), and SCA 6 ($P < 0.05$). A significant increase in pod size was observed under water stress for clone CL19/10 ($P < 0.05$) however a significant decrease was observed for ICS 1 ($P < 0.05$). A significant interaction between water treatment and CO_2 treatment was also observed for ICS 1 ($P < 0.05$) representing an increase in pod size when a water stressed tree was under high CO_2 conditions. This trend of water stress effect alleviation under high CO_2 was also noted for Pound 7/B however this was not significant. The smallest mean pod size was for the genotype

SCA 6 at 214.8cm^3 and 323.3cm^3 in harvest 1 and 2 respectively. The largest mean pod size was for the genotype ICS 1 at 507.6cm^3 and 671.0cm^3 in harvest 1 and 2 respectively.

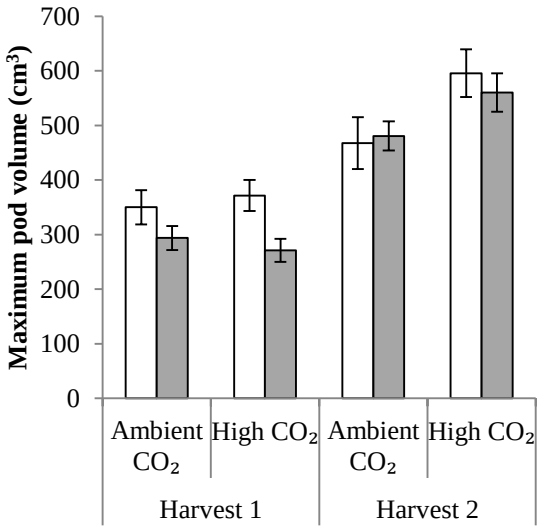

Figure 5.8 Mean maximum pod size of trees grown under two concentrations of CO_2 and two water treatments. Data combined across six genotypes.
Maximum pod size (sum of regression values 'A' and 'C') was averaged across all genotypes for each treatment. □ - Well-watered. ■ - Water-stressed. Error bars display standard error.

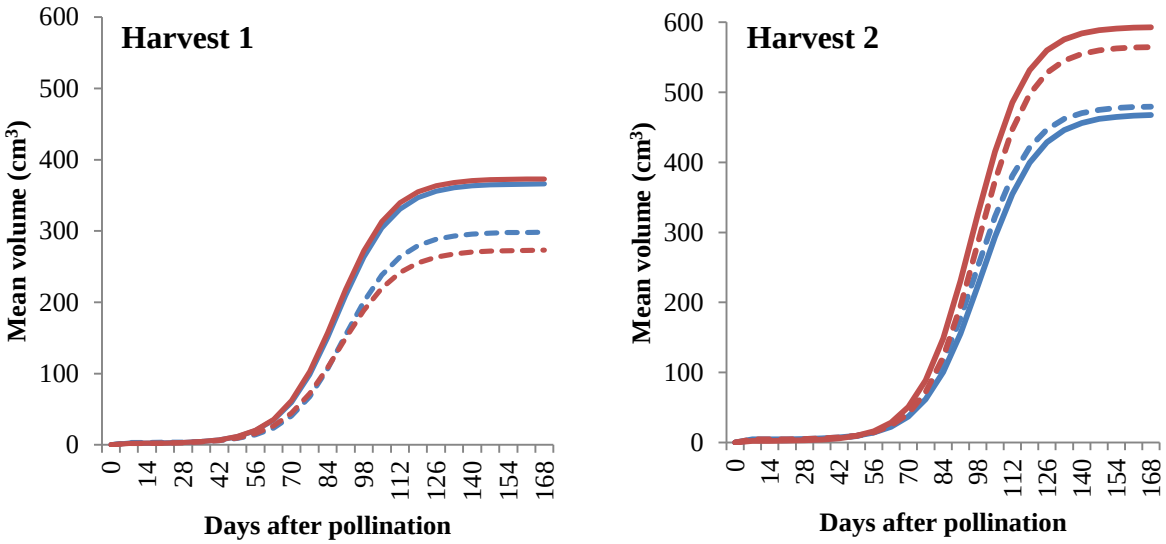

Figure 5.9 Mean regression curves of trees grown under two concentrations of CO_2 and two water treatments. Data combined across six genotypes.
Plots based on the GenStat standard logistic regression equation (Equation 5.2), where individual parameters (A, C, B and M) were taken from each pod regression and averaged using a weighted means calculation. Averaged parameters were fed into the equation using a hypothetical timeline creating an average curve. Well-watered (solid line), Water stressed (dashed line), Ambient CO_2 (●) and High CO_2 (●).

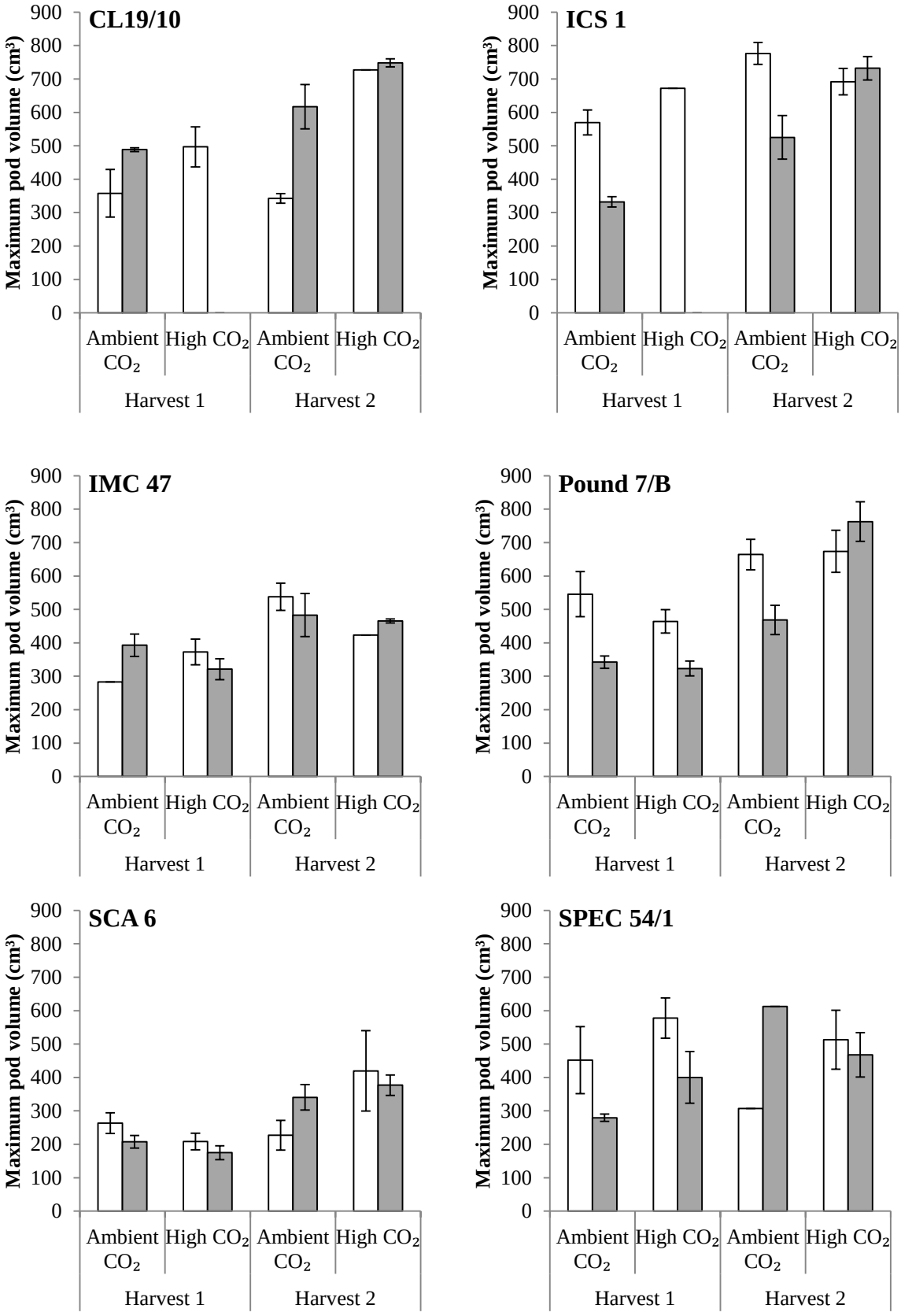

Figure 5.10 Mean maximum pod size of six cocoa genotypes grown at ambient and elevated CO_2 and under two watering treatments.
Maximum pod size (sum of regression values 'A' and 'C') was averaged for each genotype for each treatment. □ - Well-watered. ▒ - Water-stressed. Error bars display standard error.

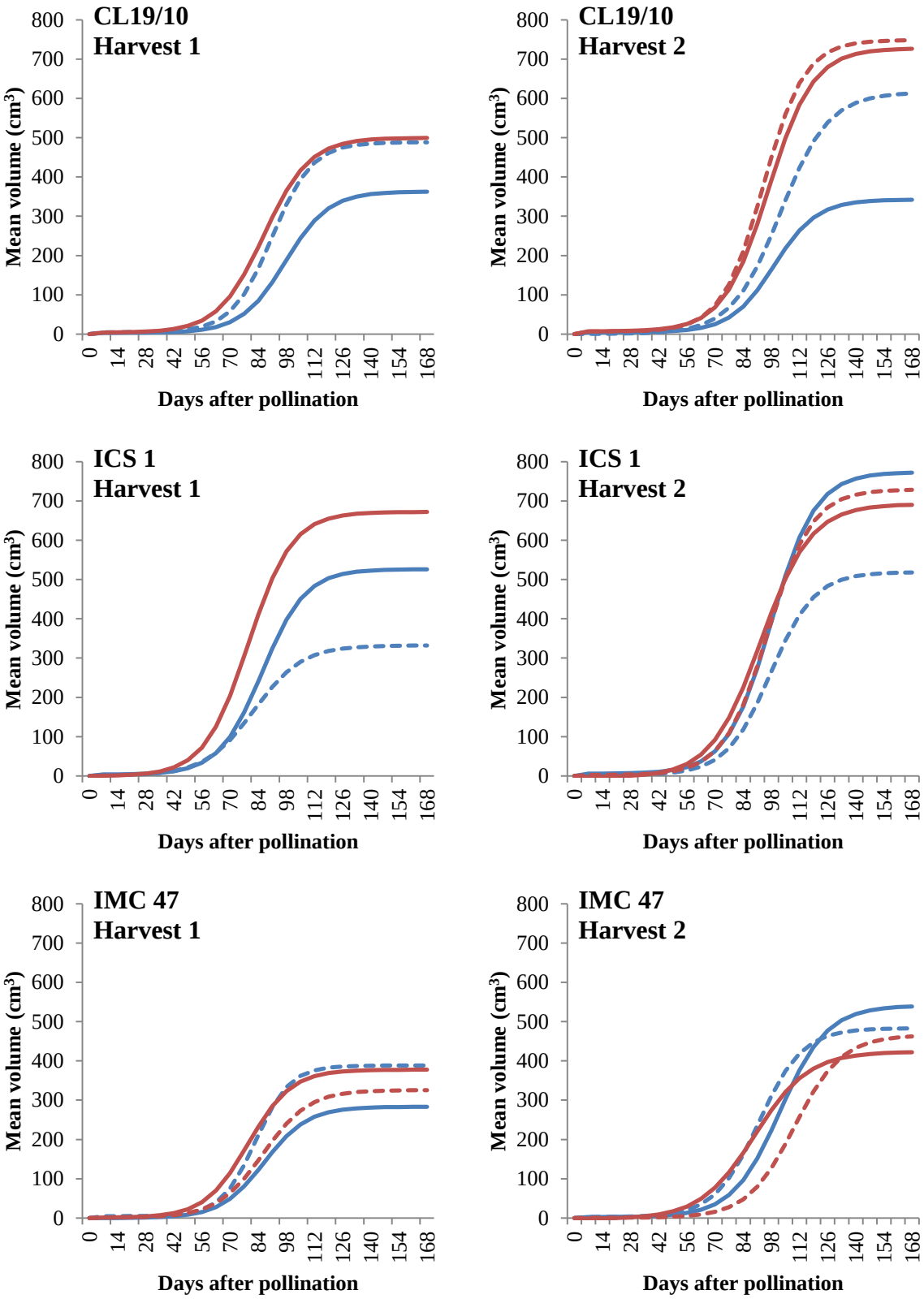

Figure 5.11 Mean regression curves of six cocoa genotypes grown at ambient and elevated CO_2 and under two watering treatments.
Plots based on the GenStat standard logistic regression equation (Equation 5.2), where individual parameters (A, C, B and M) were taken from each pod regression and averaged using a weighted means calculation. Averaged parameters were fed into the equation using a hypothetical timeline creating an average curve. Well-watered (solid line), Water stressed (dashed line), Ambient CO_2 (●) and High CO_2 (●).

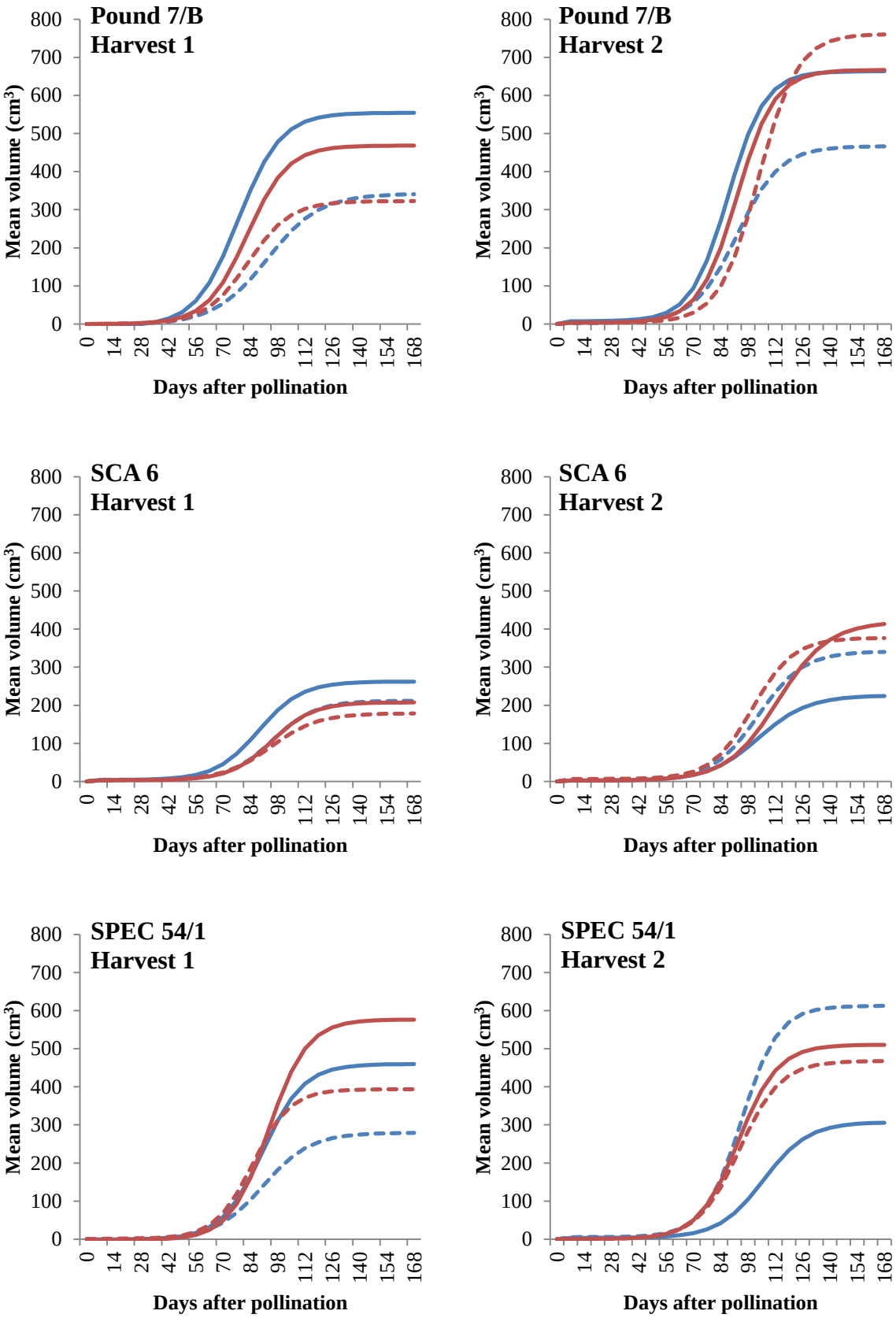

Figure 5.11 continued
Well-watered (solid line), Water stressed (dashed line), Ambient CO_2 (●) and High CO_2 (●).

5.3.4 Maximum rate of pod growth

The mean maximum rate of pod growth represents the maximum rate of increase in pod volume estimated from the fitted logistic function. This can be seen in Figure 5.12 for all genotypes combined. In harvest 1, 'WS' treatments reduced the maximum rate of growth significantly ($P < 0.001$). There were also significant differences between genotypes ($P < 0.001$) ranging from an average maximum growth rate of 4.9 cm^3 day^{-1} for the clone SCA 6, to 12.2 cm^3 day^{-1} for the clone ICS 1. There was an interaction between genotype and water availability ($P < 0.01$). A significant reduction of maximum rate of growth was observed under water stress for the clones Pound 7/B ($P = 0.01$) and SCA 6 ($P < 0.05$) (Figure 5.13). At harvest 2 the effect of water availability was not seen although a significant increase in maximum growth rate was observed under high CO_2 conditions ($P < 0.001$). Again, the effect of genotype was significant ($P < 0.001$) ranging from an average maximum growth rate of 6.9 cm^3 day^{-1} for the clone SCA 6, to 15.1 cm^3 day^{-1} for the clone ICS 1. Finally, in harvest 2 interactions were seen between water availability and genotype ($P < 0.05$), and between genotype, water availability and CO_2 treatment ($P < 0.05$). A significant increase in the rate of growth was observed under high CO_2 for the clones CL19/10 ($P < 0.01$), Pound 7/B ($P < 0.05$), and SCA 6 ($P < 0.01$).

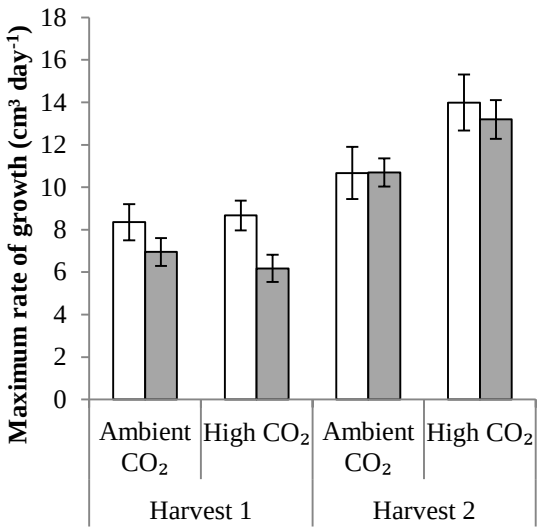

Figure 5.12 Mean maximum rate of pod growth of trees grown under two concentrations of CO_2 and two water treatments. Data combined across six genotypes.
Maximum rate of growth was averaged across all genotypes for each treatment. □ - Well-watered. ▨ - Water-stressed. Error bars display standard error.

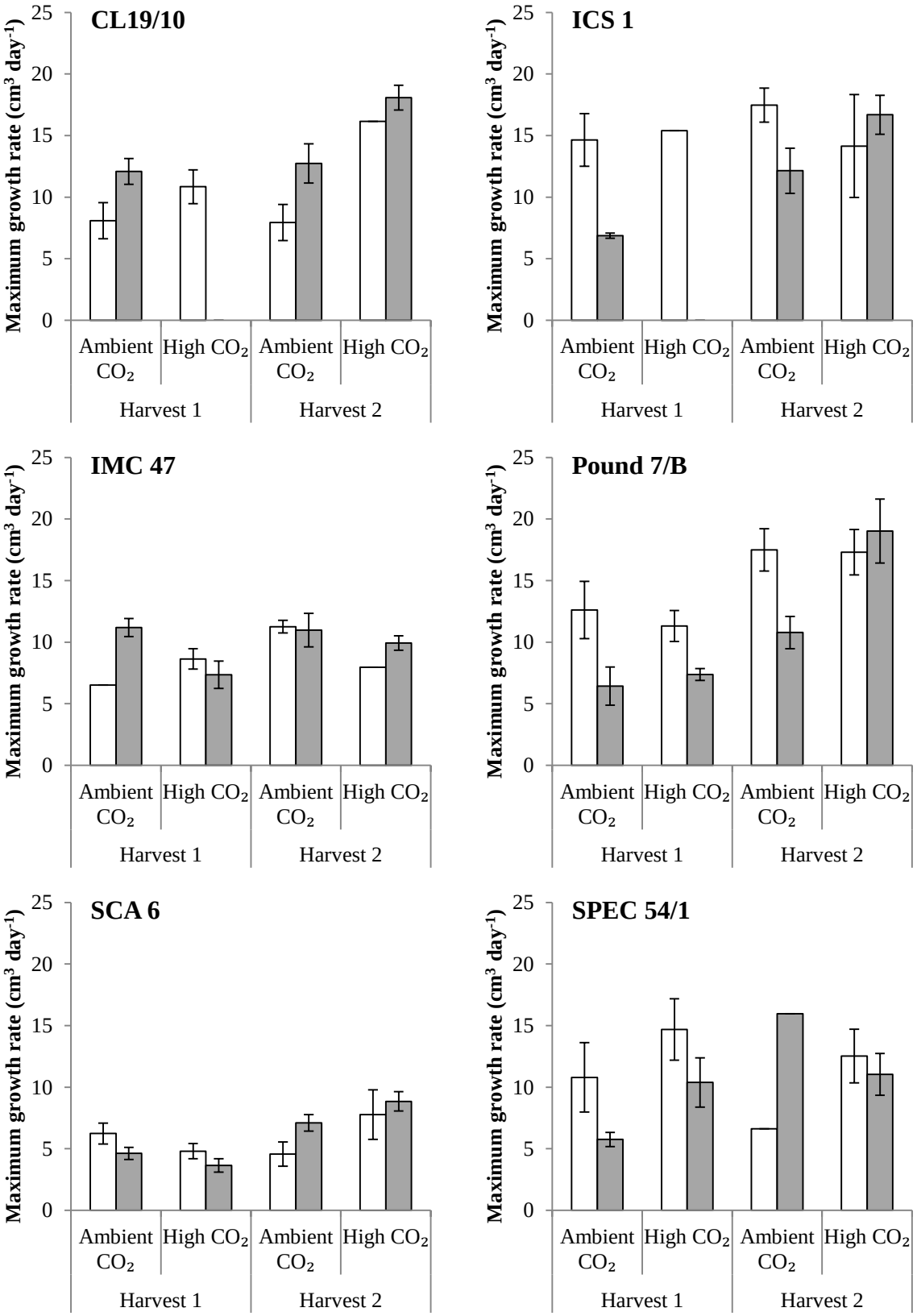

Figure 5.13 Mean maximum rate of pod growth of six cocoa genotypes grown at ambient and elevated CO_2 and under two watering treatments.
Maximum rate of growth was averaged for each genotype for each treatment. □ - Well-watered. ▨ - Water-stressed. Error bars display standard error.

5.3.5 Time to reach maximum growth rate

The mean number of days to reach maximum rate of pod growth across all genotypes is shown in Figure 5.14. This was determined by the time to reach the steepest gradient on the growth curves in Figure 5.9. There was no significant effect of CO_2 or water treatment during harvest 1; however there was a significant interaction between water treatment, CO_2 treatment, and genotype during harvest 2 ($P < 0.05$). In harvest 1, a significant reduction in time to reach maximum pod growth was observed under water stress for the genotype ICS 1 ($P < 0.05$); however an increase was observed for IMC 47 ($P < 0.05$) (Figure 5.15). A significant reduction in time to maximum growth rate is observed under high CO_2 in genotypes CL19/10 ($P = 0.001$) and ICS 1 ($P < 0.05$). A significant interaction between water treatment and CO_2 treatment is observed for the genotype Pound 7/B ($P < 0.05$). This interaction represents an increase in time to maximum growth rate under water stressed conditions but only when under ambient CO_2 conditions. In harvest 2, a significant increase in time to maximum growth rate is found for Pound 7/B in response to the water stress treatment ($P < 0.05$). A significant interaction between water treatment and CO_2 treatment is also found for IMC 47 ($P < 0.01$). This interaction represents a decrease in time to maximum growth in response to water stress under ambient CO_2, but an increase in response to water stress and high CO_2. There were significant differences between genotypes during both harvests (both $P < 0.001$). The longest time to maximum growth was observed for SCA 6 at 93.8 days and 103.8 days in harvest 1 and 2 respectively, whereas the earliest maximum growth rate was for ICS 1 in harvest 1 at 83.5 days and for Pound 7/B in harvest 2 at 92.9 days after pollination. Furthermore, there was a noticeable increase in time to reach maximum pod growth in harvest 2.

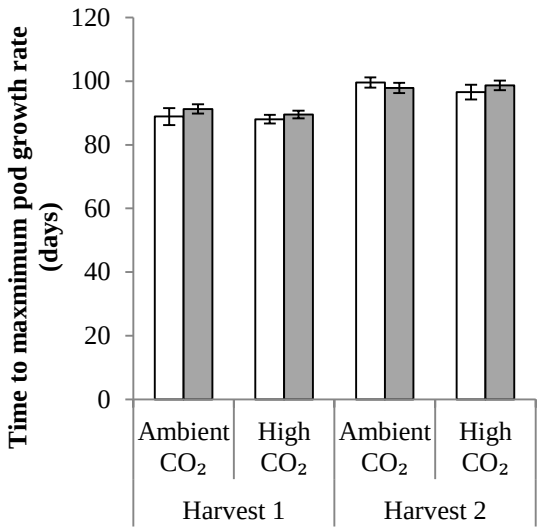

Figure 5.14 Mean number of days post-pollination at which the highest rate of growth occurred of trees grown under two concentrations of CO_2 and two water treatments. Data combined across six genotypes.

The day after pollination at which maximum growth occurred was averaged from all genotypes for each treatment. □ - Well-watered. ▩ - Water-stressed. Error bars display standard error.

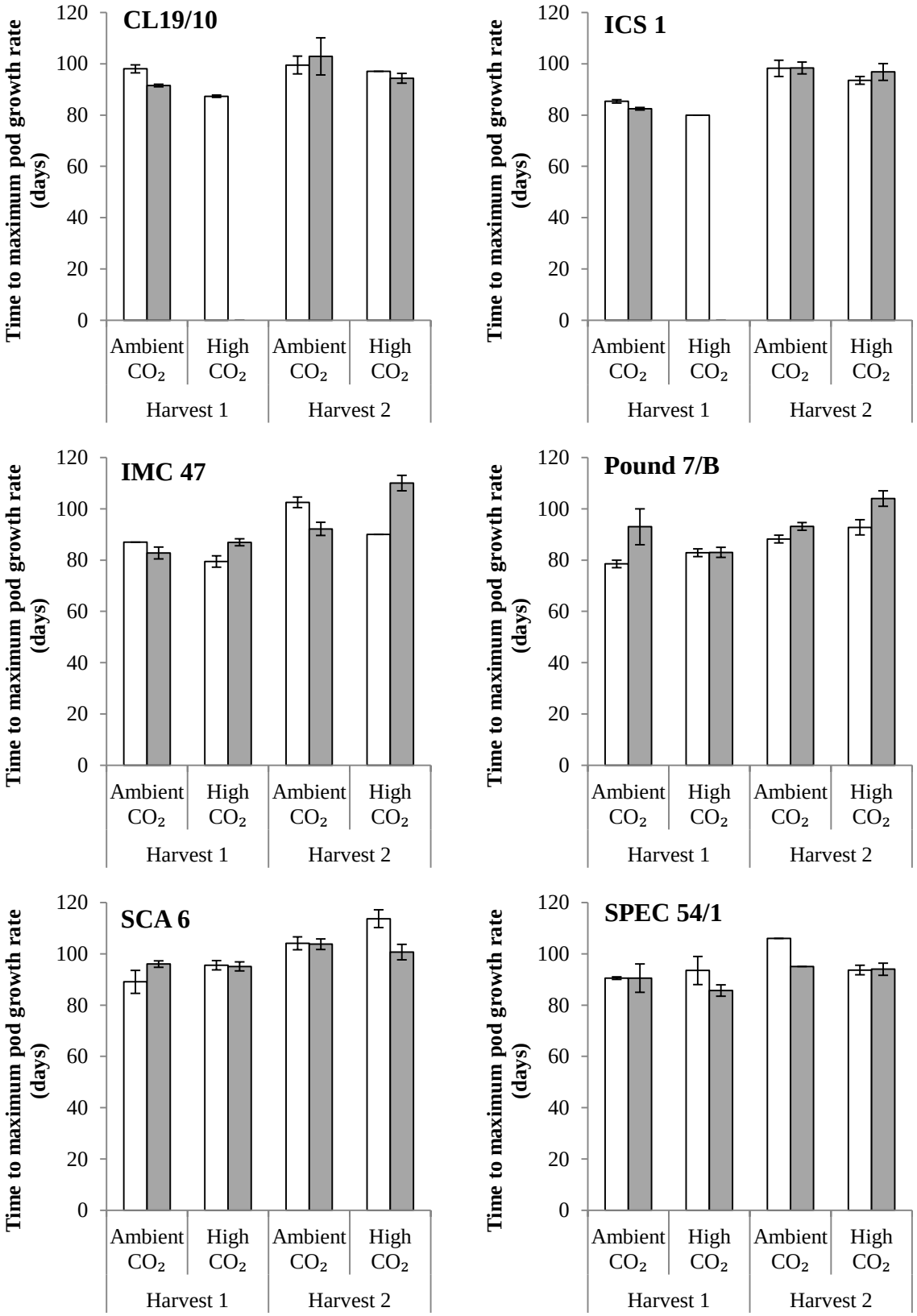

Figure 5.15 Mean number of days post-pollination at which the highest rate of growth occurred of six cocoa genotypes grown at ambient and elevated CO_2 and under two watering treatments.

The day after pollination at which maximum growth occurred was averaged for each genotype for each treatment. □ - Well-watered. ▩ - Water-stressed. Error bars display standard error.

5.3.6 Time to reach 95% maximum size

The number of days post-pollination to reach 95% of maximum pod size was used as a measure of duration of pod growth. Data for all genotypes combined is shown in Figure 5.16. Overall there were no significant effects of water stress or high CO_2 in either harvest, although duration to 95% was generally longer in harvest 2. A significant interaction between water treatment, CO_2 treatment, and genotype was observed in harvest 1 ($P < 0.05$). In harvest 1, a significant reduction in time to reach 95% maximum pod size was observed under high CO_2 for the genotype CL19/10 ($P < 0.05$) (Figure 5.17). A significant reduction in time to 95% maximum size was also observed under water stress for the genotype SPEC 54/1 ($P < 0.001$). An interaction between water treatment and CO_2 treatment was also observed for the clones SCA 6 ($P < 0.05$) and SPEC 54/1 ($P < 0.01$). For SCA 6 this interaction represents an increase in time to 95% maximum pod size when water stress was combined with high CO_2. However, in SPEC 54/1 the interaction indicates the opposite effect of a reduction in time to 95% maximum size when water stress was combined with high CO_2. In harvest 2, a significant increase in time to 95% maximum size was observed under high CO_2 for Pound 7/B ($P < 0.05$), whereas a significant reduction was observed for SPEC 54/1 ($P < 0.05$). A significant increase in time to 95% maximum size was observed under water stress for Pound 7/B ($P < 0.05$). Genotype had a significant effect on time to 95% maximum size in both harvest 1 and harvest 2 ($P < 0.01$). The longest average time to 95% maximum size is observed in clone IM 47 at 124 and 139 days in harvest 1 and 2 respectively. The shortest average time to 95% maximum size in harvest 1 is observed in clone ICS 1 at 116 days, whereas in harvest 2 it is Pound 7/B at 124 days.

A regression analysis was carried out to identify a possible relationship between duration of pod growth and maximum pod size. The analysis was carried out for genotypes individually due to the genotypic variation in pod size and duration of growth. No significant relationships were identified in either harvest for any genotype.

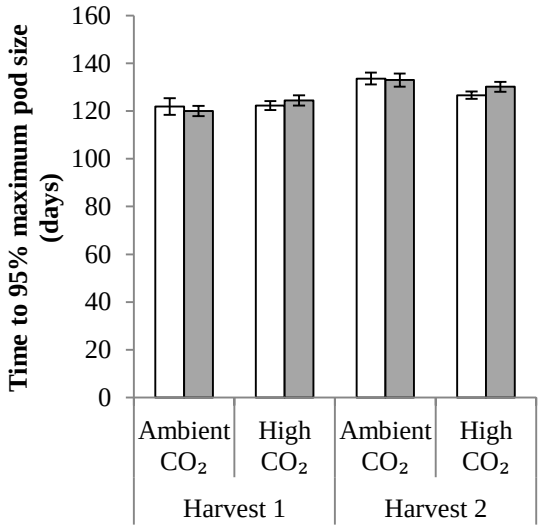

Figure 5.16 Mean number of days post-pollination to reach 95% of maximum pod size of trees grown under two concentrations of CO_2 and two water treatments. Data combined across six genotypes.
The day at which 95% of maximum pod volume was reached was averaged from all genotypes for each treatment. □ - Well-watered. ▩ - Water-stressed. Error bars display standard error.

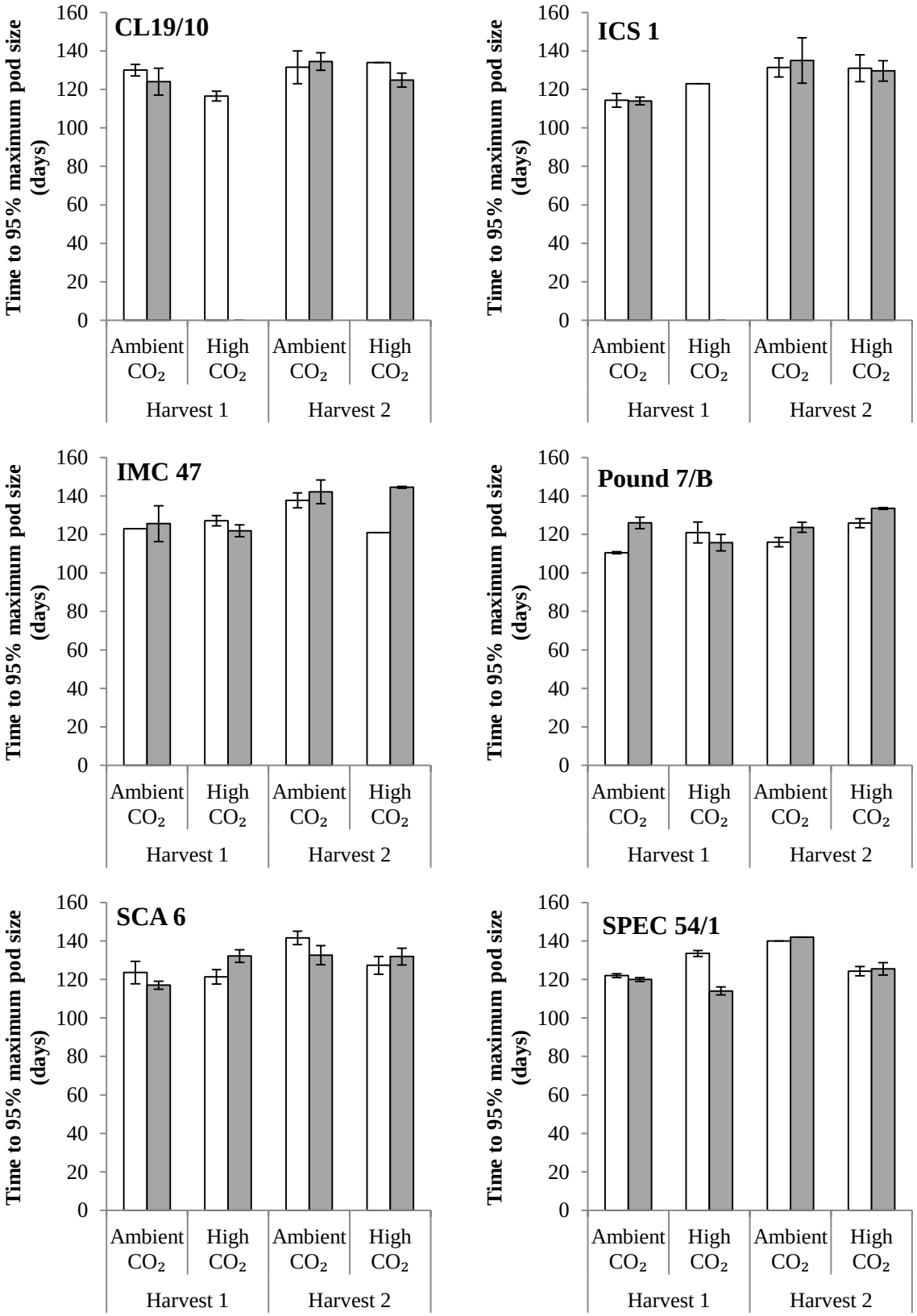

Figure 5.17 Mean number of days post-pollination to reach 95% of maximum pod size of six cocoa genotypes grown at ambient and elevated CO_2 and under two watering treatments.

The day at which 95% of maximum pod volume was reached was averaged for each genotype for each treatment. □ - Well-watered. ▨ - Water-stressed. Error bars display standard error.

5.3.7 Effect of block design

In order to identify whether there was any effect of greenhouse position on pod growth, the experiment was analysed with the following block designs:

- Block design 1: Block 1 – House 2 and 3. Block 2 – House 5 and 6.
- Block design 2: Block 1 – House 2 and 5. Block 2 – House 3 and 6.

A significant effect was observed for block design 2 at harvest 1 for maximum pod size ($P < 0.001$) and for maximum growth rate ($P < 0.001$). In harvest 2, a significant effect of block design 2 was identified for day of maximum growth rate ($P < 0.001$). Further analysis of treatment effect with each block design in isolation revealed no effect of greenhouse location on the treatment effects observed overall. Any treatment effect trends reported above were present in both blocks.

5.4 Discussion

Overall, there was a shift in response of pollination success to treatments between the two harvest periods (Figure 5.2). Initially, trees grown under high CO_2 had a significantly higher pollination success rate, whilst a trend of a lower pollination success rate was observed for 'WS' trees. However, this response was reversed in harvest 2 when a reduced pollination success rate was observed at high CO_2 (although not significant) and 'WS' resulted in a significant increase in pollination success rate. This pattern was visible in some individual genotypes but not others. The enhancement of pollination success under high CO_2 in harvest 1 may be a reflection of the increased photosynthetic rates observed in these trees at elevated CO_2 (Lahive, 2015). Fruit setting in cocoa is identified as an indicator of a tree's fruiting potential based on the availability of assimilates (Valle et al., 1990). Furthermore, a link between assimilate/carbohydrate availability and fruit set in Satsuma mandarins (*Citrus*

unshiu (Mak.) Marc.) has been observed as stem-injected sucrose supplementation resulted in 10% increases in fruit set (Iglesias et al., 2003). The increase in pollination success may be indicative of a larger fruit carrying capacity under elevated CO_2. In addition, water stress resulted in a reduction in pollination success, although this was not significant. Water stress reduced the photosynthetic rate of these trees (Lahive, 2015) further suggesting a link between assimilate availability and pollination success. As the donor pollen was not taken from experimental trees, the variation in pollination success rate can only be attributed to floral health and viability of maternal trees, or manual pollination technique. The manual pollination procedure was standardised to ensure all attempts were a reflection of maternal tree health and not the application of control pollen. Elevated CO_2 may also provide an advantage to pollen germination performance as identified in Aneja et al. (1992), where enclosed pollinations in vivo allowed for an accumulation of CO_2. This resulted in fruit set at close to 100% of the manual pollinations performed. This might further explain the increase in pollination success under high CO_2 in harvest 1; however the CO_2 concentrations in this study are much lower than those of Aneja et al. (1992).

The shift in observed response to treatment in harvest 2 suggests a change in plant behaviour over a prolonged exposure to treatments. Elevated CO_2 resulted in an increased overall tree biomass (Chapter 7). It is already known that new flush leaves act as a stronger assimilate sink than flowers and early developing pods (Alvim, 1954), suggesting that vegetative growth holds priority over reproductive development. A general reduction in pollination success was observed during harvest 2 in response to high CO_2. Prolonged exposure to the elevated CO_2 conditions increased the growth of vegetative material and as a result may have increased the competition for assimilates within the tree. Individual leaf (Lahive, 2015) and total leaf weight (Chapter 7) did not change in response to elevated CO_2, nor did the flush interval (Lahive, 2015), suggesting any change in assimilate competition was due to the changes in

other forms of vegetative growth such as wood and roots. A reduced allocation of assimilates to reproduction would explain the reduction in pollination success. The explanation as to why greater pollination success was observed under water stress in harvest 2 is less clear. It would be expected that through reducing rates of photosynthesis, water-stress would have detrimental effects on pollination success. However, it has previously been demonstrated by Sale (1970b) and Alvim and Alvim (1977), that water-stress reduces floral opening in cocoa, whilst re-watering or rains after a period of water stress stimulates floral opening. These examples represent an increase in assimilate allocation to reproductive development once a stress period ends, potentially similar to the re-water events in the water stress schedule of this study. An increase in assimilate allocation to flowers may provide explanation as to why pollination success was seen to increase. Flushing in cocoa is stimulated at the end of a period of water stress (Alvim, 1977; Sale, 1970b). However, if water stress persists, new flush growth withers, area per leaf and net leaf area is decreased, and leaf duration on the tree is reduced (Sale, 1970b). As previously discussed, new shoots are a stronger resource sink over developing fruits. If the vegetative growth of the water stressed trees was inhibited, this may result in increased resource allocation to reproductive growth. Further evidence to support this was found in the destructive harvest (Chapter 7) where water stress significantly reduced the total leaf weight, total wood weight and total leaf area of a tree.

The contrasting pollination success results prompted closer inspection of the treatment conditions surrounding the pollination periods for harvest 1 and 2. The methods for maintaining a water stress treatment had changed between pollinations in harvest 1 (beginning February 2014) and the pollinations in harvest 2 (beginning November 2014). Initial methods for maintaining water stress became unsuitable as the rate of water use increased with tree age (Chapter 2). As a result, the irrigation method was changed and ‘WS’ trees were switched to a dripper system which gave a more continuous low level irrigation.

This allowed for more accurate control over water stress conditions. During the pollinations of harvest 1, the old irrigation methods resulted in regular soil moisture conditions of below 10% (the point at which the risk of leaf wilt and drop was greatly increased) (range 8.5 – 15.6%, mean 11.1%). However, during harvest 2 the water stress treatments were under much tighter control (range 11.7 – 17.2%, mean 14.9). If the more severe stress in harvest 1 resulted in adaptation to water stress, it is possible that during the more controlled water stress method of harvest 2, the treatment had less of an impact. Adaptations to water stress can include an improved root-shoot ratio in which the plant invests more in root development to enhance water uptake (Kozlowski and Pallardy, 2002). Osmotic adjustment to improve leaf turgor and molecular changes are also potential mechanisms to improve a plants resistance to water stress. For example, dehydrin and ubiquitin can prevent denaturation and damage to proteins in water-stressed conditions. Additionally, aquaporin water channels can result in increased water flow across membranes and reduce soil to plant resistance (Kozlowski and Pallardy, 2002). As observed in loblolly pine, the severity of the induced water stress alters the gene expression which follows (Watkinson et al., 2003). The more severe water stress of harvest 1 may have resulted in additional adaptation responses within the plant which could decrease the effect of the more controlled water stress treatment. It has been observed in grapevines that the degree of stress affects the speed of photosynthetic recovery. Once extreme stress has occurred, the previous maximum rate of photosynthesis may not be recovered straight away (Flexas et al., 2006). Further study would be required to identify any varied responses of tree adaptation to different severities of water stress.

Statistical analysis of percentage cherelle wilt revealed no significant responses to elevated CO_2 or water stress. This contradicts research in Valencia Orange (*citrus sinensis* (L.) Osbeck) which identified a 70% increase in fruit retention in trees grown at elevated CO_2 compared to control trees (Downton et al., 1987). Additionally, the water stress response in

this study is contradictory to prior research in cocoa observing higher incidence of cherelle wilt under soil moisture stress (Sale, 1970b). In well-watered conditions, although not significant, there was a trend for elevated CO_2 to consistently increase the proportion of cherelle wilt. Elevated CO_2 increased the rate of photosynthesis in these trees (Lahive, 2015) and therefore would have an increased production of assimilates. However, additionally the trees grown under elevated CO_2 demonstrated an increase in vegetative growth representing a larger vegetative sink. This may have outcompeted pod development for assimilates (Valle et al., 1990; Alvim, 1954) and resulted in the increased wilt which is observed. An additional explanation as to why a general trend for increased wilt under elevated CO_2 may be the observed increased rate of pod growth seen under elevated CO_2 in harvest 2. Faster pod growth rates and larger pods under high CO_2 will have increased assimilate demand and competition between pods in harvest 2. This may have resulted in the increase in wilt observed. An overall reduced percentage of wilt in the second harvest suggests increased reproductive capacity with tree age, potentially representing an increase in allocation of assimilates to reproduction as the trees grew older. However, this may also be a reflection of generally increased photosynthetic capacity and assimilate production as tree canopies grew larger and more productive.

Overall, both maximum pod size and maximum rate of pod growth demonstrated the same responses to treatment. In harvest 1, water stress reduced maximum size and rate of growth whilst CO_2 had no effect. However in harvest 2, 'WS' had no effect and high CO_2 resulted in an increase in size and rate. Additionally, in harvest 2 there is a general increase in growth rate and final pod size. A reduction due to water stress is to be expected if the stress is having a detrimental effect on photosynthetic rates and thus assimilate availability. Equally, elevated CO_2 increased photosynthetic rate and would therefore increase assimilate production leading to the enhanced size and rate demonstrated in harvest 2. The absence of 'WS' effect in

harvest 2 may be indicative of improved water use efficiency (WUE) with prolonged exposure to treatment. Increased WUE was observed by Lahive (2015) in response to the water stress treatment. Additionally, as discussed earlier the severity of stress in harvest 1 may have induced adaptations to water stress lessening the effects of stress in harvest 2. Trees would have grown and developed larger root systems naturally over time; however water stress may have further stimulated root growth (Kozlowski and Pallardy, 2002). The lack of a response to high CO_2 in harvest 1 may be due to a requirement for a longer exposure time to elevated CO_2 before any assimilate increases become allocated to reproductive development. However, increases in yield of Valencia Orange (*citrus sinensis* (L.) Osbeck) in response to elevated CO_2 occurred in the first 12 months of exposure (Downton et al., 1987). The speed at which the effects of elevated CO_2 are observed in the fruiting of trees may vary between species.

The number of days to reach a maximum rate of growth and the days to reach 95% of maximum pod size did not show any response to treatment in either harvest. However, in both cases the second harvest showed maximum rate occurred later and duration of growth was longer compared to harvest 1. In all instances the variations in pod growth between genotypes was significant.

Genotypes varied in their pod size, rate of growth, point of maximum growth rate and duration of pod growth. Additionally there were significant interactions between genotypes and treatments indicating not all genotypes respond equally to water stress and elevated CO_2. Richness in the genetic diversity in the responses to water stress and elevated CO_2 will be a key factor in breeding cocoa varieties for future climates. Tailoring cocoa varieties to suit their growing locations will help to maximise yields and protect livelihoods in a changing environment.

Finally, the block effects which were observed on maximum pod size, maximum rate of growth, and time to maximum growth rate, were all significant for block design 2. This identifies houses 3 and 6 in one block and houses 2 and 5 in the other. Houses 3 and 6 have exterior side walls exposed to direct sunlight for prolonged periods during the day. Houses 2 and 5 mainly received sunlight from above. No differences in the temperatures are observed between the greenhouses; however the internal light levels were not monitored. The sun exposed walls may have resulted in higher light levels within houses 3 and 6. Although these differences may have resulted in changes to the degree of treatment response, closer examination of the data shows no contrasting responses to treatment in the different blocks. Overall there is no evidence to suggest greenhouse location changed the tree response to treatment.

5.5 Conclusion

Water-stress exhibited detrimental effects on pollination, maximum pod size and maximum rate of pod growth; however these effects did not persist when trees were older and had been exposed to the stress conditions for a prolonged period of time. Elevated CO_2 increased the success rate of manual pollination attempts; however this did not persist into harvest 2 where vegetative growth may out-compete fruit setting. Additionally, a trend of increased wilt under high CO_2 further suggested increased internal resource competition. Instances of water-stress appeared to counter-act the increase in wilt under high CO_2, potentially through suppression of vegetative growth decreasing sink competition. Finally, once pods passed beyond the 'wilt period', fruits appeared to benefit from the increased assimilates brought about by high CO_2, as pod size and rate of growth increased.

Chapter 6. The effects of elevated CO_2 and water stress on the yield components and bean quality of cacao

6.1 Introduction

The fruits/pods of *Theobroma cacao* are described as an indehiscent drupe which, after wilting or reaching maturity, remains attached to the tree. Generally, the pods take the form of a tough, fleshy husk surrounding the beans in the centre. The beans are surrounded by a mucilaginous pulp and each bean connects to a central placenta (Toxopeus, 1985). Typically the number of beans per pod is between 30-40 (Toxopeus, 1985) and bean size also varies depending on the genotype. Both of these factors contribute to genotypic variation in 'pod index' which indicates the number of pods required to obtain 1kg of dried beans (Turnbull and Hadley, 2015). The three main characteristics of cocoa beans which are of greatest commercial interest are the bean weight, shell (testa) percentage, and the fat content. The bean weight can also be expressed as a 'bean count' which represents the number of beans in 100g dry weight. Ideal bean weight is 1g or above, therefore bean count should be 100 or less (Wood, 1985c). Bean size is negatively correlated to shell percentage (Toxopeus and Wessel, 1970). The shell percentage can range between 11-18% and, as the shell is of little value, a lower percentage is preferred in bean processing. However, if shell percentage is too low (often as a result of washing) beans are more vulnerable to microbial contamination (Wood, 1985c). Finally, bean fat content is also a measure of bean quality. Fat content ranges between 50-58% within the fermented bean and is the most valuable component of the crop (Wood, 1985c). The total fat content has been shown to vary in response to environmental conditions with lower fat yields in the dry season and higher levels during the monsoon (Wood, 1985c; Toxopeus and Wessel, 1970).

In addition to the quantity of fat obtained, the hardness of cocoa butter is an important factor to manufacturers, with harder butters being preferred for processing (Wood, 1985c). The hardness is dependent on the composition of the cocoa butter which can be altered by the growing conditions in which the beans develop (Wood, 1985c). Cocoa butter is comprised of a mixture of triglycerides which are formed of a glycerol bonded to a combination of three fatty acids. Within cocoa butter there are two main saturated fatty acids: C16:0 Palmitic acid and C18:0 Stearic acid; and there are two main unsaturated fatty acids: C18:1 (n-9) cis Oleic acid (polyunsaturated), and C18:2 (n-6) cis Linoleic acid (monounsaturated) (Wood, 1985c). The three most common (approximately 70%) triglycerides formed from these fatty acids are the mono-unsaturated compounds: Oleodipalmitin (POP), Oleodistearin (SOS) and Oleopalmitostearin (POS) (Wood, 1985c). The remaining triglyceride content is comprised of di-unsaturated triglycerides such as Dioleopalmitin (POO) and Dioleostearin (SOO) (Liendo et al., 1997; Wood, 1985c). Higher proportions of di-unsaturated triglycerides disrupt the formation of mono-unsaturated triglycerides resulting in a reduced melting point and a softer cocoa butter (Liendo et al., 1997; Chaiseri and Dimick, 1989). Different growing regions have been identified as factors in the hardness of cocoa butter with South America producing generally the softest, North and Central America and Africa having medium hardness, and Asia and Oceana producing generally harder butters (Chaiseri and Dimick, 1989). Fluctuations in the proportion of unsaturated fatty acids, and consequently the softness of the cocoa butter in Brazil have been linked to the temperatures in which the beans develop. Beans which formed in cooler temperatures had a lower ratio of saturated to unsaturated fatty acids and thus produced a softer butter, whereas beans produced in March (hot season) had harder cocoa butter (Wood, 1985c; Berbert, 1976). Changes in fat content and fatty acid compositions have been linked to temperatures for other crops including sunflower

(Rondanini et al., 2003) and soybean (Oliva et al., 2006), the latter of which demonstrated genotypic variability to treatment responses.

Generally, the beans contribute 32.0 - 44.5% to the total pod biomass and show a degree of variation between genotypes (Daymond et al., 2002a). As may be expected, assimilate availability, altered either through changes in light levels or amount of vegetative growth, has been linked to changes in yield (Daymond and Hadley, 2008; Daymond et al., 2002a). The difference in canopy growth between genotypes is identified as a cause of variation in assimilate production, thus affecting resource allocation for reproductive development (Yapp and Hadley, 1994). With links to assimilate availability already established, it is natural that other environmental factors which alter photosynthetic rates are also likely to have an effect on yield. A physiological production model for cocoa has suggested that over 70% of changes in bean yield can be explained by the radiation levels across the year plus the rainfall patterns from the two driest months of the year (Zuidema et al., 2005). In a drought study by Moser et al. (2010a), rainfall was intercepted by large roofs which were installed around field-grown cocoa trees to reduce the rain throughfall by approximately 80%. Trees demonstrated resilience to drought in most aspects of growth despite water levels approaching wilting point. However, bean yield showed particular sensitivity to drought and was significantly reduced compared to trees without rainfall interception. This bean yield reduction was largely attributed to a reduction in pod set and the event of pod ripening during the driest months between November (2007) and March (2008). Furthermore, drought associated with El Niño-Southern Oscillation (ENSO) has been shown to severely reduce bean yields by up to 62% (Keil et al., 2008).

As atmospheric concentrations of carbon dioxide (CO_2) have been increasing and are set to increase further in the coming years (IPCC, 2014), the influence on plant growth and yield are also of interest for many economically important crops. As previously discussed,

assimilate production rates in cocoa, as a direct result of rate of photosynthesis, has an important bearing on yield. Although elevated CO_2 studies on *T. cacao* are limited, elevated CO_2 has been previously demonstrated to increase rates of photosynthesis (Lahive, 2015; Baligar et al., 2008) as is common in most C_3 plants (Ziska et al., 1991). Therefore, yield increase would be expected as a result. Increases in photosynthesis, seed number, and seed dry weight were observed in Kidney bean when grown under elevated CO_2 conditions of 700ppm (Vara Prasad et al., 2002). The increases in overall yield were largely attributable to increases in seed number. Additionally, increases in seed number per pod, and pods per plant resulted in 15% increases in yield of soybean in a free-air carbon dioxide experiment (FACE) which created CO_2 concentrations of around 550ppm (Ort et al., 2006). Similar results may be expected in cocoa, however as genetic variability has already been identified in yield partitioning (Daymond et al., 2002a) certain genotypes may respond better than others. As new vegetative growth in cocoa has been identified as a strong assimilate sink (Valle et al., 1990; Mckelvie, 1956; Alvim, 1954), new leaf or branch/trunk growth stimulated by elevated CO_2 may reduce assimilate availability for reproductive growth. Furthermore, an interaction between the effects of elevated CO_2 and water-stress may also occur as elevated CO_2 is observed to reduce stomatal conductance in many plant species (Long et al., 2004). However, a previous study of the cocoa trees in the experiments described in this thesis revealed no change in stomatal conductance (Lahive, 2015). Improvements in water use efficiency (WUE) were observed, although this was attributed to the increases in photosynthetic rate (Lahive, 2015). Any effect of CO_2 which improves water use efficiency may help to alleviate detrimental effects of water stress.

In this study, the effects of water-stress and elevated CO_2 on each cocoa pod component were analysed from two periods of pod harvest across six genotypes of *T. cacao*. In a future climate of increased atmospheric CO_2, precipitation patterns are expected to change (IPCC,

2014). From this study, results should indicate how various genotypes will respond to these conditions, with a view to aiding in future breeding programmes.

6.2 Materials and methods

Trees of six genotypes of *T. cacao* (CL19/10, ICS 1, IMC 47, Pound 7/B, SCA 6 and SPEC 54/1) were grown in climate controlled greenhouses at the University of Reading for 2 years. They were maintained under ambient (averaging 437ppm across the whole experimental period) or elevated (700ppm) CO_2 conditions, and were irrigated under a well-watered (WW) or water-stressed (WS) conditions. Experimental methods and treatments are described in full in Chapter 2.

There were two periods of pod growth throughout the experiment (dates represent the time from first pollination to last pod harvest):

- Harvest 1 – 14.02.2014 to 07.11.2014
- Harvest 2 – 26.11.2014 to 18.07.2015

The pollinations were manual and used pollen from trees of the genotype Amelonado which were not maintained under experimental conditions (Chapter 2). For harvest 1, pod harvesting occurred between 01.09.2014 and 07.11.2014. Initially, pods were harvested when a colour change occurred however bean germination was often observed within the pod. Due to a lack of visible cues of pod ripening, 27% of the pods harvested contained germinated beans. As these pods have depleted water content, broken beans and degraded interior walls, they were not included in the harvest analysis. Subsequently, the pod age at point of harvest from harvest 1 was used to determine the optimum harvest point for each genotype in each treatment. This was then used to estimate harvest times for the pods at harvest 2. As a result,

only 6% of the pods at harvest 2 were excluded from analysis due to germinated beans. Pod harvesting at harvest 2 took place between 27.05.2015 and 18.07.2015.

6.2.1 Pod harvest

Pods were removed from the tree by cutting the pod stalk as close to the top of the pod as possible. This ensured extra weight was not added to the pod from the stalk. The pod was weighed in its entirety to give total pod fresh weight (KERN scales, model DE150K20D *KERN & SOHN*, Balingen, Germany). The husk was then cut in half whilst care was taken to avoid making any cuts to the beans (see Figure 6.1). The beans, husk and placenta were separated, and digital callipers were used to measure the thickness of the wall of the husk on either side, half way down the pod (lengthways). The two thickness values were later averaged to give a mean husk thickness per pod. The pulp was removed from the beans using paper towel. Finally, a razor blade was used to score the testa (skin) of each bean which allowed the testa to be removed. The husk was weighed on a large balance (KERN scales, model DE150K20D *KERN & SOHN*, Balingen, Germany) and the other components were weighed on a higher sensitivity balance (KERN scales, model PCB250-3, *KERN & SOHN*, Balingen, Germany) then placed into individual foil wallets. All components from the pod were then placed into a large paper bag and placed in a ventilated drying oven (*Heraeus oven*, Model UT 6760, *Heraeus*, Hanau, Germany) at 70°C until the weight of the components remained constant. This took between 4-7 days depending on the pod size. The dry weights of each of the components were later recorded using the high sensitivity balance. Bean to husk ratio was calculated by dividing the total bean dry weight per pod by the total husk dry weight per pod.

Figure 6.1 Image of pod dissection. Pod I.D: 5O1
Pods husks were split lengthways without damaging the beans inside. Half way down the length of the husk, the width of the husk wall was measured on either side.

6.2.2 Bean fat analysis

Due to a limitation in treatment representation, quantity of beans, and the need of specialist equipment and procedures, the dried cocoa beans were sent to a testing services laboratory: Eurofins Scientific (Eurofins Food Testing, Wolverhampton, UK), for fat analysis. At harvest 1, SCA 6 provided a high number of pods per treatment and was therefore the only genotype analysed. All other genotypes were either missing representation of a treatment or contained too few beans to enable testing (a minimum of 10g of dried beans per pod was required). At harvest 2, sample sizes allowed fat analysis of beans from Pound 7/B and SCA 6.

Total fat content

Total fat was measured using the British Standards Institute guide for the determination of total fat content (BS-4401, 1970). This involved the digestion of dry unfermented bean sample in hydrochloric acid. The sample was then filtered and dried, then extracted with petroleum ether. Detailed methodology was considered commercially sensitive information by Eurofins Scientific, therefore limited procedural information is provided.

Fatty acid profile

To create a fatty acid profile, the bean fat was extracted from the sample using the Bligh and Dyer technique (Kirk and Sawyer, 1991). The extracted fat was then trans-esterified using methanolic sodium methoxide to form the fatty acid methyl esters (FAMEs). The methyl esters were analysed by gas chromatography with a column designed to separate the cis and trans-isomers. Methods are based on McCance and Widdowson (2014), ISO (2002), and AOAC (1990). Eurofins Scientific accreditation reference is BS EN ISO/IEC 17025:2005 UKAS 0342 (Eurofins, 2016). Detailed methodology is considered commercially sensitive information by Eurofins Scientific, therefore limited information is provided.

Sugar content

For harvest 2, samples were also analysed for total sugar (as sucrose) content by Eurofins Scientific. The method used was for the determination of reducing sugars, expressed as glucose, and total sugars, expressed as sucrose, in feeding stuffs and food. Sugars were extracted with aqueous ethanol. The solution was then clarified and the sugars were determined. Reducing sugars were determined before acid inversion, and total sugars were

determined after acid inversion through the reducing action of glucose on copper (II). The unused copper (II) was reacted with iodide to create iodine. The quantity of iodine and hence the amount of sugar, was then determined by titration with thiosulphate. The methods above are based on the following sources; commission regulation EC-No.152 (2009), standard operating procedure (SOP) SAMP/019 – Procedures for clean sample preparation, SOP SAMP/015 - Procedures for sample preparation (non-soil), and SOP H/007 - Determination of residual moisture in plant material and animal feeding stuffs. Once again the precise procedures used by Eurofins Scientific are commercially sensitive and therefore the information provided here is the information provided by the company.

6.2.3 Statistical Analysis

Effects of treatment on all pod components, fat content and sugar content were analysed using GenStat 15th edition statistical software (GenStat, VSN International Ltd., Hemel Hempstead, UK) using an unbalanced design analysis of variance (ANOVA). Two block designs were created to test for any effect of greenhouse.

6.3 Results

6.3.1 Pod weight

Mean pod fresh weight was calculated for different treatments using pods from all genotypes (Figure 6.2). At harvest 1 a significant reduction in pod weight was observed under water-stress ($P < 0.01$) and there was a significant interaction between CO_2 treatment and genotype ($P < 0.01$). When analysing all pod harvest results for individual genotypes, it should be noted that mature pods were not obtained from the treatments shown in Table 6.1. The

genotypes CL19/10, Pound 7/B, SCA 6 and SPEC 54/1 did not show any significant responses to treatment at harvest 1 ($P > 0.05$) (Figure 6.3). ICS 1 showed a significant reduction in fresh weight under water stress ($P < 0.05$) and also an increase in response to elevated CO_2 ($P < 0.05$), however IMC 47 showed the opposite response to CO_2 with a reduction in fresh weight under high CO_2 ($P < 0.05$). At harvest 2, elevated CO_2 resulted in a significant increase in pod weight ($P < 0.001$). Also at harvest 2, there was a significant interaction between water availability and genotype ($P < 0.05$). There was also a significant interaction between CO_2 treatment and genotype ($P < 0.01$). At harvest 2, significant increases in fresh weight under high CO_2 were observed for CL19/10 ($P < 0.05$), Pound 7/B ($P < 0.01$), and SCA 6 ($P < 0.01$). ICS 1, IMC 47 and SPEC 54/1 showed no significant response to high CO_2 ($P > 0.05$). Both CL19/10 and ICS 1 showed a significant interaction between CO_2 and water treatments ($P < 0.05$ and $P < 0.01$ respectively). For CL19/10 the fresh weight increased under 'WS' but only under ambient CO_2. For ICS 1 a lower fresh weight was observed under 'WS' but only in the ambient CO_2 treatment. Under high CO_2 an increase in fresh weight was observed under the 'WS' treatment. There was a significant effect of genotype in both harvests ($P < 0.001$ for both) with ICS 1 having consistently heavier pods, averaging 401.8g and 525.5g at harvest 1 and 2 respectively. SCA 6 had consistently the lightest pod fresh weight at 144.7g and 233.0g at harvest 1 and 2 respectively. There was also an overall increase in pod weight at harvest 2.

Similar treatment effects were observed for total dry weight as for fresh weight. Total dry weight was calculated by the sum of the weight of each dried component. This was then averaged across all genotypes per treatment (Figure 6.2). At harvest 1, there was a significant reduction in dry weight in response to water-stress ($P < 0.001$). There was a significant interaction between CO_2 treatment and genotype ($P < 0.05$). There was also an interaction between water treatment and genotype ($P < 0.05$). At harvest 1, ICS 1 and SCA 6 showed a

reduction in pod dry weight in response to water-stress (both $P < 0.05$) (Figure 6.4). The response to elevated CO_2 differed between genotypes. ICS 1 showed a significant increase in pod dry weight under elevated CO_2 ($P < 0.05$), and SCA 6 showed a significant decrease in pod dry weight under elevated CO_2 ($P < 0.05$). No significant effects of treatment were observed for the remaining genotypes at harvest 1. At harvest 2, there was a significant increase in dry weight in response to elevated CO_2 ($P < 0.001$). There were also a significant interaction between CO_2 treatment and genotype ($P < 0.05$). There was an additional interaction between water treatment and genotype ($P < 0.05$). At harvest 2, elevated CO_2 significantly increased pod dry weight in CL19/10 ($P < 0.001$), ICS 1 ($P <0.05$), Pound 7/B ($P <0.01$), and SCA 6 ($P <0.01$). A significant increase in pod dry weight was recorded for CL19/10 under water stress ($P < 0.05$), whereas in ICS 1 a reduction in pod dry weight under 'WS' was recorded ($P <0.01$). The increase in pod dry weight in response to elevated CO_2 in ICS 1 was largely attributable to a low 'Ambient-WS' average. The 'Ambient – WW' average is equal to that of the elevated CO_2 treatments. No interaction between water and CO_2 treatment was observed. There was a significant effect of genotype at both harvests (both $P < 0.001$). Again ICS 1 had the heaviest dry weights averaging 92.8g and 117.4g for harvest 1 and 2 respectively, and SCA 6 had the lowest dry weights averaging 29.3g and 42.7g for harvest 1 and 2 respectively.

A significant effect of block design 2 was found for total pod fresh and dry weight in harvest 1 (both $P < 0.001$). In both cases this represented an overall reduction in pod weight in houses 3 and 6; however the trend in response to treatment was the same in both blocks.

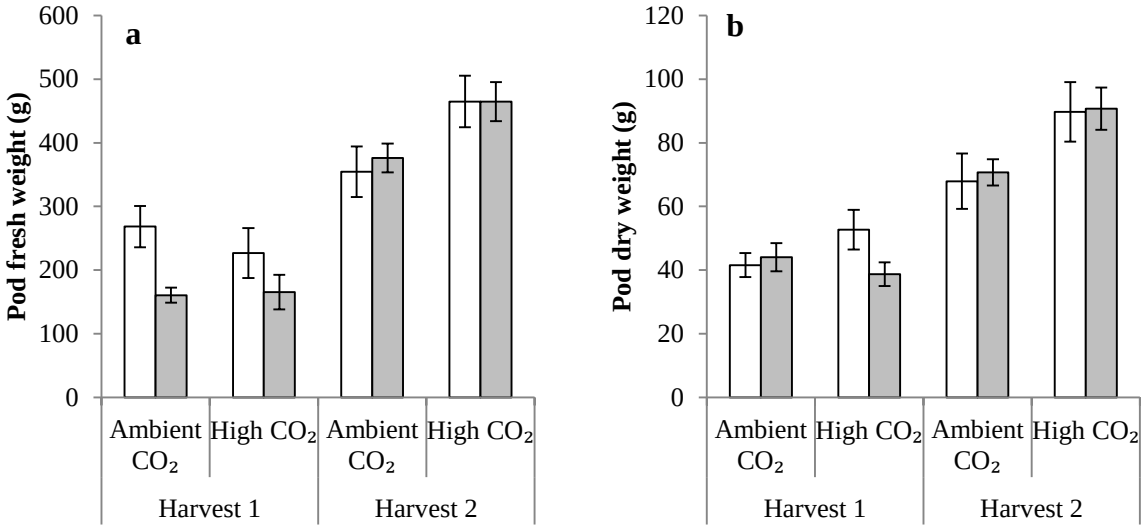

Figure 6.2 Mean pod fresh (a) and dry (b) weight from trees grown at two concentrations of CO_2 and two watering treatments. Data are combined across six genotypes.
Mean pod dry weight was calculated by the sum of the weight of the individual dried pod components. □ - Well-watered. ▒ - Water-stressed. Error bars show standard error of the mean.

Table 6.1 Treatments which did not harvest mature pods

Genotype	Harvest 1	Harvest 2
CL19/10	High CO_2 - WS	-
ICS 1	High CO_2 - WS	-
IMC 47	Ambient CO_2 - WW	-
Pound 7/B	-	High CO_2 - WW
SCA 6	-	-
SPEC 54/1	-	Ambient CO_2 - WW

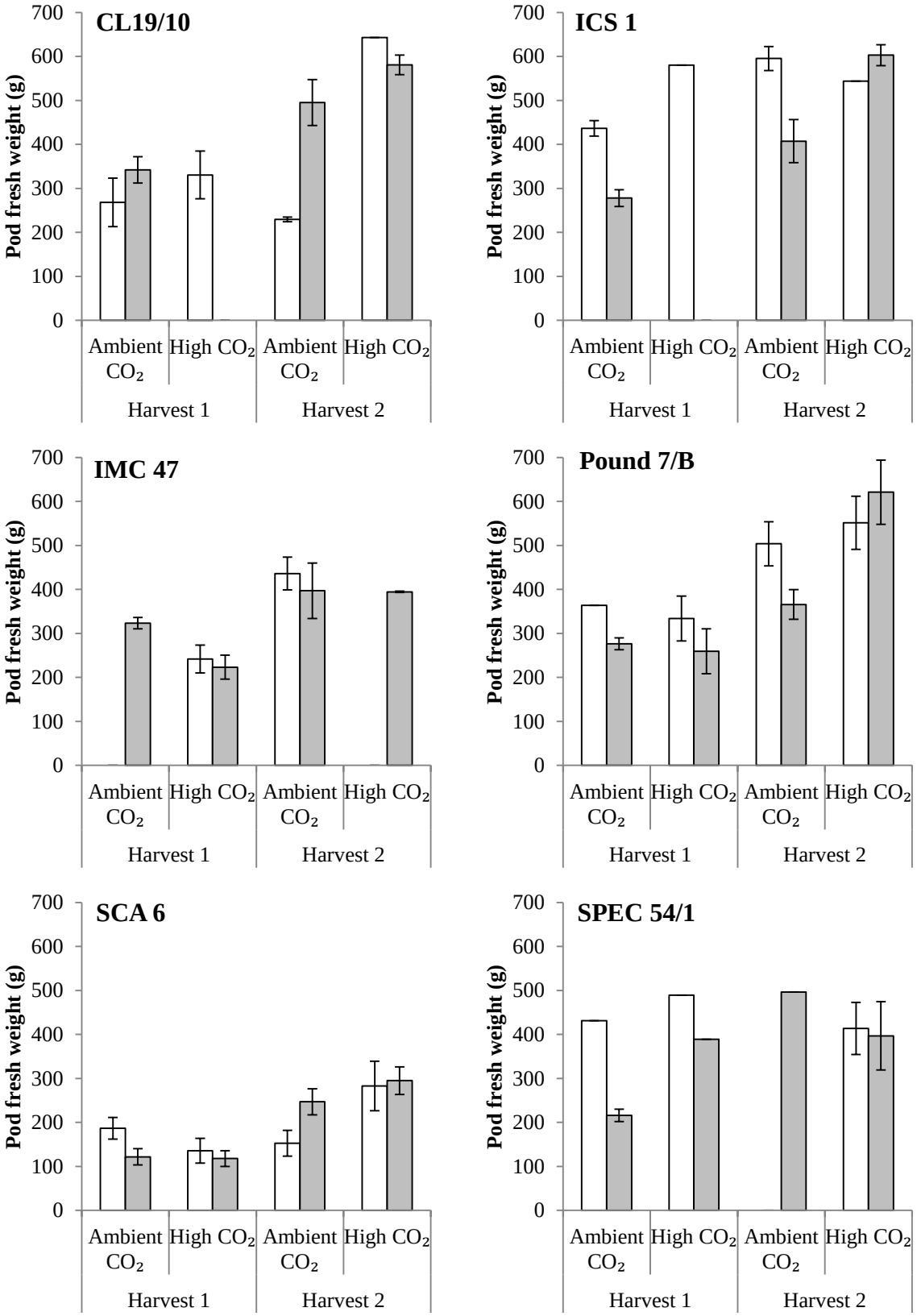

Figure 6.3 Mean pod fresh weight of six cocoa genotypes grown at ambient and elevated CO_2 and under two watering regimes.
□ - Well-watered. ▒ - Water-stressed. Error bars show standard error of the mean.

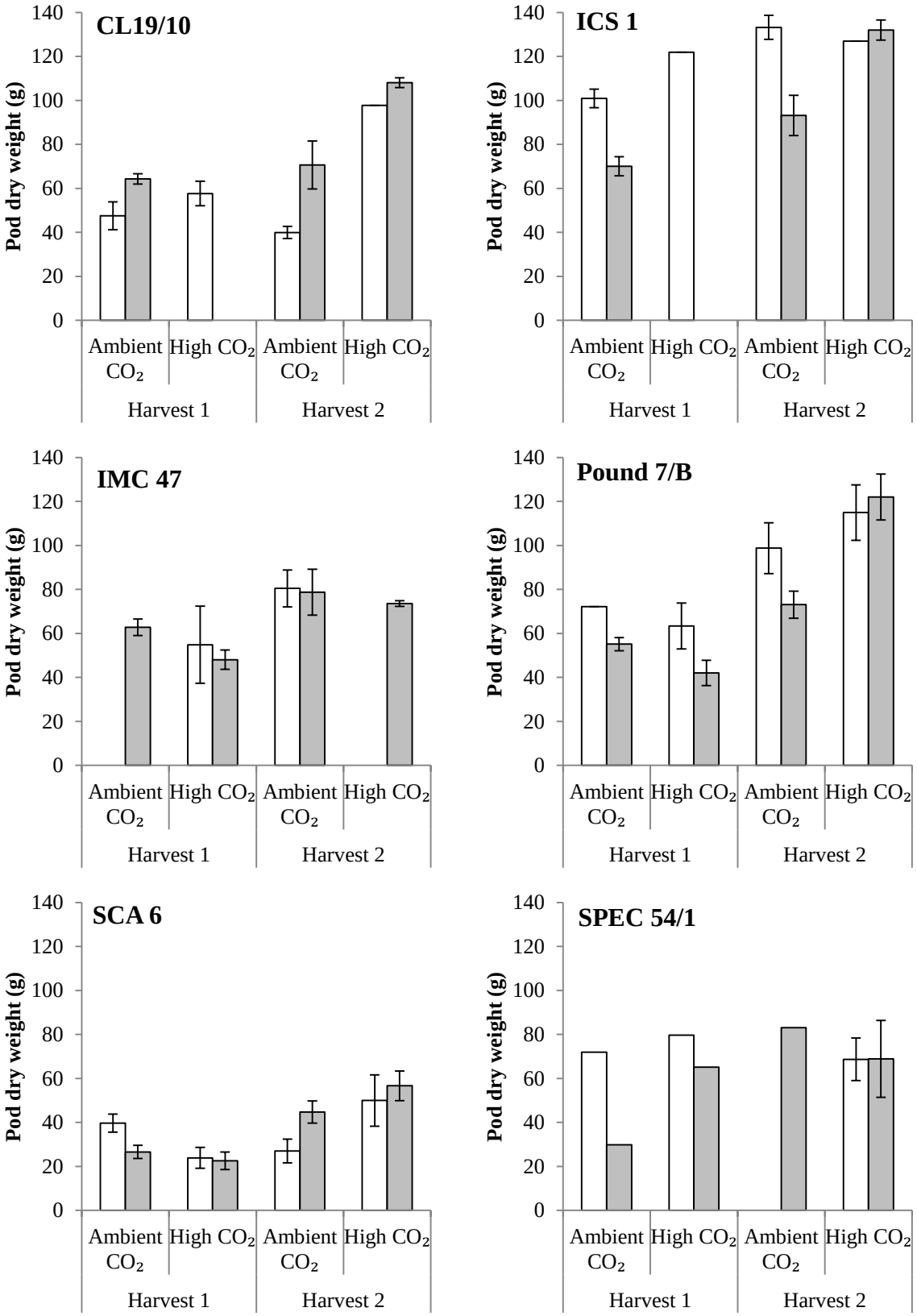

Figure 6.4 Mean pod dry weight of six cocoa genotypes grown at ambient and elevated CO_2 and under two watering regimes.
Mean pod dry weight was calculated by the sum of the weight of the individual dried pod components. □ - Well-watered. ▒ - Water-stressed. Error bars show standard error of the mean.

6.3.2 Husk weight

Mean husk fresh weight for all genotypes is shown in Figure 6.5. At harvest 1, a significant reduction in husk fresh weight was observed under water stress ($P < 0.01$) and there was a significant interaction between CO_2 treatment and genotype ($P < 0.001$). Mean husk fresh weight for individual genotypes is shown in Figure 6.6. At harvest 1, only ICS 1 and IMC 47 showed significant responses to the treatments. An increase in husk fresh weight was observed under elevated CO_2 for ICS 1 ($P <0.05$), whereas the opposite trend was seen for IMC 47 with a reduced husk weight under elevated CO_2 ($P < 0.05$). A significant reduction in weight was also observed for ICS 1 in response to water stress ($P < 0.05$). At harvest 2 a significant increase in husk fresh weight was observed under elevated CO_2 ($P <0.001$) in addition to significant interactions between water treatment and genotype ($P < 0.05$). There was also an interaction between water treatment, CO_2 treatment and genotype ($P < 0.01$). Furthermore, husk weight was generally greater in harvest 2 compared to harvest 1. At harvest 2, a significant increase in husk fresh weight under elevated CO_2 was recorded in CL19/10 ($P < 0.01$), Pound 7/B ($P < 0.01$) and SCA 6 ($P <0.01$). CL19/10 also showed a significant increase in husk fresh weight under water stress ($P < 0.05$). Finally, a significant interaction between water availability and CO_2 treatments was observed for CL19/10 and ICS 1 (both $P < 0.05$). For CL19/10, increased husk weight was observed under water stress but only under ambient CO_2, whereas ICS 1 showed reduced weight under water stress but only under ambient CO_2. A significant effect of genotype was observed in both harvests (both $P < 0.001$). SCA 6 had consistently the lowest mean husk fresh weight (100.5g and 172.8g at harvest 1 and 2 respectively). ICS 1 had the heaviest husk fresh weight at harvest 1 averaging 262.9g and CL19/10 had the heaviest at harvest 2 averaging 394.5g.

Mean husk dry weight for all genotypes is shown in Figure 6.5. As with the fresh weight data, a significant reduction in husk weight was recorded under water stress at harvest 1 ($P < 0.01$) and a significant increase was observed under elevated CO_2 at harvest 2 ($P < 0.001$). A significant interaction was seen between CO_2 treatment and genotype ($P < 0.05$) and an interaction between water treatment and genotype in harvest 1 ($P < 0.01$). Mean husk dry weight for individual genotypes is shown in Figure 6.7. At harvest 1, a significant effect of treatment was only observed for the clones ICS 1 and IMC 47. For ICS 1, dry weight significantly increased under elevated CO_2 ($P < 0.05$), and under water stress, dry weight significantly decreased ($P < 0.01$). In IMC 47, the opposite effect of elevated CO_2 was observed as dry weight decreased under elevated CO_2 ($P < 0.05$). At harvest 2 there was a significant interaction between CO_2 treatment and genotype ($P < 0.01$), an interaction between water treatment and genotype ($P < 0.05$), and an interaction between water treatment, CO_2 treatment and genotype ($P < 0.05$). At harvest 2, there was a significant increase in husk dry weight under elevated CO_2 for CL19/10 ($P < 0.001$), ICS 1 ($P < 0.05$), Pound 7/B ($P < 0.001$) and SCA 6 ($P < 0.001$). A significant increase in dry weight under water stress was also observed in CL19/10 ($P < 0.05$) along with a significant interaction between water and CO_2 treatment ($P < 0.05$). Here an increase in husk dry weight occurred under 'WS' but only under ambient CO_2. Under elevated CO_2, 'WS' had little effect. Finally, for ICS 1 there is also a significant reduction in dry weight under 'WS' ($P < 0.05$). A significant effect of genotype occurred in both harvests (both $P < 0.001$). Again SCA 6 had the lowest mean husk dry weight (15.4g and 24.7g for harvest 1 and 2 respectively). ICS 1 had the highest mean husk dry weight (48.4g and 61.4g at harvest 1 and 2 respectively).

A significant effect of block design 2 was found for husk fresh and dry weight in harvest 1 (both $P < 0.001$). In both cases this represented an overall reduction in husk weight in houses 3 and 6; however the trend in response to treatment was the same in both blocks.

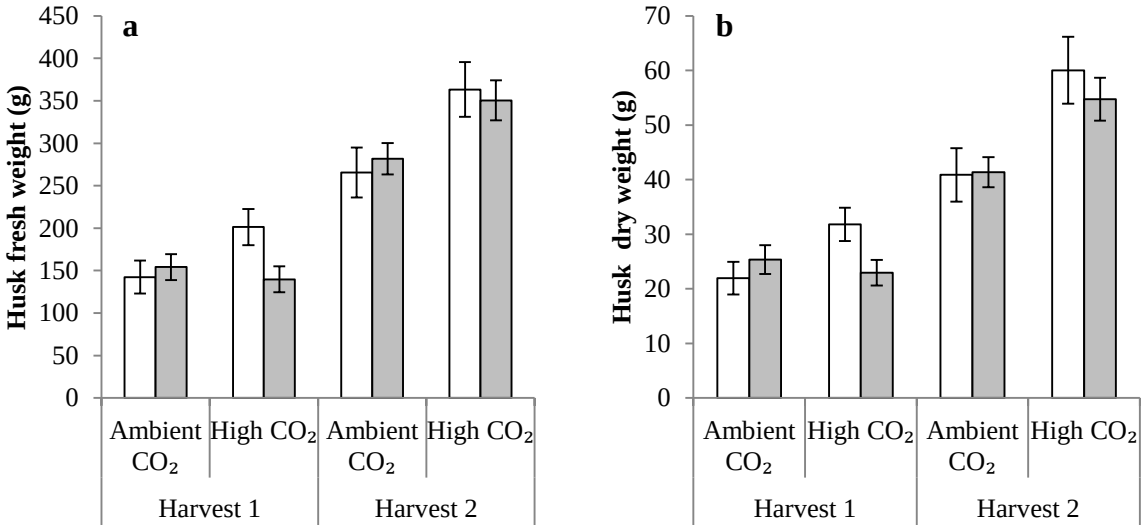

Figure 6.5 Mean husk fresh (a) and dry (b) weight from trees grown at two concentrations of CO_2 and two watering treatments. Data are combined across six genotypes.
□ - Well-watered. ▒ - Water-stressed. Error bars display standard error of the mean.

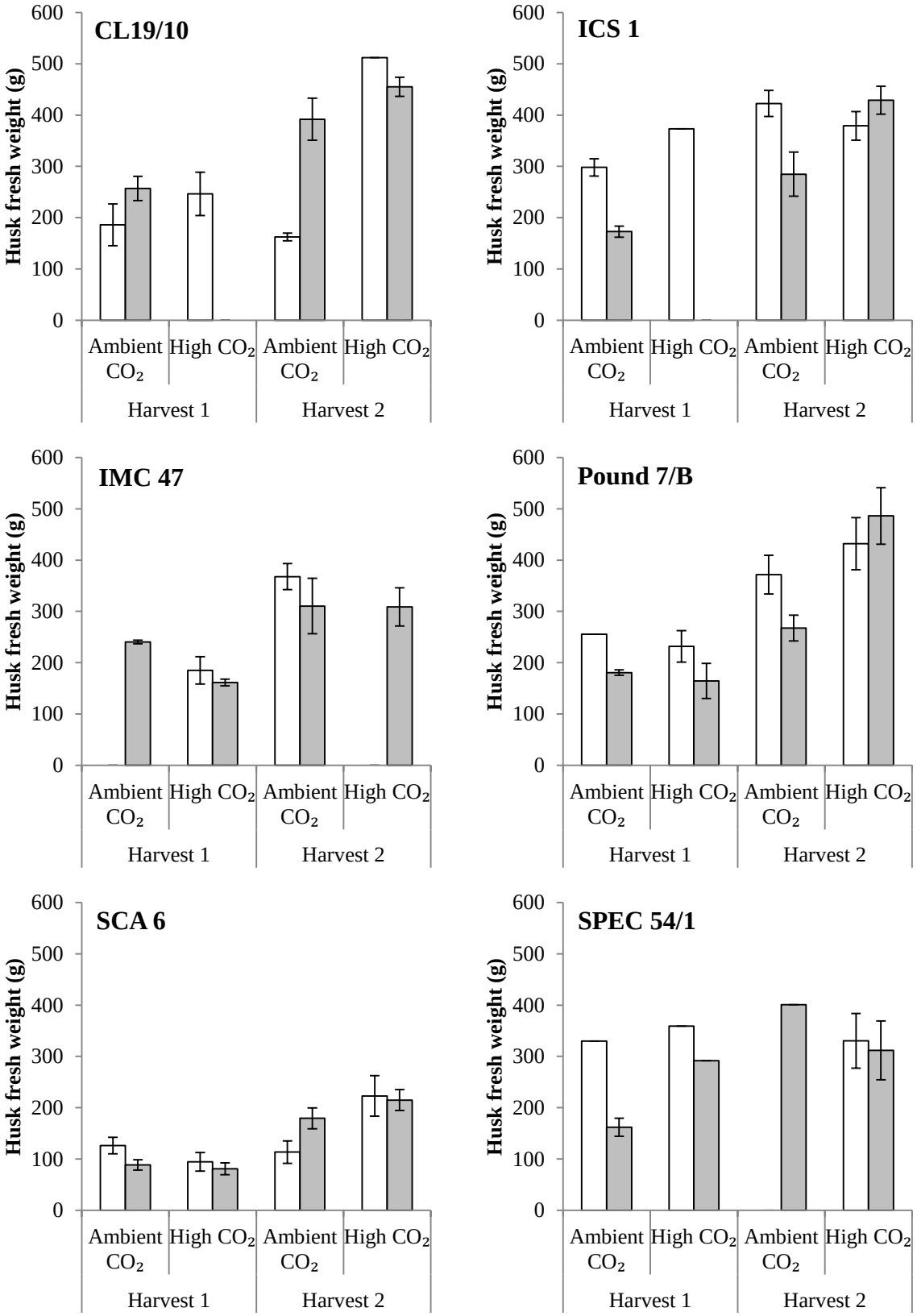

Figure 6.6 Mean husk fresh weight of six cocoa genotypes grown at ambient and elevated CO2 and under two watering regimes.

□ - Well-watered. ▒ - Water-stressed. Error bars show standard error of the mean.

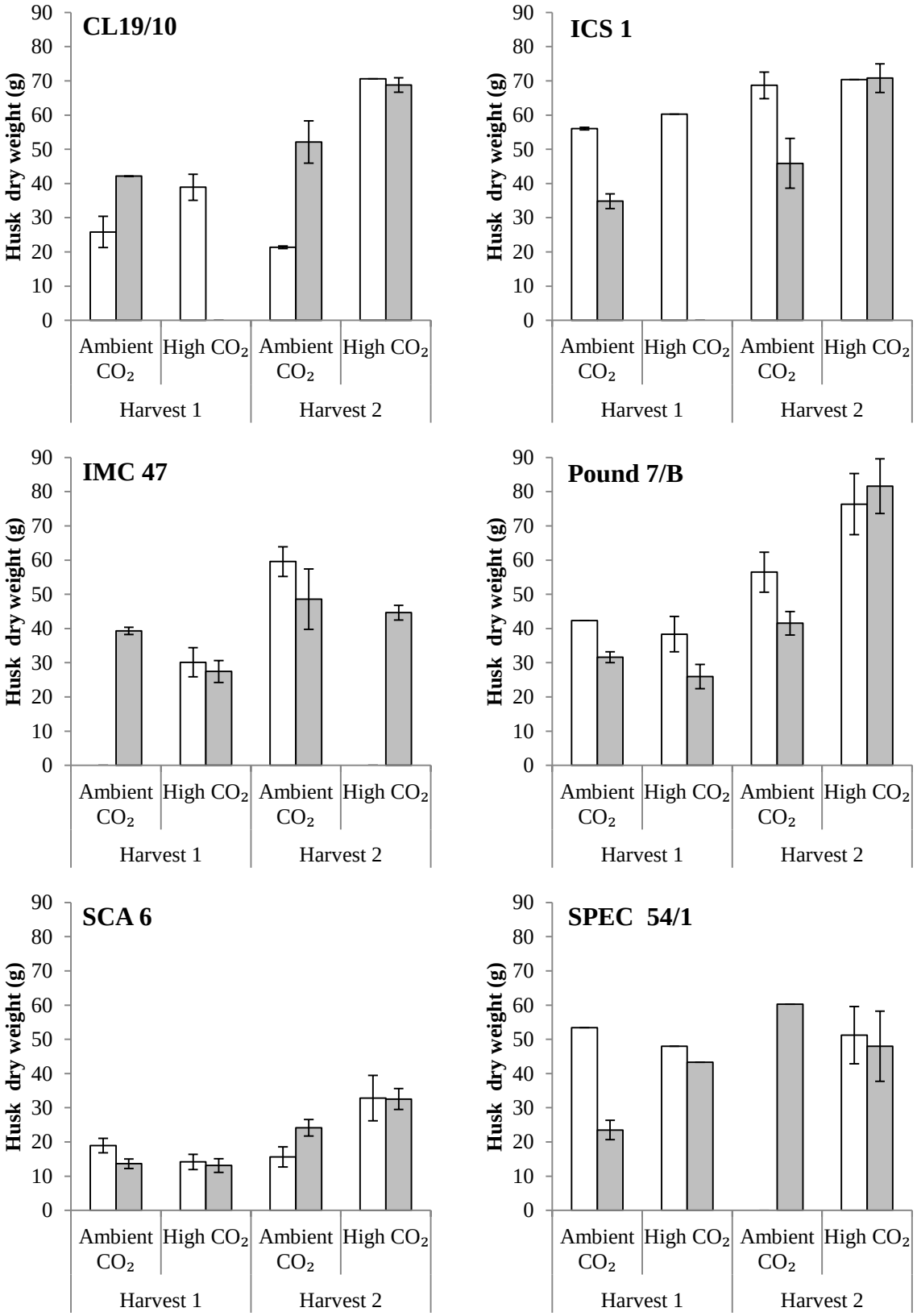

Figure 6.7 Mean husk dry weight of six cocoa genotypes grown at ambient and elevated CO2 and under two watering regimes.

□ - Well-watered. ▒ - Water-stressed. Error bars show standard error of the mean.

6.3.3 Husk thickness

At harvest 1 no significant differences in thickness were observed between treatments ($P > 0.05$) (Figure 6.8). However at harvest 2, pod walls were significantly thicker under elevated CO_2 ($P < 0.05$). Additionally, husks were generally thicker at harvest 2 compared to harvest 1. There were no significant interactions between genotype and treatment.

A significant effect of block design 2 was found for husk thickness in harvest 1 ($P = 0.01$). This represented an overall reduction in thickness in houses 3 and 6.

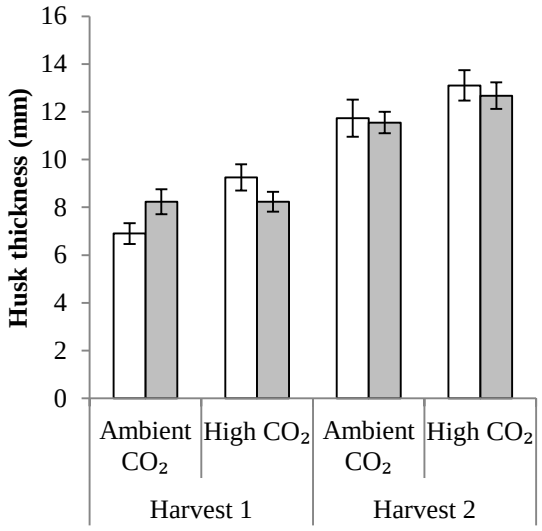

Figure 6.8 Mean husk fresh thickness from trees grown at two concentrations of CO_2 and two watering treatments. Data are combined across six genotypes.
□ - Well-watered. ▒ - Water-stressed. Error bars show the standard error of the mean.

6.3.4 Total bean weight

Mean bean fresh weight per pod for all genotypes combined is shown in Figure 6.9. At harvest 1 there was a significant interaction between genotype and CO_2 treatment ($P < 0.01$). Mean bean fresh weight per pod for each genotype is shown in Figure 6.10. At harvest 1, a significant increase in bean fresh weight under elevated CO_2 was observed for SPEC 54/1 ($P < 0.05$). Also for SPEC 54/1, there was a significant reduction in bean fresh weight under 'WS' ($P < 0.05$). There were no other significant treatment effects at harvest 1. At harvest 2

there was a significant increase in bean fresh weight under elevated CO_2 (P <0.05). There were also significant interactions between water availability and genotype (P <0.05). At harvest 2 there was a significant increase in bean weight under elevated CO_2 for CL19/10 (P < 0.05). For SCA 6, bean weight increased significantly (P < 0.05) under WS. Finally, for ICS 1 there was a significant interaction between CO_2 and water availability (P < 0.01) whereby under 'WS' bean fresh weight declined under ambient CO_2 but increased under elevated CO_2. There was a significant effect of genotype in both harvests (both P < 0.001). SCA 6 had the lowest bean fresh weight averaging 19.9g and 24.1g for harvest 1 and 2, respectively. ICS 1 had the highest bean fresh weight averaging 63.3g and 76.7g for harvest 1 and 2, respectively.

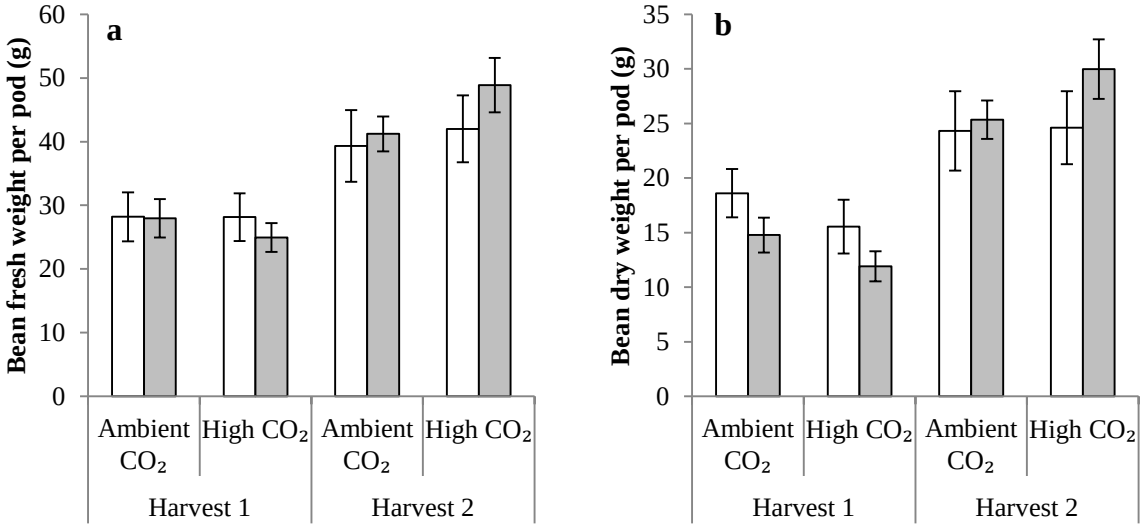

Figure 6.9 Mean bean fresh (a) and dry (b) weight per pod from trees grown at two concentrations of CO_2 and two watering treatments. Data are combined across six genotypes.
□ - Well-watered. ▒ - Water-stressed. Error bars show standard error of the mean.

Mean bean dry weight per pod for all genotypes is shown in Figure 6.9. A reduction in bean dry weight was observed under elevated CO_2 and under water stress at harvest 1 (P < 0.05 and P < 0.01 respectively). There was also a significant interaction between CO_2 treatment and genotype. Mean bean dry weight per pod per genotype is shown in Figure 6.11. There

were no significant responses to treatment at harvest 1 except for a reduction in bean dry weight under elevated CO_2 in SCA 6 ($P < 0.01$). At harvest 2 there was a significant interaction between water treatment and genotype ($P < 0.05$). At harvest 2, pod bean weight was generally greater than in harvest 1. A significant increase in dry weight under elevated CO_2 was observed for CL19/10 and ICS 1 (both $P < 0.05$). A significant increase was also observed in SCA 6 under 'WS' ($P < 0.05$). A significant interaction between CO_2 and water availability ($P < 0.05$) was observed for ICS 1; a decrease in bean weight was observed under 'WS' in ambient CO_2 but an increase under elevated CO_2. There was a significant effect of genotype in both harvests (both $P < 0.001$). Again SCA 6 had the lowest dry bean weight per pod averaging 10.5g and 14.78g at harvest 1 and 2 respectively. ICS 1 had the largest dry bean weight per pod averaging 37.7g and 47.5g at harvest 1 and 2 respectively.

A significant effect of block design 2 was found for total bean fresh and dry weight in harvest 1 (both $P < 0.001$). In both cases this represented an overall reduction in bean weight in houses 3 and 6; however the trend in response to treatment was the same in both blocks.

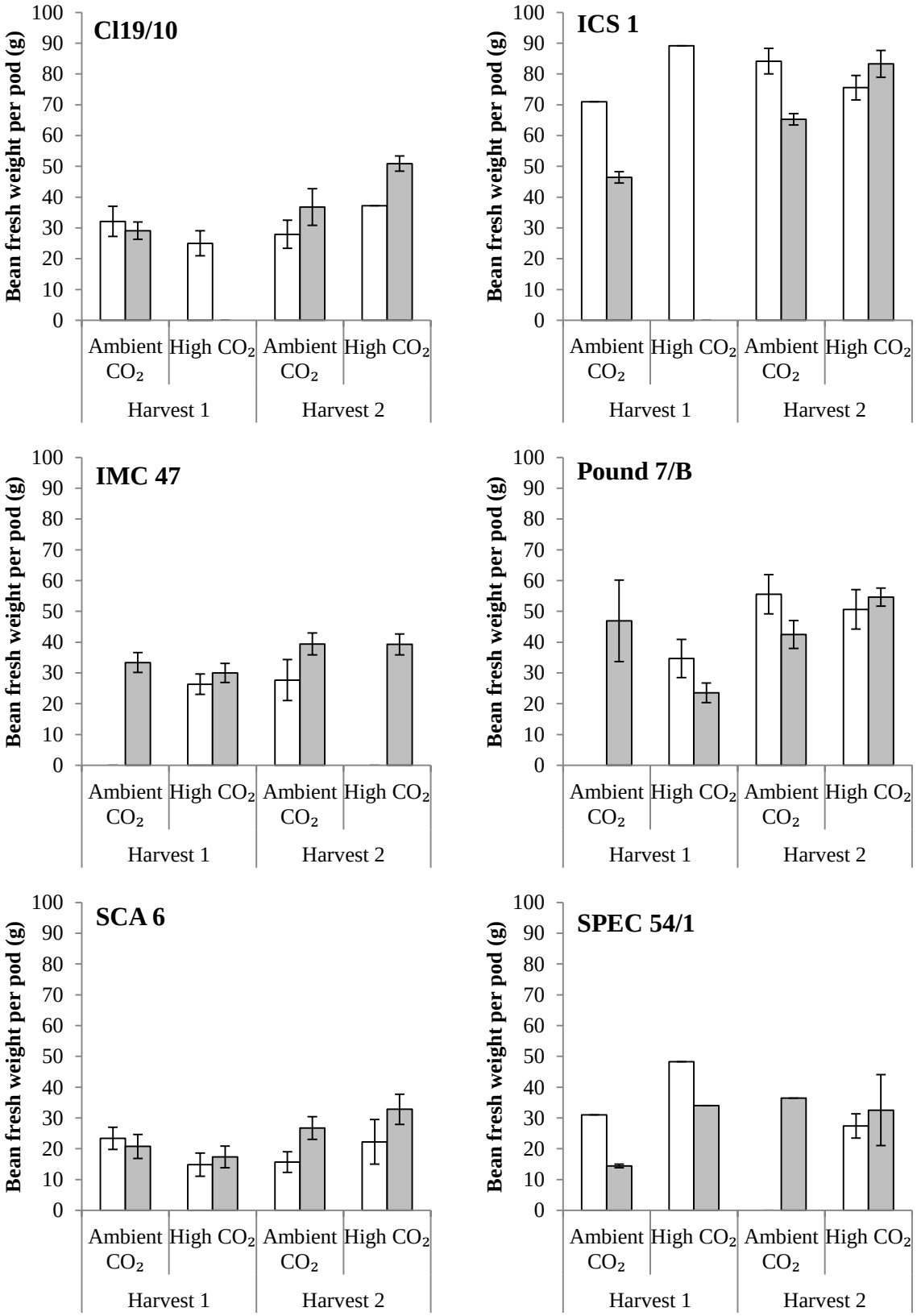

Figure 6.10 Mean bean fresh weight per pod of six cocoa genotypes grown at ambient and elevated CO_2 and under two watering regimes.
□ - Well-watered. ▒ - Water-stressed. Error bars show standard error of the mean.

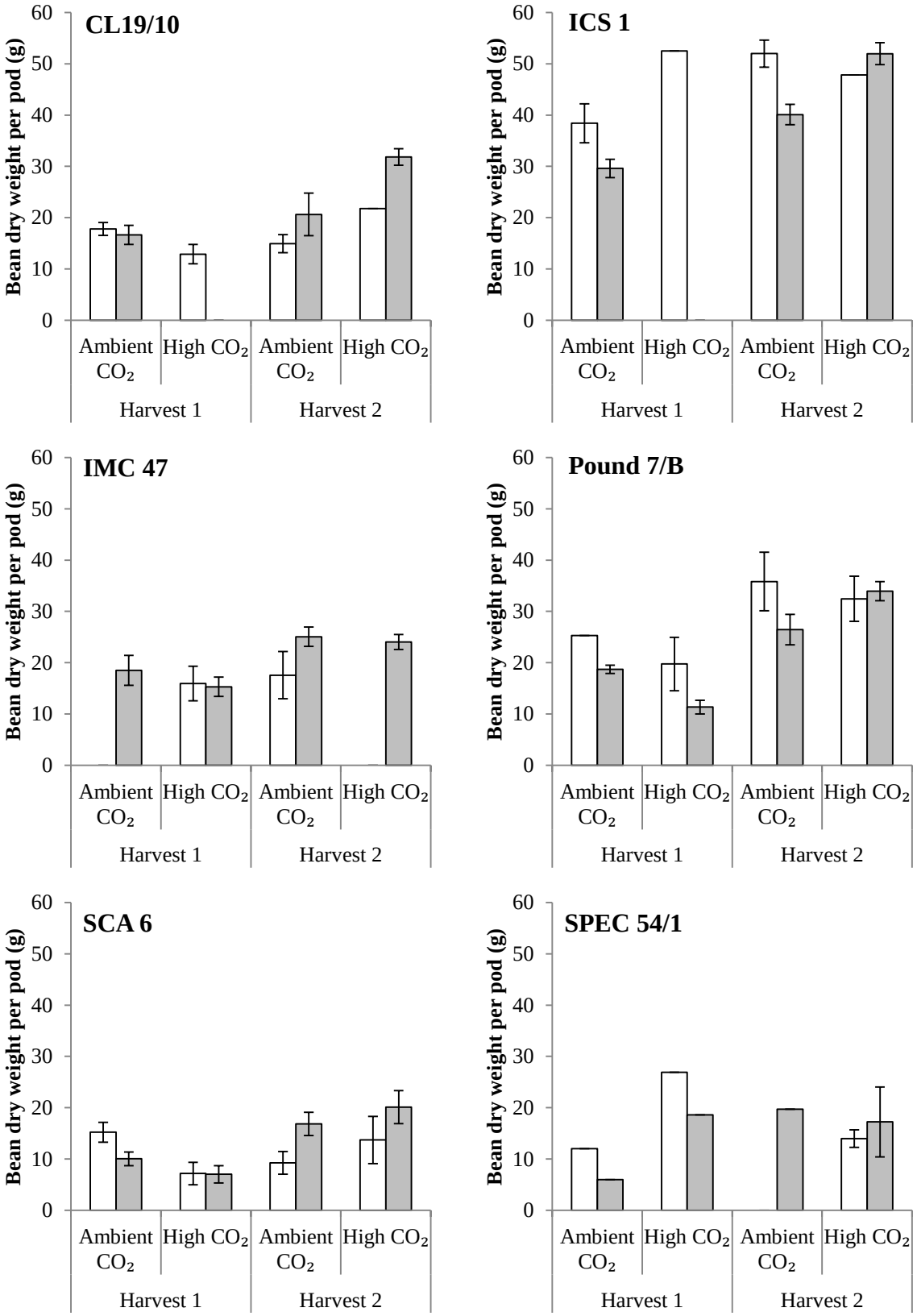

Figure 6.11 Mean bean dry weight per pod of six cocoa genotypes grown at ambient and elevated CO_2 and under two watering regimes.
□ - Well-watered. ▒ - Water-stressed. Error bars show standard error of the mean.

6.3.5 Bean number

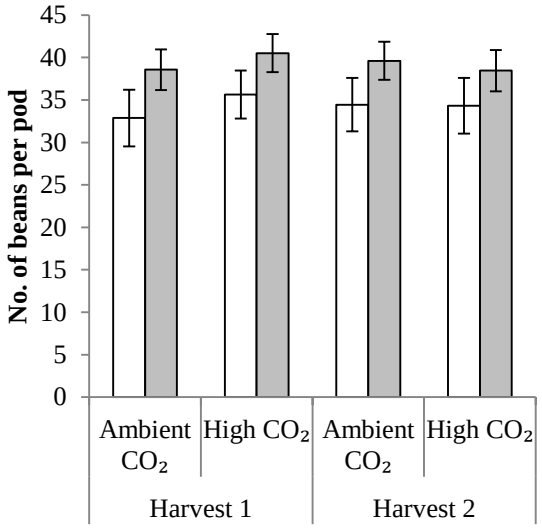

Figure 6.12 Mean number of beans per pod from trees grown at two concentrations of CO_2 and two watering treatments. Data are combined across six genotypes.
□ - Well-watered. ▒ - Water-stressed. Error bars show standard error of the mean.

Mean number of beans per pod for all genotypes is shown in Figure 6.12. A significant increase in bean number under water stress was observed at harvest 2 ($P < 0.05$) however this trend is also evident at harvest 1. For both harvests there was a significant effect of genotype on bean number ($P < 0.001$). SPEC 54/1 had the lowest mean bean number per pod (averaging 23.9 and 22.4 at harvest 1 and 2 respectively). Pound 7/B had the highest mean bean number per pod (averaging 45.9 and 45.0 for harvest 1 and 2 respectively). There were no significant interactions between genotype and treatment.

A significant effect of block design 2 was found for bean number in harvest 1 ($P < 0.05$). This represented an overall reduction in bean number in houses 3 and 6.

6.3.6 Individual bean weight

Mean individual fresh bean weight across all genotypes is shown in Figure 6.13. At harvest 1 there was a significant decrease in individual bean fresh weight under elevated CO_2 ($P < 0.01$) and under water stress ($P < 0.01$). Additionally, significant interactions were observed between CO_2 treatment and genotype ($P < 0.01$), water availability and genotype ($P < 0.01$), and water availability, CO_2 and genotype ($P < 0.05$). Mean individual bean fresh weight was also analysed for individual genotypes (Figure 6.14). At harvest 1, a significant decrease in bean weight under elevated CO_2 was recorded for ICS 1 and IMC 47 (both $P < 0.05$) and the same trend was seen for CL19/10 and Pound 7/B although these were not significant ($P > 0.05$). Additionally, a decrease in individual bean weight under water stress was observed for ICS 1 and SPEC 54/1 ($P < 0.01$ and $P < 0.05$ respectively). Finally, for SPEC 54/1, a significant interaction between water and CO_2 was observed ($P < 0.05$); a smaller decrease in bean weight under water stress was observed under elevated CO_2 compared with ambient CO_2. At harvest 2, there was a significant increase in individual bean fresh weight under elevated CO_2 ($P < 0.001$). There were also interactions between water availability and genotype ($P < 0.001$), and between water availability, CO_2 and genotype ($P < 0.001$). At harvest 2, elevated CO_2 resulted in an increase in individual bean weight for CL19/10 ($P < 0.05$), ICS 1 ($P < 0.01$) and Pound 7/B ($P < 0.001$). The effect of water stress varied between genotypes; for CL19/10 ($P < 0.01$), an increase in water stress was only observed under ambient CO_2 ($P<0.05$ for the interaction between CO_2 and watering treatment). In ICS 1, there was a significant ($P < 0.001$) interaction between water and CO2 such that, under the water stress treatment, lower bean weight was recorded under ambient CO_2, but higher bean weight was recorded under elevated CO_2. In both harvests there were significant differences in individual bean fresh weight between genotypes (both $P < 0.001$). SCA 6 had consistently the lowest mean individual bean fresh weight (0.57 and 0.83g for harvest 1 and 2,

respectively). ICS 1 had the largest individual bean fresh weight (1.65 and 1.88g for harvest 1 and 2, respectively).

Individual bean dry weight is shown in Figure 6.13. At harvest 1, elevated CO_2 and water stress resulted in a reduced individual bean dry weight (both $P < 0.001$). There was also a significant interaction between CO_2 and genotype ($P < 0.01$). Mean individual bean dry weight for each genotype is shown in Figure 6.15. At harvest 1, elevated CO_2 resulted in significantly lower individual bean weights for CL19/10 ($P < 0.05$) and SCA 6 ($P < 0.001$). Additionally, SCA 6 showed a significantly reduced bean weight under water stress ($P < 0.01$). At harvest 2, bean weights were generally larger than in harvest 1. Additionally, there was a significant increase in individual bean weight under elevated CO_2 ($P < 0.001$) and a significant interaction between water availability and genotype ($P < 0.004$). At harvest 2, there was a significant increase in bean weight under elevated CO_2 for CL19/10 and ICS 1 (both $P < 0.05$), and Pound 7/B ($P < 0.01$). A significant increase in bean weight was also observed for CL 19/10 under water stress ($P < 0.05$). A significant interaction between water and CO_2 was observed for ICS 1 ($P < 0.05$); bean weight declined under water stress at ambient CO_2 but the opposite response to water stress was observed at elevated CO_2. For both harvests there was a significant effect of genotype (both $P < 0.001$). SCA 6 had the lowest mean bean dry weight (0.28 and 0.51g at harvest 1 and 2, respectively). ICS 1 had the largest mean individual bean weight (0.98 and 1.16g at harvest 1 and 2, respectively).

At harvest 1, a significant effect of block design 1 and block design 2 was found for individual bean fresh weight ($P < 0.05$ and $P < 0.01$ respectively) and individual bean dry weight ($P < 0.05$ and $P < 0.001$ respectively). In both cases, this represented an overall reduction in bean weight in houses 2 and 3 (block design 1), and houses 3 and 6 (block design 2). However the trend in response to treatment was the same in both blocks, regardless of block design. A significant effect of block design 1 was observed in harvest 2 ($P < 0.01$).

This represented a reduction in bean weight in houses 5 and 6; however the trend in response to treatment was the same in both blocks.

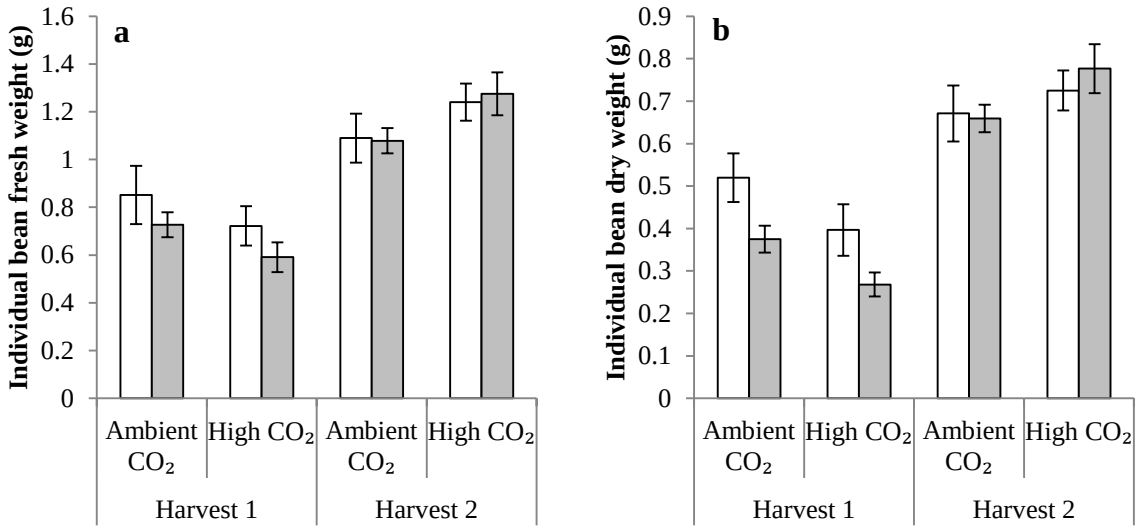

Figure 6.13 Mean individual bean fresh (a) and dry (b) weight from trees grown at two concentrations of CO_2 and two watering treatments. Data are combined across six genotypes.
Calculated by dividing the total bean weight by the number of beans for each given pod. □ - Well-watered. ▩ - Water-stressed. Error bars show standard error of the mean.

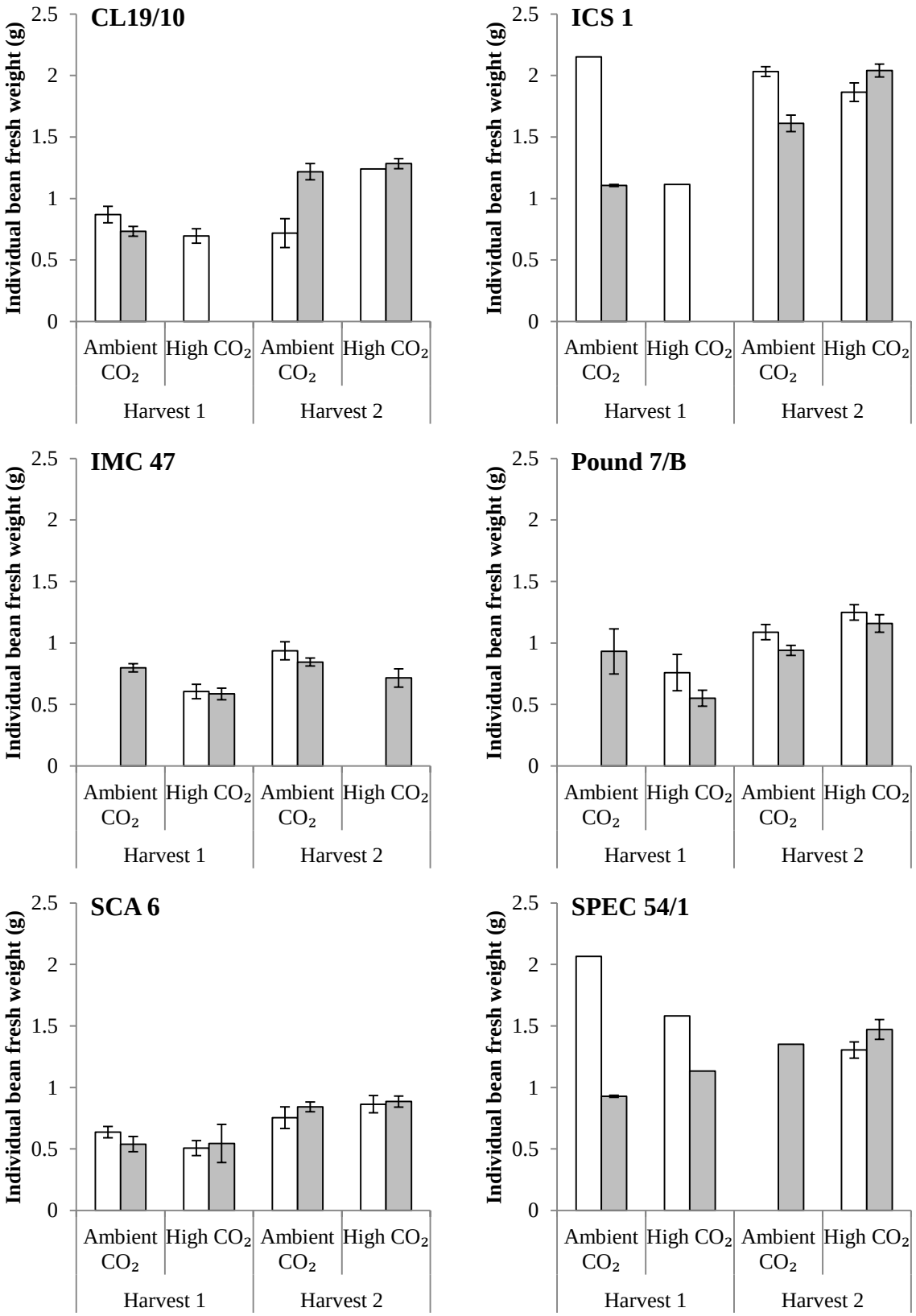

Figure 6.14 Mean individual bean fresh weight of six cocoa genotypes grown at ambient and elevated CO_2 and under two watering regimes.
Calculated by dividing the total bean fresh weight by the number of beans for each given pod. □ - Well-watered. ▒ - Water-stressed. Error bars show standard error of the mean.

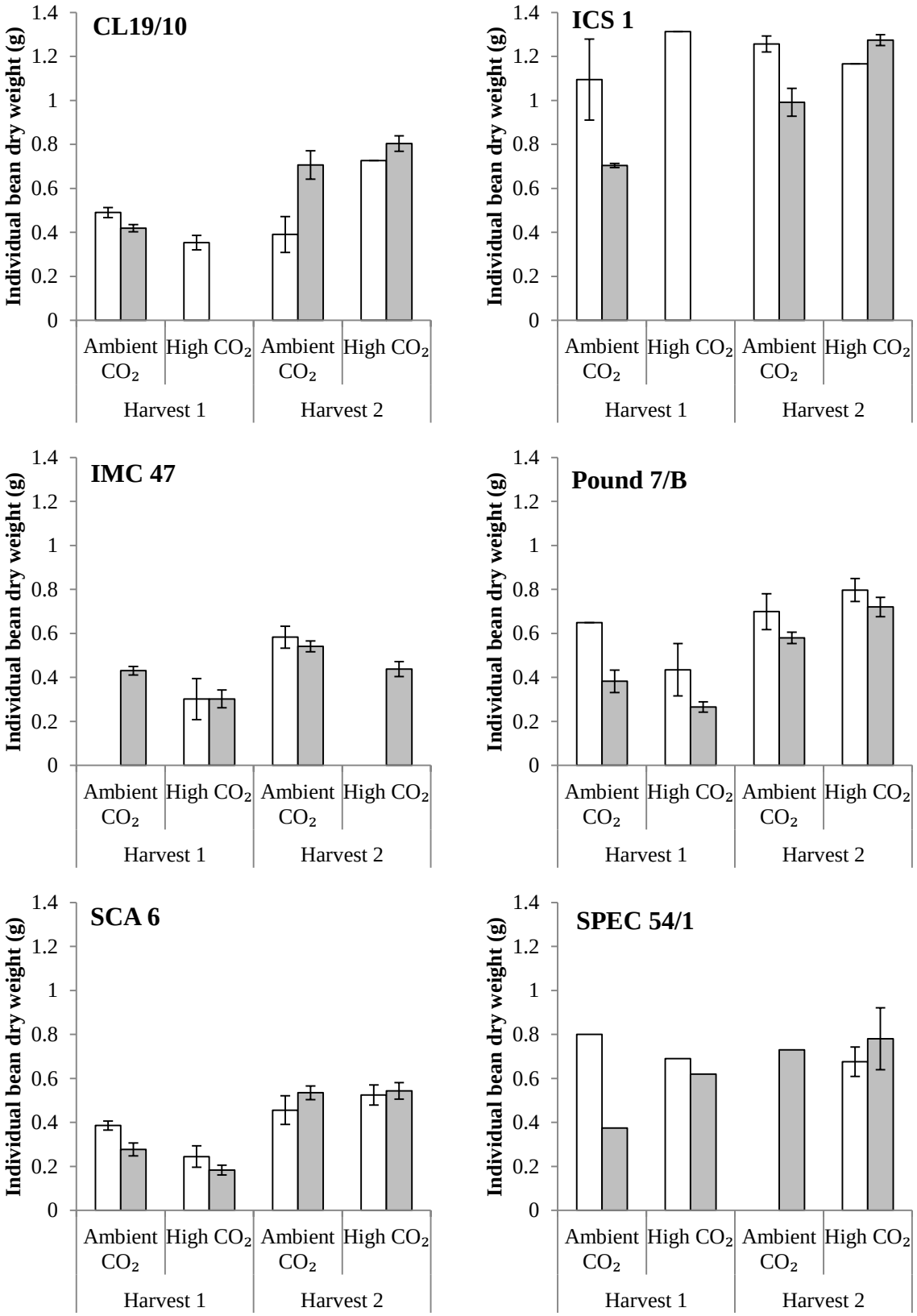

Figure 6.15 Mean individual bean dry weight of six cocoa genotypes grown at ambient and elevated CO_2 and under two watering regimes.
Calculated by dividing the total bean dry weight by the number of beans for each given pod. □ - Well-watered. ▒ - Water-stressed. Error bars show standard error of the mean.

6.3.7 Bean moisture content

Bean moisture content is shown for all genotypes combined in Figure 6.16. At harvest 1, the only significant response to treatment was an increase in moisture content under high CO_2 ($P < 0.05$). At harvest 2, bean moisture content was stable with the only significant variation was due to genotype ($P < 0.01$). The highest mean bean moisture content was found in clone SPEC 54/1 (52.7% and 47.8% in harvest 1 and 2 respectively). The lowest mean bean moisture content in harvest 1 was in clone ICS 1 (38.6%). In harvest 2, the lowest moisture was found in clone IMC 47 (37.1%). There were no significant interactions between genotype and treatment.

A significant effect of block design 1 was found for bean moisture content in harvest 2 ($P < 0.001$). This represented an overall reduction in moisture content in houses 2 and 3.

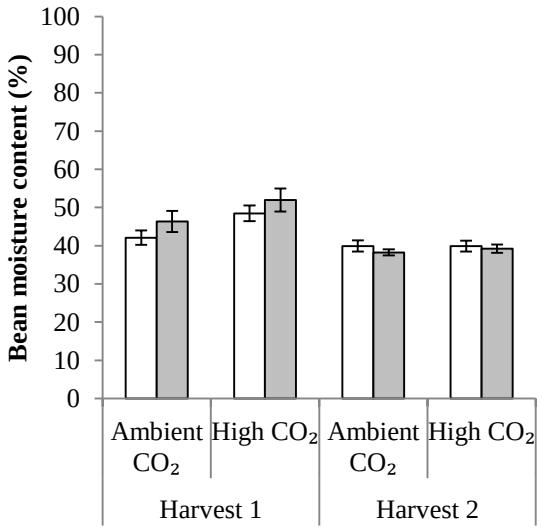

Figure 6.16 Mean bean moisture content from trees grown at two concentrations of CO_2 and two watering treatments. Data are combined across six genotypes.
Calculated from the weight loss from bean drying. □ - Well-watered. ▒ - Water-stressed. Error bars show standard error of the mean.

6.3.8 Bean to husk ratio

The bean to husk ratio using data from all genotypes is shown in Figure 6.17. At harvest 1, there was a significant reduction in bean to husk ratio under elevated CO_2 conditions ($P < 0.001$). Additionally, there was a significant interaction between water treatment and genotype ($P = 0.05$). The mean bean to husk ratio is shown for individual genotypes in Figure 6.18. A significant decrease in bean to husk ratio under elevated CO_2 treatments was observed for the genotypes CL19/10 and SCA 6 in harvest 1 (both $P < 0.01$). A significant decrease in bean to husk ratio was observed under water stress in the genotype CL19/10 ($P < 0.05$). At harvest 2 a significant decrease in bean to husk ratio was recorded under elevated CO_2 ($P < 0.001$) in addition to a significant increase in bean to husk ratio under water stress ($P < 0.05$). A significant decrease in bean to husk ratio under elevated CO_2 treatment was observed for Pound 7/B in harvest 2 ($P < 0.01$). A significant increase in bean to husk ratio was observed under water stress for IMC 47 ($P < 0.05$). Finally, a significant interaction was observed between water availability and CO_2 in harvest 2 for the genotype CL19/10 ($P < 0.05$). ICS1 and SPEC 54/1 showed no response to treatment across the 2 harvests. At both harvests, there was a significant effect of genotype. ICS 1 consistently had the highest mean bean to husk ratio (0.79 and 0.83 at harvest 1 and 2, respectively), whereas SPEC 54/1 consistently had the lowest mean bean to husk ratio (0.38 and 0.31 at harvest 1 and 2, respectively).

A significant effect of block design 2 was found for bean to husk ratio in harvest 1 and 2 ($P < 0.05$ and $P < 0.01$ respectively). In both cases this represented an overall reduction in ratio in houses 3 and 6; however the trends in response to treatment were the same in both blocks.

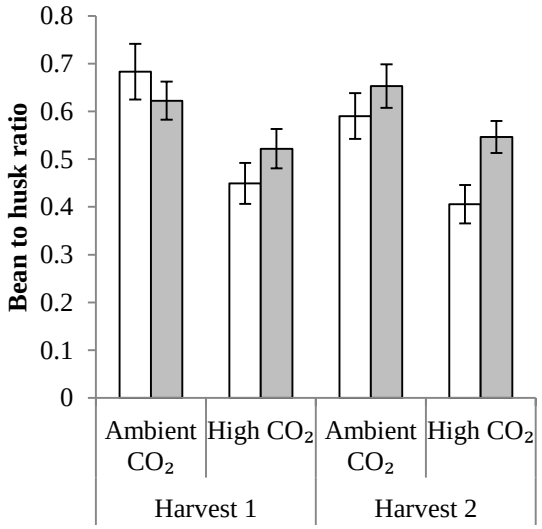

Figure 6.17 Mean bean to husk ratio from trees grown at two concentrations of CO_2 and two watering treatments. Data are combined across six genotypes.
Calculated using the total bean dry weight per pod and total husk dry weight per pod. □ - Well-watered. ▨ - Water-stressed. Error bars show standard error of the mean.

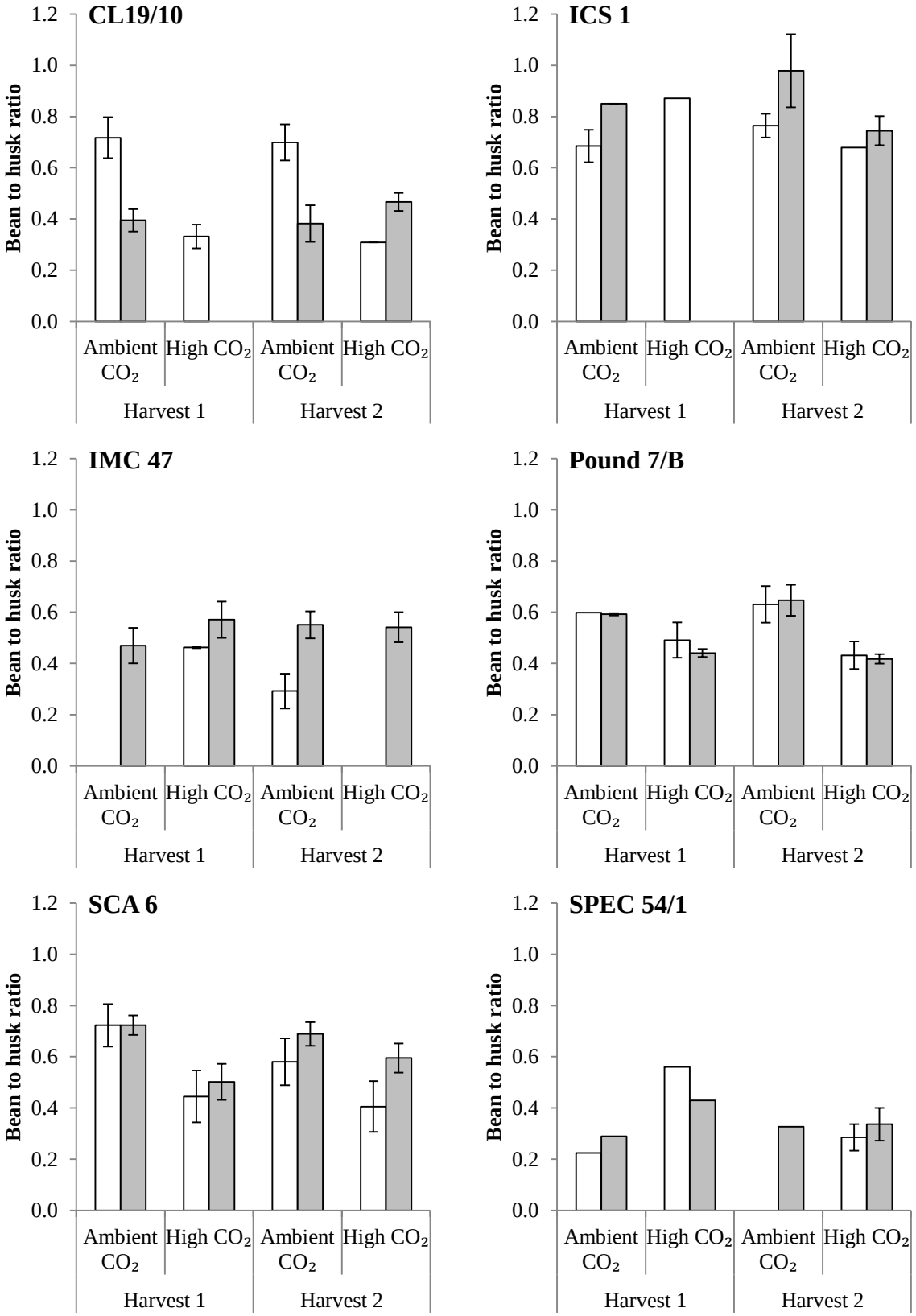

Figure 6.18 Mean bean to husk ratio of six cocoa genotypes grown at ambient and elevated CO_2 and under two watering regimes.
Calculated using the total bean dry weight per pod and total husk dry weight per pod. □ - Well-watered. ▨ - Water-stressed. Error bars show standard error of the mean.

6.3.9 Shell percentage

For all genotypes combined, at harvest 1 there was a significant increase in shell percentage under high CO_2 conditions ($P < 0.01$) (Figure 6.19). At harvest 2 there was only a significant effect of genotype ($P < 0.001$). The lowest overall mean shell percentage was recorded in ICS 1 (10.0%) and the highest was CL19/10 (13.7%) for harvest 2. There were no significant interactions between genotype and treatment. No significant effect of block design was observed.

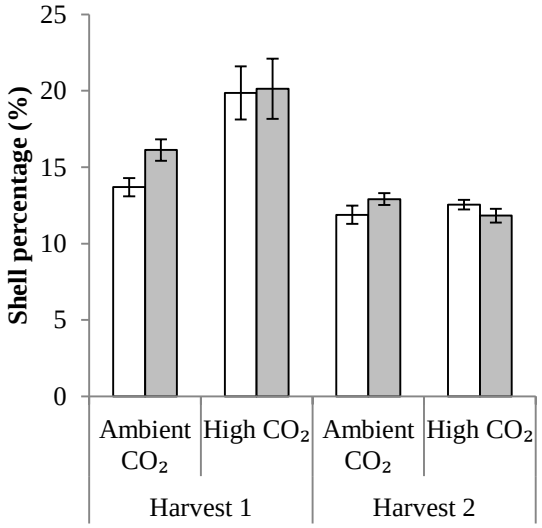

Figure 6.19 Mean shell (testa) percentage from trees grown at two concentrations of CO_2 and two watering treatments. Data are combined across six genotypes.
The percentage of testa dry weight from the sum of the bean and testa dry weights. □ - Well-watered. ▨ - Water-stressed. Error bars show standard error of the mean.

6.3.10 Bean fat content

Bean fat content (expressed as g/100g of sample) is shown in Figure 6.20 for the genotypes Pound 7/B and SCA 6. For Pound 7/B data were only available for harvest 2 and results show a significant interaction between CO_2 and water treatment ($P < 0.05$). Fat content declined under ambient CO_2 and well-watered treatment. However, this did not occur under elevated CO_2 and well-watered conditions. For the clone SCA 6, a significant reduction in fat content

occurred under elevated CO_2 ($P < 0.05$) at harvest 1 but not at harvest 2. No significant effects of treatments were observed at harvest 2.

A significant effect of block design 1 was observed for fat content in Pound 7/B at harvest 2 ($P < 0.05$). This represented a reduction in fat content in houses 5 and 6. A significant effect of block design 1 was observed for fat content in SCA 6 at harvest 1 ($P < 0.05$). This represented a reduction in fat content in houses 5 and 6; however trends in response to treatment were the same in both blocks.

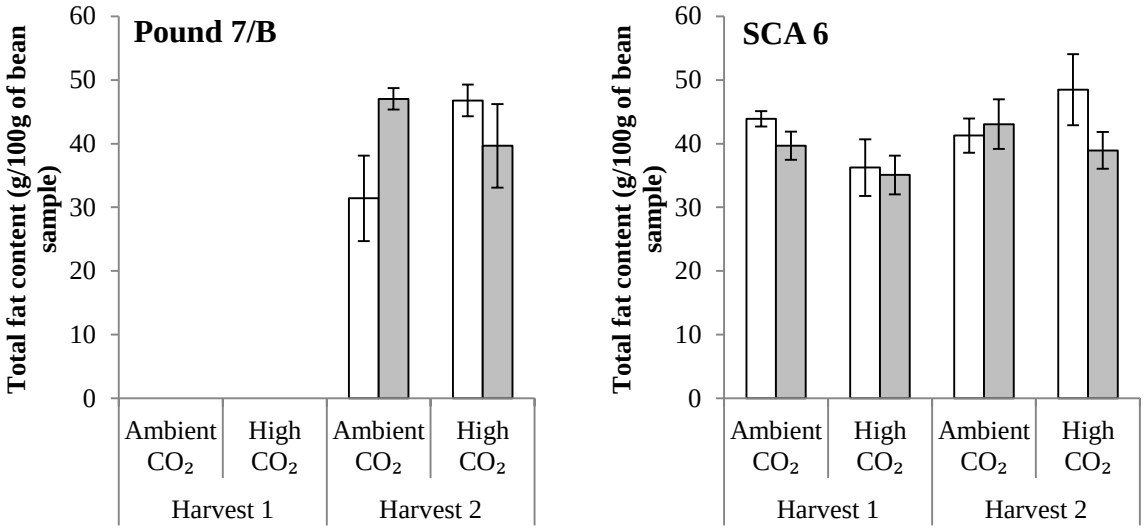

Figure 6.20 Mean fat content of beans from genotypes Pound 7/B and SCA 6 grown at ambient and elevated CO_2 and under two watering regimes.
The mean total bean fat content expressed as g/100g of sample. Data was only obtained from harvest 2 for Pound 7/B. □ - Well-watered. ▩ - Water-stressed. Error bars show standard error of the mean.

6.3.11 Bean fatty acid content

The fatty acid content for Pound 7/B is shown in Figure 6.21. A significant interaction between CO_2 and water treatment was observed for palmitic acid ($P < 0.05$) and stearic acid ($P < 0.05$). In both cases the fat content declined under the ambient CO_2 and well-watered treatments. However, this did not occur under elevated CO_2 and well-watered conditions.

The fatty acid content for SCA 6 is shown in Figure 6.22. At harvest 1 there was a significant reduction of steric acid ($P < 0.05$), cis oleic acid ($P < 0.01$) and cis linoleic acid ($P < 0.05$) under elevated CO_2. However, at harvest 2 there were no significant effects of treatments.

A significant effect of block design 1 was observed for palmitic, oleic, and linoleic acid content (all $P < 0.05$) for Pound 7/B at harvest 2. In all cases, this represented a reduction in content in houses 5 and 6. No effect of block design was observed for SCA 6.

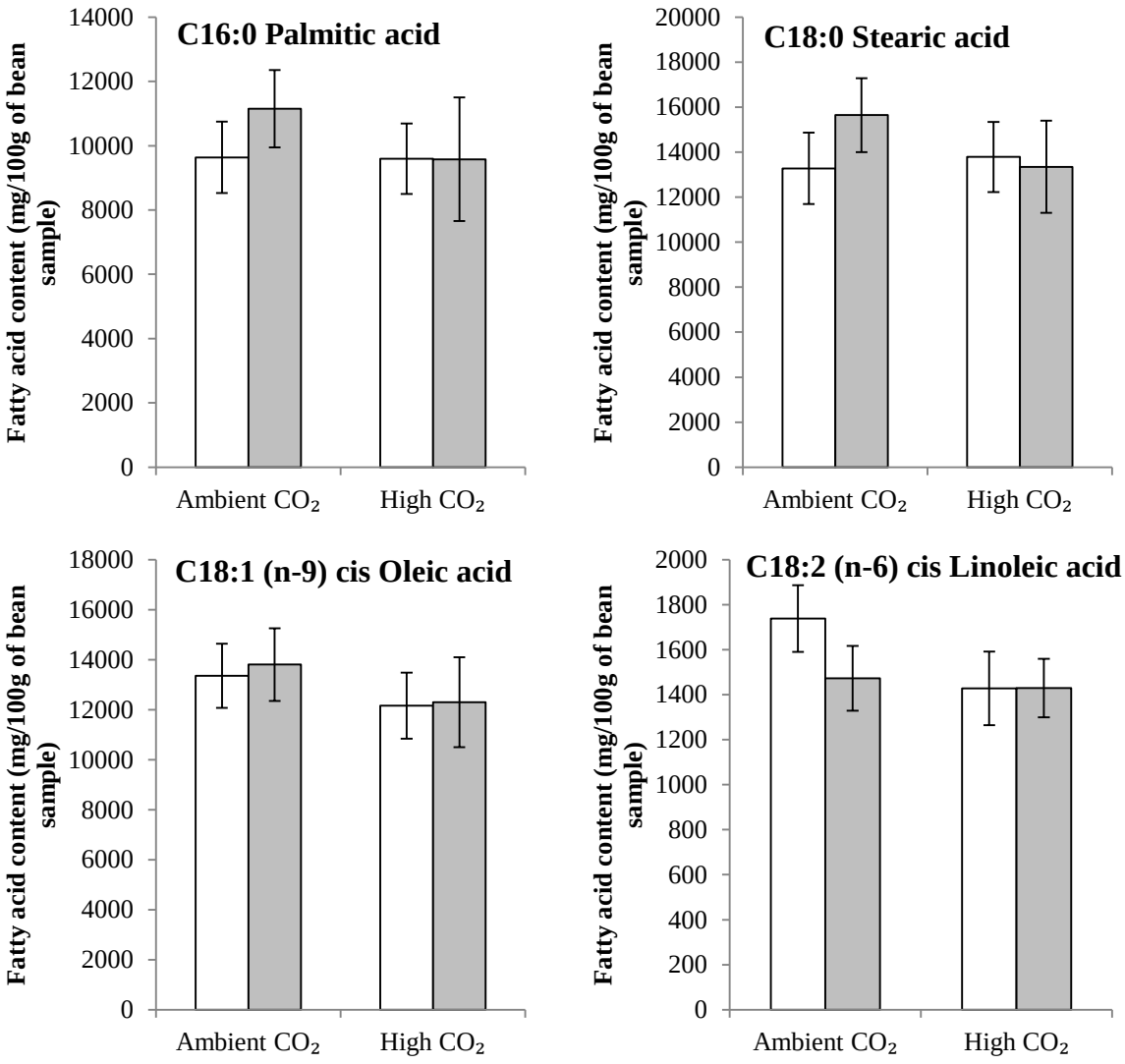

Figure 6.21 Mean fatty acid content of beans from genotype Pound 7/B grown at ambient and elevated CO_2 and under two watering regimes.
Mean bean fatty acid content expressed as mg/100g of sample. Pound 7/B was analysed for harvest 2 only. □ - Well-watered. ▒ - Water-stressed. Error bars show standard error of the mean.

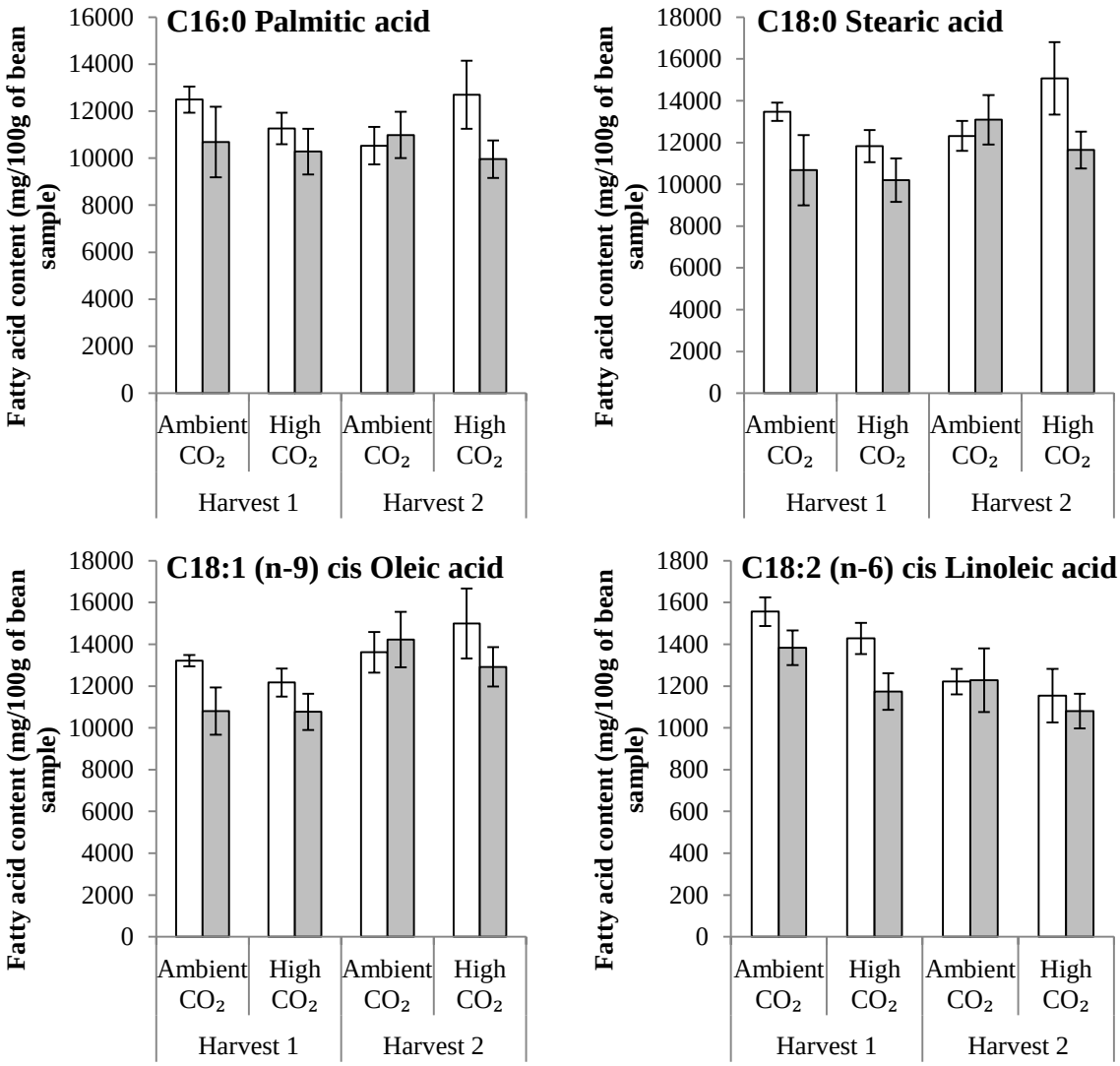

Figure 6.22 Mean fatty acid content of beans from genotype SCA 6 grown at ambient and elevated CO_2 and under two watering regimes.
Mean bean fatty acid content expressed as mg/100g of sample. □ - Well-watered. ▩ - Water-stressed. Error bars show standard error of the mean.

6.3.12 Unsaturated fatty acid content

Mean unsaturated fatty acid content of the cocoa butter is shown in **Figure 6.23** for Pound 7/B and SCA 6. Pound 7/B showed a significant reduction in unsaturated fatty acid content under elevated CO_2 conditions ($P < 0.05$). SCA 6 showed no significant response to treatment in both harvests ($P > 0.05$).

A significant effect of block design 2 was observed for mean unsaturated fatty acids content for Pound 7/B and SCA 6 (both $P < 0.05$) at harvest 2. In both cases, this represented an increase in percentage in houses 3 and 6; however trends in response to treatment were the same in both blocks.

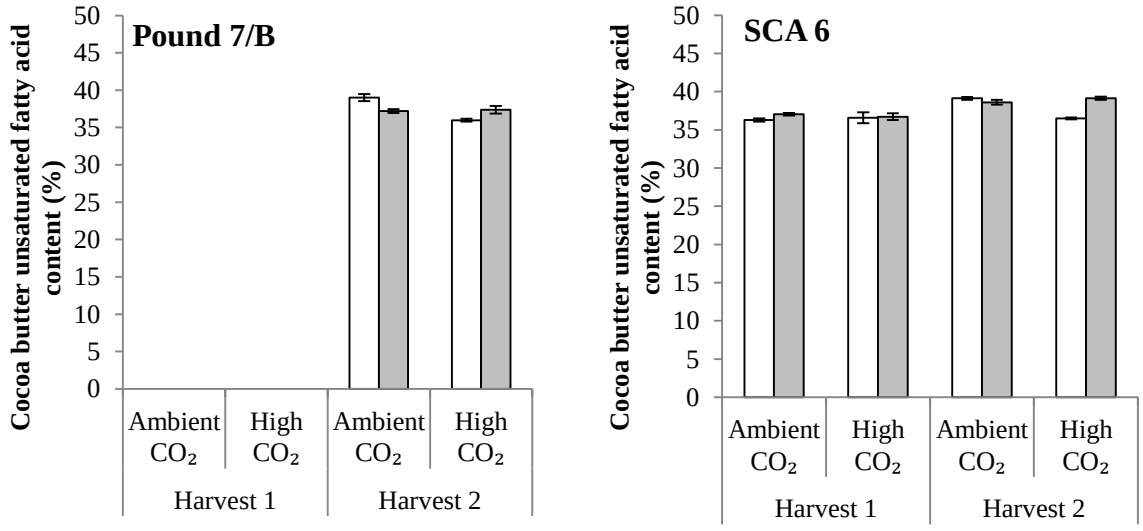

Figure 6.23 Mean cocoa butter unsaturated fatty acid content of beans from genotypes Pound 7/B and SCA 6 grown at ambient and elevated CO_2 and under two watering regimes.
Calculated using the values for total mono-unsaturated, poly-unsaturated and saturated fatty acids. □ - Well-watered. ▩ - Water-stressed. Error bars show standard error of the mean.

6.3.13 Bean sucrose content

Mean total bean sucrose content for Pound 7/B and SCA 6 is shown in Figure 6.24. Pound 7/B showed no significant responses to treatment or interactions ($P > 0.05$) despite a large reduction in sucrose content under 'Ambient CO_2 – WS', and a higher content achieved under elevated CO_2. Similarly, SCA 6 showed no significant effect of treatment or interactions ($P > 0.05$) despite contrasting responses to water availability under elevated CO_2.

A significant effect of block design 2 was observed for bean sucrose content for Pound 7/B at harvest 2 ($P < 0.01$). In both cases, this represented a decrease in bean sucrose content in houses 3 and 6. No effect of block design was observed for SCA 6.

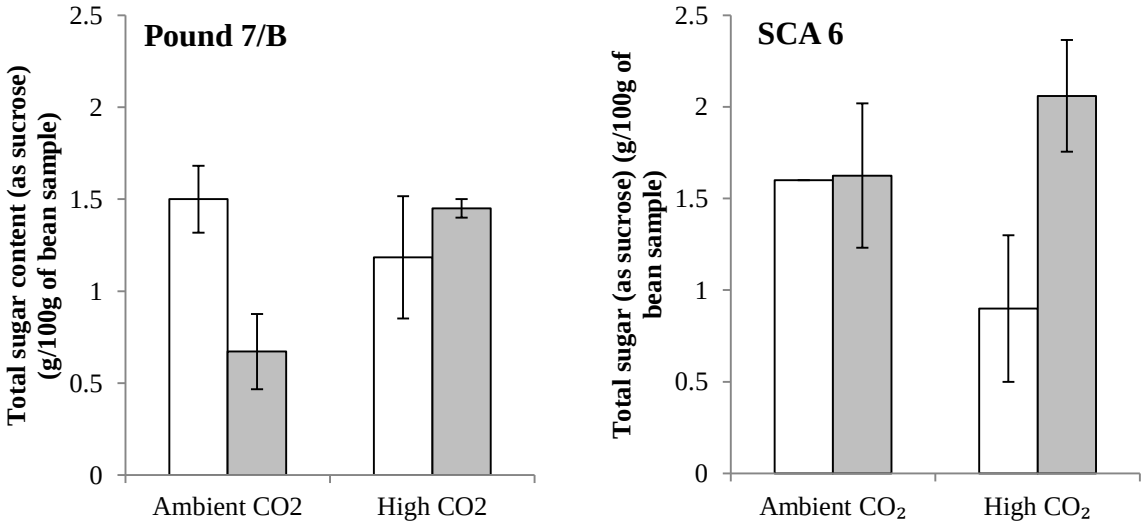

Figure 6.24 Mean total sugar content (as sucrose) of beans from genotypes Pound 7/B and SCA 6 grown at ambient and elevated CO_2 and under two watering regimes.
Total sugar was measured at harvest 2 only. □ - Well-watered. ▩ - Water-stressed. Error bars show standard error of the mean.

6.4 Discussion

Overall, responses to treatments differed between the two harvests. At harvest 1, the most consistent trend observed was in response to water stress. Overall pod weight (fresh and dry), husk weight (fresh and dry), bean dry weight per pod, and individual bean weight (fresh and dry) all decreased in response to water stress. This may be expected as water stress reduces photosynthetic rates in cocoa (Lahive, 2015) and, as a result, assimilate availability for pod development would be reduced. Although husk weight was reduced, the husk thickness did not significantly change suggesting a reduced density of the fibrous material which forms the pod husk. This may result in a softer, less tough husk. Softer or weaker husks may be more susceptible to cocoa pod borer (Daymond et al., 2002a; Azhar et al., 1995). Bean dry weight per pod also declined under water stress, however, bean fresh weight per pod did not, indicating that any reduction in bean mass is compensated for by increased moisture content. This conclusion is reflected in the non-significant trends observed in bean moisture content analysis suggesting increased moisture content under water stress in harvest 1 (Figure 6.16).

An additional trend at harvest 1 was a reduction in bean dry weight per pod, and mean individual bean weight (fresh and dry), in response to elevated CO_2. An increase in photosynthetic rate has been observed in *T. cacao* grown at elevated CO_2 (Lahive, 2015) which would be expected to increase the assimilate availability to reproductive development such as bean growth. However, reproductive development has been shown to be out-competed as a carbon sink by new vegetative growth (Alvim, 1954; Mckelvie, 1956). In the destructive harvest of this study (Chapter 7), elevated CO_2 significantly increased the woody material weight. Additionally, an increase in flush size was identified in cocoa seedlings grown at elevated CO_2; however it should be noted that the enhanced flush size was not observed in adult trees (Lahive, 2015). Despite an increased assimilate supply in elevated CO_2, increased vegetative growth may have outcompeted the assimilate demand from

reproductive growth, specifically bean development which showed a particular sensitivity to elevated CO_2 in the first harvest. An additional response to elevated CO_2 was a reduction in bean to husk ratio in both harvests, representing a shift in assimilate partitioning towards husk development under elevated CO_2. Daymond et al. (2002a) identify bean to husk ratio as a key partitioning factor in the variability of yield in cocoa along with the partitioning between vegetative and reproductive growth. The reduction in bean to husk ratio observed here in response to elevated CO_2 was not observed in all the genotypes studied therefore holding strong potential for future breeding to avoid detrimental effects on yield under conditions of elevated CO_2.

The reduction in bean weight in response to elevated CO_2 coincides with an increase in shell percentage under the same conditions at harvest 1. These results reflect the negative relationship between bean size and shell percentage reported by Wood (1985c). Such a response in future climates of higher atmospheric CO_2 would be undesirable as the shell is of little value. Excess assimilate investment into shell development is partitioning resources away from other more valuable pod components. However, although the testa is of little value, it does provide the dried beans with physical barrier protection against microbial contamination and pest attack, therefore a minimum shell percentage of 11% is preferred (Wood, 1985c).

At harvest 2, the most consistent trend was an increase in pod weight (fresh and dry), husk weight (fresh and dry), husk thickness, bean fresh weight per pod, and individual bean weight (fresh and dry) in response to elevated CO_2. As previously discussed, increases in atmospheric CO_2, increase the rate of photosynthesis in *T. cacao* (Lahive, 2015; Baligar et al., 2008). Therefore it is logical that increased photosynthetic rate would increase assimilate availability to reproductive development resulting in the increases observed in this study. However, these effects were only observed in the second harvest despite the change in

photosynthetic rate in response to elevated CO_2 occurring from the start of the study (Lahive, 2015). This delayed effect of elevated CO_2 suggests that initially the additional assimilate production may have been allocated to the growth of vegetative and woody material which is known to outcompete reproductive growth through the increase in cherelle wilt during periods of vegetative flushing (Alvim, 1954). As trees matured and the vegetative growth began to slow, the investment or allocation of resources to reproductive growth would have increased. As observed in sour orange, trees grown under elevated CO_2 (700ppm) initially demonstrated heavy increases in trunk and branch volume. When this increase began to decline the investment began to increase in tree fruiting (Kimball et al., 2007). Overall, the assimilate investment of the sour orange trees into biomass did not decrease but was transferred onto reproductive growth. This would explain why changes in cocoa pod components were not observed until the second year of the study. Despite the enhanced bean weights observed under elevated CO_2 in harvest 2, a reduction in bean to husk ratio was still observed in this harvest under elevated CO_2 conditions, representing a shift in partitioning towards husk development. As discussed previously, the lack of this response in certain genotypes will be a key factor in future breeding to preserve or improve yield partitioning. Bean to husk ratio also increased in response to water stress in harvest 2, representing increased partitioning to bean development under water stress. This may be representative of reduced vegetative sink competition as leaf weight and area was reduced under water stress (Chapter 7).

An increase in bean number was observed under water stress at harvest 2 and this trend was also seen at harvest 1. There are a number of factors which could affect the number of beans which develop to maturity in a cocoa pod. Previous research has identified a strong positive relationship between the number of pollen grains deposited in pollination and the number of beans in a mature pod (Falque et al., 1995). It is assumed that all flowers in this study

received approximately the same volume of pollen as they were pollinated by the same standardised procedure (Chapter 3). Although some flowers received pollen from 2 stamens and others from 3, these two outcomes were present on every tree therefore no preferential pollination attempts could have occurred between trees or between treatments. An additional factor in seed number is the health of gametes prior to fertilisation, and the health of the embryo post fertilisation. Donor pollen in this study was sampled from non-experimental trees therefore pollen heath, although it may have naturally fluctuated, would not have been a factor. Maternal trees were the trees exposed to the experimental treatment and therefore ovule health may have altered in response to treatment. A decline in ovule health has been identified in soybean when exposed to water deficit stress (Kokubun et al., 2001) and ovule abortion has been observed in *Arabidopsis* exposed to salt tress (Sun et al., 2004). Although salt stress includes potential additional stresses of toxicity and ion imbalances, parallels can be drawn with water stress as reduced soil water potential impairs water uptake, resulting in symptoms similar to that of water stress (Lambers et al., 2008a). Ovule abortion would decrease the potential number of beans within a pod and may have been expected to occur in the water stress treatment of this study. However, contrary to the suggestions of previous research, the number of beans which developed per pod in this study seemed to increase in response to water stress. The decrease in photosynthetic rate observed in cocoa grown under water stress (Lahive, 2015) would have reduced the assimilate availability for pod growth investment. However it was also identified in the destructive harvest of this study (Chapter 7) that water stress also reduced the vegetative growth of trees. Vegetative growth is a strong assimilate sink and is observed to outcompete reproductive growth. This is most clearly identified through an increase in cherelle wilt during periods of vegetative flushing (Alvim, 1954). As the vegetative growth was reduced under water stress, the vegetative sink would have reduced and increased levels of assimilates may have been available for bean

development, despite a reduced photosynthetic rate. Additionally, pollination success rates were also improved under water stress in this study (Chapter 5), this may further indicate reduced vegetative sink competition with maternal flowers under water stress. Finally, although the pollination procedure was standardised, a count of pollen grains deposited on the style was not monitored here. To gain a clearer relationship between environmental stress such as water deficit and bean number, the volume of pollen used in germination will need to be considered in future research.

In almost all cases, there was significant natural variation between genotypes in the yield parameters measured, suggesting potential to breed for yield improvements. The exception to this genetic variation was husk thickness which showed similar thicknesses across all genotypes. Furthermore, shell percentage at harvest 1 did not differ between genotypes; however shell percentage became more varied at harvest 2.

Due to a restricted number of pods per genotype and a minimum weight of beans required for fat analysis, SCA 6 (harvest 1 and 2) and Pound 7/B (harvest 2 only) were the only genotypes used for fat analysis. Overall fat content and composition remained unaffected by water stress in both harvests for SCA 6. However, there were responses to elevated CO_2. Total fat content, stearic acid, oleic acid and linoleic acid all declined significantly under elevated CO_2 at harvest 1. Both oleic and linoleic acids are unsaturated fatty acids and decreases in their production would result in reduced formation of triglycerides with unsaturated fatty acid tails, ultimately leading to harder cocoa butters which are more desirable for processing (Wood, 1985c). However, a decrease in saturated fatty acids such as stearic acid could potentially have the opposite effect if the stearic acid is replaced by additional unsaturated fatty acids. However as no significant increases in other major fatty acids were observed it appeared that a shift in quantities was not due to increases elsewhere, but as a result of a decrease in overall fat content. The reason why total fat content was reduced was unclear, therefore at harvest 2,

analysis of the total sugar content was conducted to identify if the loss in fat content was counterbalanced by an increase in sugar content, potentially as a direct response to increased assimilate availability under elevated CO_2. No significant changes to sugar content were observed. Furthermore, any significant results found at harvest 1 were not repeated at harvest 2. No changes in the percentage of unsaturated fatty acids were observed in both harvests for SCA 6. It may be that under elevated CO_2, the earlier harvest coincided with increased vegetative growth due to increased assimilate production with higher photosynthesis rates (Lahive, 2015). The young vegetative growth may have outcompeted the bean development for assimilates as has been previously documented in cocoa through the increased incidence of wilt during leaf flushing (Mckelvie, 1956; Alvim, 1954). This could have resulted in the detriment to fat content observed here. By harvest 2 the vegetative growth may have slowed, shifting assimilates to reproductive growth as observed in sour orange (Kimball et al., 2007).

Pound 7/B was not analysed at harvest 1, however at harvest 2 interactions between water and CO_2 treatments were observed for total fat and for the content of palmitic and stearic fatty acids such that decreases in fat content were observed under well-watered and ambient CO_2 treatments. Additionally, if the well-watered treatments are compared for both CO_2 treatments, an increase in content was also observed under elevated CO_2. This may be explained by an increase in assimilates available for bean development through enhanced photosynthesis at elevated CO_2. The increase under water stress for ambient CO_2 treatments seems to go against what may be expected as water stress will have reduced photosynthesis and therefore assimilate availability. As vegetative growth in cocoa is known to be a dominant assimilate sink over reproductive growth (Alvim, 1954; Valle et al., 1990; Mckelvie, 1956), the enhanced fat content may be a result of reduced vegetative competition brought about by reduced vegetative growth under water stress. Further support for this is evident in the destructive harvest (Chapter 7) in which reduced wood, leaf and leaf area was

observed in response to water stress. The enhanced photosynthetic rates observed under elevated CO_2 may have removed this effect of vegetative sink as assimilates were supplemented through enhanced rates of photosynthesis. An increase in the production of palmitic and stearic fatty acids under water stress for Pound 7/B as is suggested here would increase the saturated fatty acid content, increasing the production of monounsaturated triglycerides such as Oleodipalmitin (POP), Oleodistearin (SOS) and Oleopalmitostearin (POS). This is further supported here through a significant decrease in unsaturated fatty acids for Pound7/B under elevated CO_2. This would decrease the formation of polyunsaturated fatty acids through increased palmitic and stearic acid availability (Liendo et al., 1997). Overall, the melting point of the butter would increase, improving its quality for processing (Wood, 1985c).

Despite the limited analysis of genetic variation here, the results still show contrasting genotypic responses to treatment. SCA 6 responded to high CO_2 at harvest 1 however it would seem that longer exposure to high CO_2 and more established trees may remove any impacts on cocoa butter production. However, effects of CO_2 treatment were observed for Pound 7/B despite equal exposure and tree age. The results do not suggest a detrimental effect of water stress and elevated CO_2 in future climates based on these two genotypes.

Testing for the effect of greenhouse location using block designs identified an influence of houses 3 and 6 which are exposed to direct sunlight for much of the day. Further investigation as to the effects of greenhouse location revealed no alteration of the treatment responses observed here. One exception was the mean individual bean weight which was reduced under water stress in houses 2 and 5, but was increased under water stress in houses 3 and 6. Generally houses 3 and 6 received higher levels of direct sunlight throughout the day. When the data from the two blocks were analysed in isolation for treatment effect; houses 3 and 6 showed a generally lower individual bean weight under well-watered

conditions compared to houses 2 and 5. The trees exposed to higher light levels may have exhibited altered growth and leaf development which in turn may have affected their reproductive growth. Exposure to high irradiance in cocoa seedlings has been shown to increase leaf flushing, increase assimilate partitioning to root development, and increase transpiration rates (Galyuon, 1994), all of which could impact on reproductive growth. Despite this observed effect of greenhouse location, all other parameters measured displayed consistent responses to treatment across both blocks.

6.5 Conclusions

An overall shift in response to treatments was observed between the two harvests. Tree investment in pod components generally decreased under water stress at harvest 1 with little effect of elevated CO_2. However, at harvest 2 assimilate investment in pod components increased under high CO_2 conditions. In general, the results suggest a developmental tolerance of water stress with prolonged exposure, while beneficial effects of elevated CO_2 appear to occur after an initial delay period in which any increases in assimilate production is not allocated to reproductive growth. SCA 6 showed an overall reduction in bean cocoa butter content in response to high CO_2 at harvest 1. These detrimental effects also seem to diminish with prolonged exposure as no effect of treatment was observed at harvest 2. Pound 7/B responded differently suggesting potential increases in total fat and saturated fatty acid content dependent on the environmental conditions. Overall, there was strong genetic variability in response to CO_2 and water stress suggesting potential for yield protection and breeding programmes for climate change resilience.

Chapter 7. The effects of elevated CO_2 and water stress on canopy development and photosynthesis of cacao

7.1 Introduction

The focus of this long term study was to investigate the effects of elevated carbon dioxide and water deficit stress on the reproductive development of *Theobroma cacao* using controlled environment glasshouses. Many of the responses have been discussed within the context of assimilate productivity and partitioning within the tree. Assimilate production occurs through photosynthesis in the leaves. Variation in leaf area and photosynthetic rates will partially account for differences in tree vigour and assimilate investment into reproductive growth (Alvim, 1977). To gain further insights into the reproductive responses observed in the study, an assessment of vegetative growth was carried out at the end of the experimental period.

It is generally observed that the rate of photosynthesis increases in C_3 plants when the atmospheric concentration of carbon dioxide (CO_2) is elevated (Long et al., 2004). This photosynthetic response occurs as the activity of Rubisco, the enzyme responsible for the first step in carboxylation (Smith et al., 2010), is limited by current atmospheric concentrations of CO_2 (Long et al., 2004). Therefore an increase in the concentration of CO_2 increases the rate of carboxylation. Additionally, Rubisco plays a role in the oxygenation of RuBP as part of photorespiration. This process is in direct competition with carboxylation. The affinity of Rubisco for CO_2 is greater than that of oxygen therefore increases in CO_2 concentration reduces the rate of oxygenation, further increasing rates of photosynthesis (Long et al., 2004). The instantaneous effects of elevated CO_2 on the photosynthetic responses of cocoa have been tested on seedlings using an infrared gas analyser (Baligar et al., 2008). Measurements

at several CO_2 concentrations (between 850 and 85ppm) revealed increases in photosynthetic rate by up to 33% with increases in CO_2, in addition to a strong reduction in stomatal conductance (65%). Additional research on cocoa seedlings growing in 700ppm CO_2 for 57 days revealed significant increases in the dry weight of leaves, stems and roots (Baligar et al., 2005). The photosynthetic responses to elevated CO_2 of the six mature cacao genotypes in this study were investigated by Lahive (2015). Maximum photosynthetic rate (A_{max}) was significantly increased in all genotypes by an average of 54%. Mean light saturation point increased under elevated CO_2; however this was only significant under well-watered conditions. Lahive (2015) found no significant reduction of stomatal conductance in response to elevated CO_2 in adult cocoa genotypes which contradicts the findings of Baligar et al. (2008), however this may be due to different genotypes used in both studies, in addition to the differences in the tree age as Baligar used cacao seedlings. The work by Lahive (2015) took place prior to the completion of this project therefore a destructive harvest was not possible. No destructive harvests of adult cocoa trees under prolonged exposure to elevated CO_2 concentrations have been carried out. However there have been similar studies on citrus trees using free-air CO_2 enrichment (FACE) facilities. Eight sour orange trees (*Citrus aurantium* L.) were grown from seedlings in open-top clear chambers. Four of these chambers were enriched with an additional 300 μmol mol^{-1} CO_2 above that of ambient concentrations. These conditions were maintained for 17 years. Final measurements revealed biomass increases at elevated CO_2 of large branches, trunks, stumps and large roots by up to 55% without any effect on the root/shoot ratio. Cumulative pruned biomass was also measured and an increase of 70% was observed under elevated CO_2 conditions. Leaf area per tree increased by 10% and the cumulative fruit production across the whole study was increased by 85%. The increases in fruit biomass were brought about by increases in fruit number, not fruit size (Kimball et al., 2007).

Moisture availability plays a large role in the vegetative growth of cacao (Toxopeus, 1985). An established link between rainfall patterns and cacao flushing cycles exists with new leaf expansion occurring when rainfall returns after a period of drought (Alvim and Alvim, 1977). Additionally, the authors observed an increase in leaf drop upon the return of rains and attributed this to the rupture of abscission layers with the rehydration of the stems. Sale (1970b) also observed vigorous flushing with the return of irrigation to experimental cacao trees however the observed pattern of leaf abscission differed from that of Alvim and Alvim (1977) and occurred on the arrival of a dry period. This may be due to differences in the severity of the drought imposed as both studies used different methods to determine when water shortage was physiologically detected by the tree. The trees used by Alvim and Alvim (1977) were also older in both their greenhouse and field studies. No genotype information was provided for that study. Such variations may account for the differences in leaf drop timing. Further results from Sale (1970b) reveal net leaf area, area per leaf and duration of leaves on a tree were all reduced in response to dry moisture treatments. Photosynthetic analysis of the trees used in the present study revealed a significant reduction in A_{max} (60%) and stomatal conductance due to the water stress treatment (Lahive, 2015).

The decreases observed in the stomatal conductance of many plants under elevated CO_2 (Long et al., 2004) is consistent with the results found in cocoa seedlings by Baligar et al. (2008). Baligar et al. goes on to suggest the changes in stomatal behaviour will improve water use efficiency (WUE) through reductions in transpirational loss of water. WUE in that study increased 3-fold with increases in CO_2 from 370 to 680ppm. This could result in the amelioration of negative effects of water stress under conditions of elevated CO_2. However, Lahive (2015) did not observe any significant reductions in stomatal conductance in response to elevated CO_2 in adult cocoa trees, although, significant increases in intrinsic WUE were observed under elevated CO_2 which was attributed to increases in photosynthetic rate.

Differences in the cocoa genotypes used in either study or the difference in the age of the trees used may have contributed to the contrasting observations made. Regardless of the varied responses, there is scope for amelioration of the effects of water stress when combined with elevated CO_2.

Genetic variability in the canopy architecture of cacao has already been identified by Daymond et al. (2002b). As the importance of canopy size and the photosynthetic rate are already well established as being important factors in explaining variation in cacao tree vigour and yield (Alvim, 1977; Zuidema et al., 2005), in addition to assessing the biomass changes in the trees of this study, an estimation of canopy productivity was also carried out. For this study, the aim was to develop a method to estimate canopy productivity with enough accuracy to provide a relative comparison of photosynthetic productivity between trees. Photosynthetic light response curves had already been obtained from the experimental trees (Lahive, 2015). With the data available, the biomass of each tree in combination with averaged light interception measurements within the individual canopies, could be used to build a basic estimate of canopy assimilate production. These estimates, along with biomass measurements were used to build a more detailed picture of the responses to treatments.

7.2 Materials and methods

Six genotypes of cocoa were grown under either elevated (700ppm) or ambient carbon dioxide (averaging 437ppm), and well-watered or water stressed conditions for 23 months (see Chapter 2 for full details of materials and methods).

Throughout the experiment, minimum pruning was undertaken to minimise interference with tree growth, however as trees grew larger, pruning was necessary to manage space restrictions within the greenhouse. Generally, trees were permitted up to 3 large vertical main

stems. Lateral growth around the base of the trees was removed to allow for access to pod growth. Trees were permitted to reach a maximum height of 3 meters, above which growth was pruned. Large lateral branches were directed in an upwards direction using bamboo canes for support. Chupon growth from the tree base was removed. Full details of tree husbandry can be found in Chapter 2. Prunings and fallen leaves were not recorded and are therefore not included in the calculations of this chapter.

7.2.1 Measurement of canopy light interception

To measure the light distribution through the canopy, the canopy was segmented into defined layers which differed in their leaf density and as a result their light penetration. For example, in Figure 7.1 (a), the leaves in layer 1 would be exposed to higher levels of light than leaves in layers 2 and 3. Additionally, the leaves in layer 2 are likely to be denser than that of layer 3 which would also alter the light levels. Each tree was marked on a large, central trunk/branch to identify a boundary between distinct layers of the canopy. The number of layers ranged from 2 to 5 depending on the tree size and the variation present in the canopy structure. Once this was completed a ceptometer (Sunfleck ceptometer, *Decagon Devices Inc*., Pullman, WA, USA) was used to estimate the average light level within each canopy layer on 30th June 2015). The sensor stick of the ceptometer was inserted into the canopy so it spanned the width of the canopy and crossed near to the central point. Rotating around the centre of the canopy layer, 4 light measurements were taken (see Figure 7.1 (b)) and averaged. For each tree, the percentage light transmission through the canopy was calculated for each canopy layer. The transmitted light level was calculated as a percentage of the incident light level recorded in layer 1.

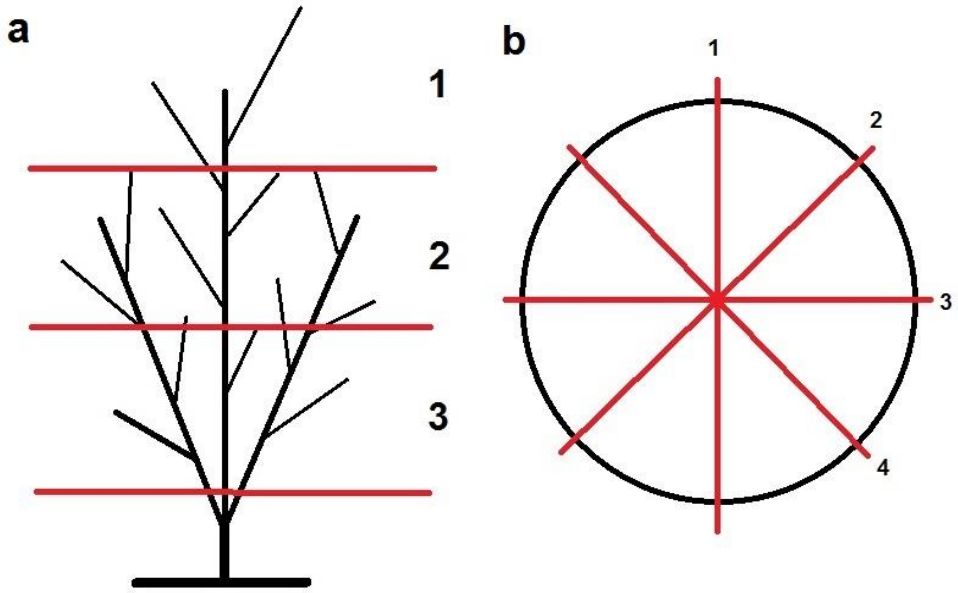

Figure 7.1 Canopy layering (a) and ceptometer measurement method (b).
Each tree's discrete canopy was layered according to leaf density. (a) represents the imagined canopy layer boundaries. (b) represents the 4 positions of the ceptometer sensor in each canopy layer (as viewed from above).

7.2.2 Destructive harvest

To identify variation in vegetative growth due to experimental treatments, all trees were harvested at the end of the 23 month period (27.07.15 to 11.08.15).

The canopy layers which were marked on each tree (as described in 7.2.1) were used here to identify distinct canopy layers for dissection. Beginning at the top layer, at the dividing marker between the top layer and the layer below, a horizontal cross-section was imagined at this point (see Figure 7.1 a). Any branches above this plane were removed and categorised as 'layer 1'. Equally, branches which may have originated below but grew upwards across this plane were cut at the point they crossed the boundary between layers. The same was true for branches which originated above but grew downwards. Once the tree was cut into its respective layers, the leaves were separated from the branches. Soft (flush) leaves were distinguished from hardened mature leaves. Total fresh weights of the woody material and

both leaf types were recorded (KERN scales, model DE150K20D *KERN & SOHN*, Balingen, Germany). From each tree layer, a small representative sample was taken of wood and both leaf types and placed into separate labelled paper bags. The remaining tree material was discarded. The weights of the respective samples were recorded. The leaf area of the hard and soft leaf samples was measured using a WD3 WinDIAS leaf image analysis system (*Delta-T Devices Ltd,* Cambridge, UK). All samples were returned to their bags and placed into ventilated drying ovens (*Heraeus oven,* Model UT 6760, *Heraeus*, Hanau, Germany) at 70°C until the weights remained stable. Finally, dry weights from each sample were recorded (KERN scales, model DE150K20D and model PCB250-3, *KERN & SOHN*, Balingen, Germany).

Using the representative sample leaf area in combination with the sample leaf weight and total leaf weight, an estimate of the total leaf area in each canopy layer was calculated (see Equation 7.1).

Equation 7.1 Calculation of total leaf area

$$Predicted\ total\ leaf\ area = \left(\frac{Sample\ leaf\ area}{Sample\ leaf\ fresh\ weight}\right)\ x\ Total\ leaf\ fresh\ weigh$$

Additionally, using the proportion of weight loss between representative sample fresh weight and dry weight, in combination with the total fresh weights, an estimation of total dry weights was calculated (see Equation 7.2).

Equation 7.2 Calculation of total dry weight

$$Total\ dry\ weight = Total\ fresh\ weight\ x\ \left(\frac{Sample\ dry\ weight}{Sample\ fresh\ weight}\right)$$

7.2.3 Estimation of canopy productivity

To gain further insight into the effects of treatment on the overall productivity of the trees, an estimation of net canopy photosynthesis was calculated. For the purposes of this study, the term 'canopy' shall be used to refer to the discrete leaf canopy of each individual tree. Although, by this stage of the experiment, there was some partial tree overlap and shading, the measurement of discrete canopies allowed trees and thus treatment effects to be assessed individually.

The measurement of photosynthetic activity using an infrared gas analyser provides a rate of photosynthesis ($\mu mol.m^{-2}.s^{-1}$). Using the total leaf area of the tree, the assimilation rate of the whole tree can be estimated as the product of leaf area and the assimilation rate. The assimilation rate fluctuates according to the light levels reaching the leaf surface. Naturally, the light levels within a tree canopy are not homogeneous; therefore the canopy was split into layers according to the distribution of light (see 7.2.1). To calculate assimilation rates at different light levels, light response curves created by Lahive (2015) were utilised.

Light response curve parameters for each genotype and treatment were used in Equation 7.3 in combination with the light levels recorded in each tree layer, to calculate an assimilation rate. To ensure the assimilation rates were comparable, the absolute light levels recorded within each canopy layer, were used to calculate the proportion of light which reached each layer. Using these percentages, a fixed hypothetical light level could be used to compare assimilation rates of trees. The hypothetical light level used was the highest light level recorded by the ceptometer within the glasshouses (1556.5 $\mu mol\ m^{-2}\ s^{-1}$).

Equation 7.3 Non-rectangular hyperbola
Where A is assimilation rate (µmol m^{-2} s^{-1}), ∅ is quantum efficiency (µmol (CO_2) $µmol^{-1}$ (photon)), Q is irradiance (µmol m^{-2} s^{-1}), A_{max} is light saturated photosynthetic rate (µmol m^{-2} s^{-1}), *k* is convexity, and R is respiration rate (µmol m^{-2} s^{-1}).

$$A = \left\{ \frac{(\emptyset Q + Amax) - \sqrt{[(\emptyset Q + Amax)^2 - 4\emptyset Q k Amax]}}{2k} \right\} - R$$

The hard leaf area of each layer was multiplied by the corresponding assimilation rate to provide a total assimilation rate per layer. The values from each layer were then integrated to provide a total net canopy assimilation rate per tree. The soft leaf areas were not included in this calculation as new flush leaves have little to no photosynthetic activity until hardened (Baker and Hardwick, 1973).

7.2.4 Statistical analysis

Effect of treatment on all destructive harvest components and canopy assimilation rate were analysed by means of unbalanced design analysis of variance (ANOVA) using GenStat 15th edition statistical software (GenStat, VSN International Ltd., Hemel Hempstead, UK). Two block designs were created to test for any effect of greenhouse.

7.3 Results

7.3.1 Total tree weight

The total biomass fresh and dry weight above the 'soil' level was averaged using data from all genotypes per treatment (see Figure 7.2). The biomass represented a combination of woody material, hard and soft leaf weight. The same significant responses to treatment were observed for both fresh and dry weights. There was significantly greater biomass under elevated CO_2 conditions ($P < 0.01$) in which an increase of 12.4% and 9.6% was observed for fresh and dry weights respectively. There was a significant reduction of biomass under water stressed conditions ($P < 0.001$ for fresh and dry weights) under both ambient and elevated CO_2. Overall there was a reduction in fresh weight of 26.7%, and a reduction in dry weight of 29.3%. In addition, there was a significant effect of genotype (both $P < 0.001$ for fresh and dry weights). CL19/10 had the highest mean total biomass fresh and dry weights at 7077g and 2418g respectively, whereas SCA 6 had the lowest mean at 3103g and 1004g respectively. For fresh and dry weights there were no significant interactions between CO_2 and water treatment and no interactions between genotype and treatment.

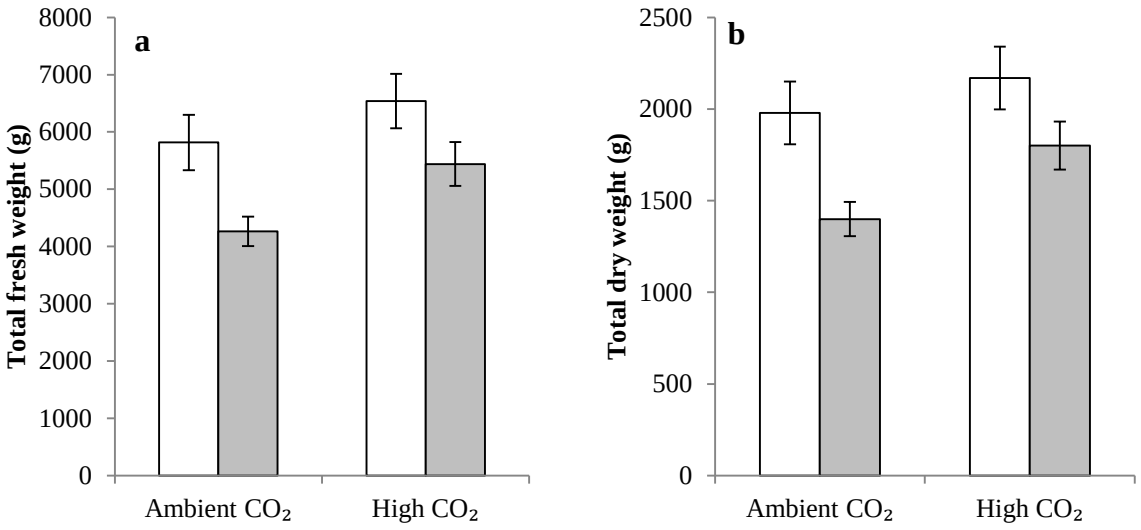

Figure 7.2 Mean total fresh (a) and dry (b) weights of all above ground biomass from trees grown at two concentrations of CO_2 and two watering treatments. Data are combined across six genotypes.
Dry weights were estimated using representative samples. □ - Well-watered. ▩ - Water-stressed. Error bars show standard error of the mean.

7.3.2 Total wood weight

The mean total fresh and estimated dry weights of woody material are shown in **Figure 7.3**. The same significant responses to treatment were observed for both fresh and dry weights. Under elevated CO_2 there was a significant increase in woody material weight (both $P < 0.001$ for fresh and dry weights). Fresh weight increased by 19.6% and dry weight increased by 17.5%. Both fresh and dry weights showed a significant reduction of woody material under water stressed conditions (both $P < 0.001$ for fresh and dry weights) under both CO_2 conditions. Fresh weight decreased by 30.0% and dry weight decreased by 31.7%. In addition, for both fresh and dry weights, the effect of genotype was significant (both $P < 0.001$ for fresh and dry weights). CL19/10 had the highest mean total woody material fresh and dry weights at 4301g and 1436g respectively whereas SCA 6 had the lowest mean at 1826g and 597g respectively. For both fresh and dry weight there were no significant interactions between CO_2 and water treatment and no interaction between genotype and treatment.

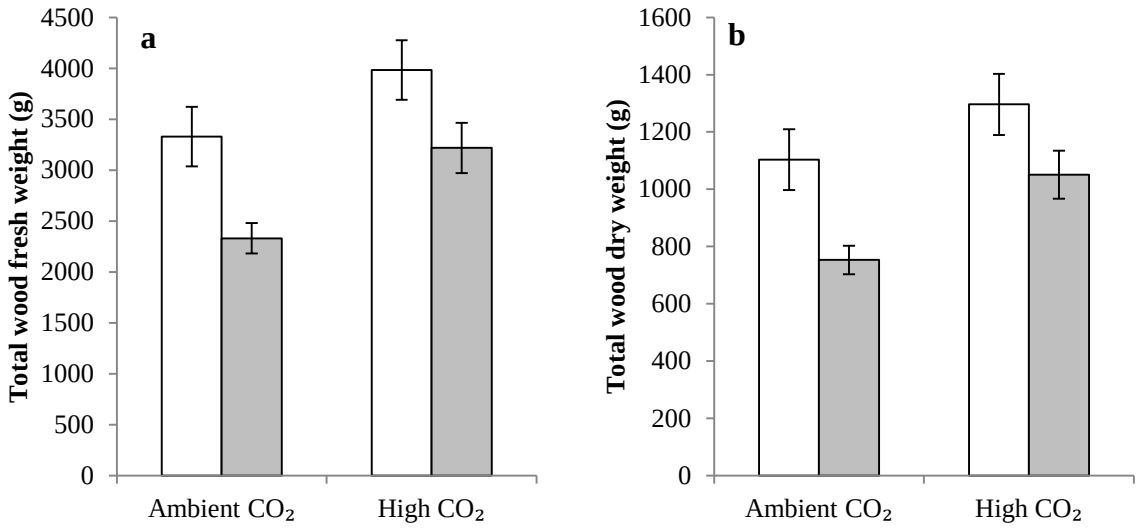

Figure 7.3 Mean total wood fresh (a) and dry (b) weight from trees grown at two concentrations of CO_2 and two watering treatments. Data are combined across six genotypes.
Dry weights were estimated using representative samples. □ - Well-watered. ▒ - Water-stressed. Error bars show standard error of the mean.

7.3.3 Percentage wood weight

There was a significant increase (3.5% fresh and 3.7% dry) in the percentage of total biomass accounted for by wood under elevated CO_2, a significant decrease (3.4% fresh and 4.5% dry) under water stress, and a significant effect of genotype for both fresh and dry weights (all $P < 0.001$) (Figure 7.4). SPEC 54/1 had the highest mean percentage of woody material fresh and dry weights at 62.7% and 61.3% respectively, whereas IMC 47 had the lowest mean at 54.7% and 52.4% respectively. For fresh and dry weight there were no significant interactions between CO_2 and water treatment and no interaction between genotype and treatment. A significant effect of block design 1 was found for wood fresh weight as a percentage of total fresh biomass ($P < 0.05$). This effect represented an overall reduction in percentage in houses 5 and 6; however the trend in response to treatment was the same in both blocks.

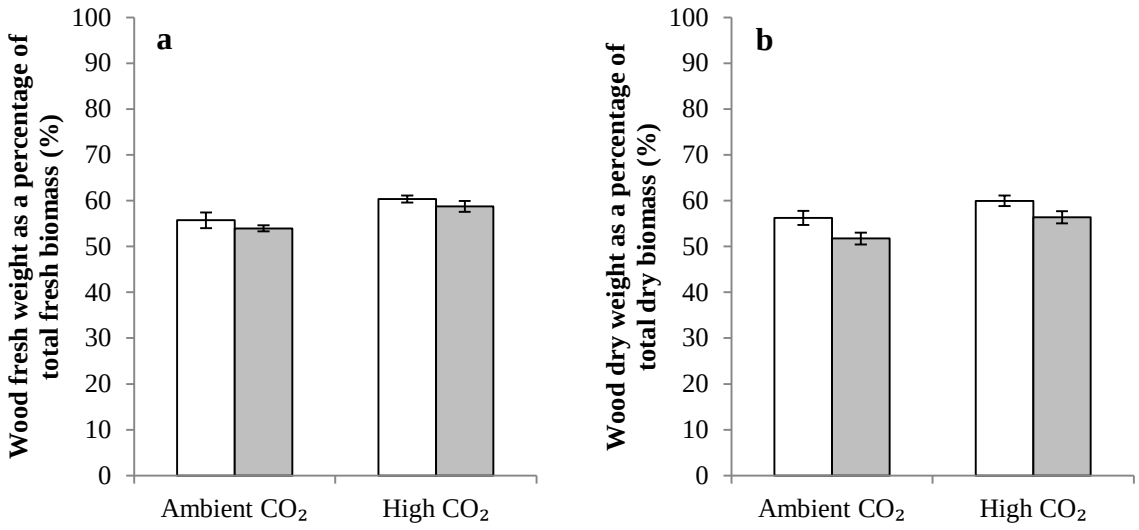

Figure 7.4 Wood fresh (a) and dry (b) weight as a percentage of total biomass from trees grown at two concentrations of CO_2 and two watering treatments. Data are combined across six genotypes.
Dry weights predicted using representative samples. □ - Well-watered. ▒ - Water-stressed. Error bars show standard error of the mean.

7.3.4 Total hard leaf weight

There was a significant reduction in leaf weight under water stressed conditions for both fresh and dry weights ($P < 0.001$ and $P < 0.01$ respectively) (**Figure 7.5**). Fresh weight decreased by 25.4% and dry weight decreased by 24.0%. There was no significant effect of CO_2. The effect of genotype was also significant for both fresh and dry weights (both $P < 0.001$). CL19/10 had the highest mean total hard leaf fresh and dry weights (2515g and 925.8g respectively), whereas SCA 6 had the lowest mean (1216g and 433.4g respectively). For fresh and dry weight there were no significant interactions between CO_2 and water treatment and no interaction between genotype and treatment.

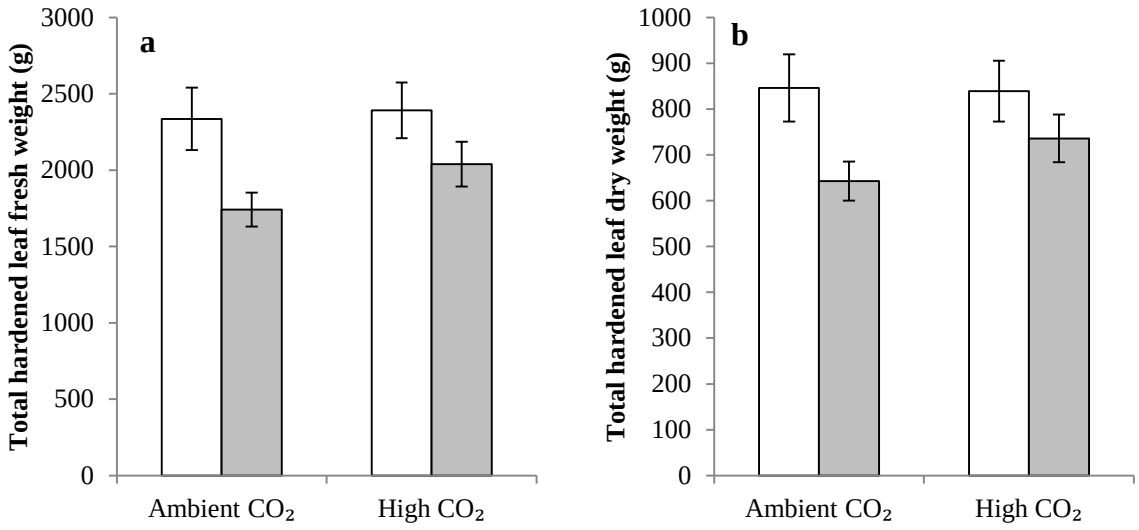

Figure 7.5 Mean total hard leaf fresh (a) and dry (b) weights from trees grown at two concentrations of CO_2 and two watering treatments. Data are combined across six genotypes.
Dry weights predicted using representative samples. □ - Well-watered. ▒ - Water-stressed. Error bars show standard error of the mean.

7.3.5 Total soft leaf weight

There were no significant effects of treatment or genotype ($P > 0.05$) on mean total fresh and predicted dry weights for soft leaves (Figure 7.6). However; there was a general trend of an increase in soft leaf weight under water stress. For fresh and dry weight there were no significant interactions between CO_2 and water treatment and no interaction between genotype and treatment. A significant effect of block design 1 was found for total soft leaf fresh weight ($P < 0.05$). This effect represented an overall reduction in weight in houses 5 and 6; however the trend in response to treatment was the same in both blocks.

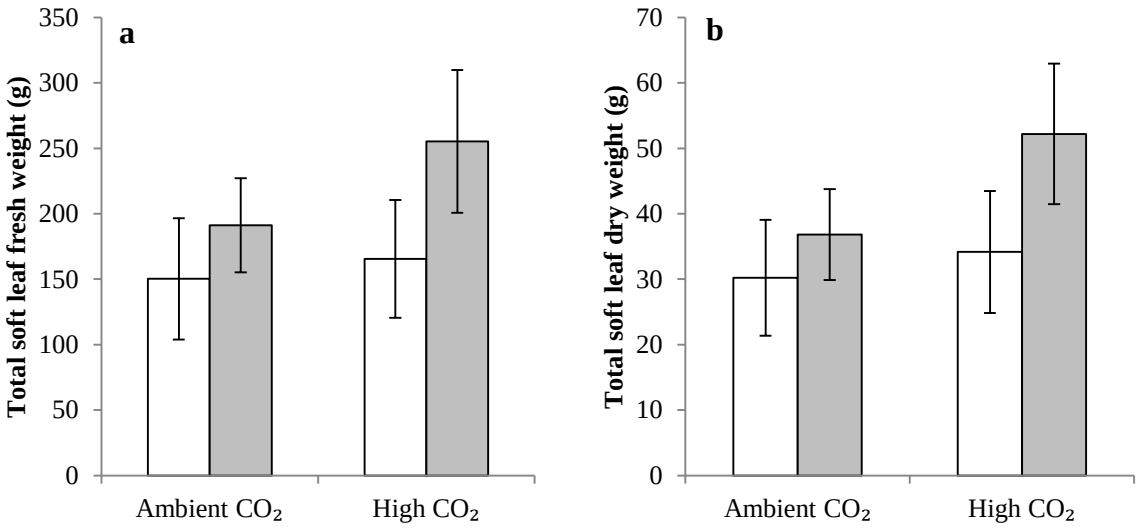

Figure 7.6 Mean total soft leaf fresh (a) and dry (b) weights from trees grown at two concentrations of CO_2 and two watering treatments. Data are combined across six genotypes.
Dry weights predicted using representative samples. □ - Well-watered. ▩ - Water-stressed. Error bars show standard error of the mean.

7.3.6 Hard leaf area

There was a significant 24.4% reduction in total leaf area in response to water stress ($P < 0.001$) (**Figure 7.7**). There was no significant effect of CO_2. Additionally, there was a significant effect of genotype ($P < 0.001$). CL19/10 had the highest leaf area (139058cm^2), whereas SCA 6 has the lowest (63395cm^2). There were no significant interactions between CO_2 and water treatments, and no interaction between genotype and treatment.

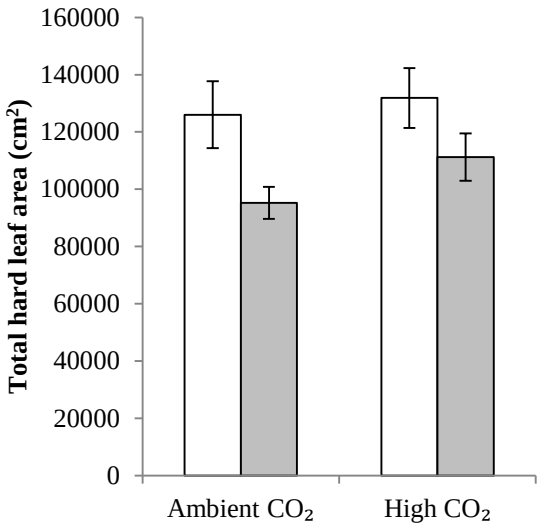

Figure 7.7 Mean estimated total hard leaf area from trees grown at two concentrations of CO_2 and two watering treatments. Data are combined across six genotypes.
Total hard leaf area was calculated using the leaf area and fresh weight of representative samples with the total hard leaf fresh weight. □ - Well-watered. ▩ - Water-stressed. Error bars show standard error of the mean.

7.3.7 Specific leaf area

There were no significant treatment responses observed ($P > 0.05$), no interactions between CO_2 and water treatment, and no interactions between genotype and treatment on specific leaf area (Figure 7.8). A significant effect of block design 1 ($P < 0.01$) and block design 2 ($P < 0.05$) was found for specific leaf area. This effect represented an overall reduction in specific leaf area in houses 2 and 3 (block design 1) and houses 3 and 6 (block design 2).

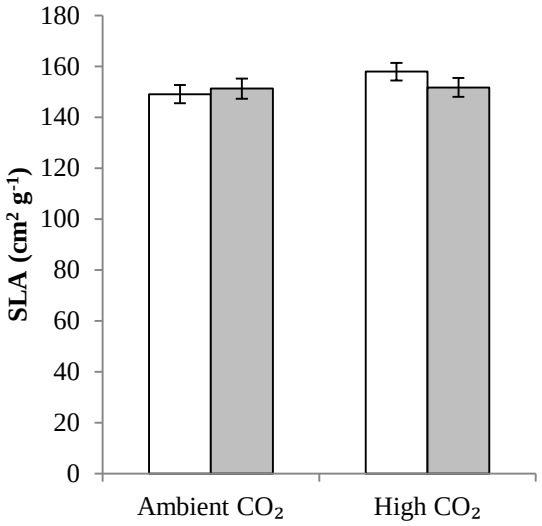

Figure 7.8 Specific leaf area of trees grown at two concentrations of CO_2 and two watering treatments. Data are combined across six genotypes.
Calculated using hard leaf area and hard leaf dry weight of representative samples. □ - Well-watered. ▨ - Water-stressed. Error bars show standard error of the mean.

7.3.8 Canopy photosynthetic rate

The estimated canopy net assimilation rate at an incident irradiance of 1556.5 μmol m^{-2} s^{-1} is shown in Figure 7.9. A significant 61.2% increase in canopy assimilation rate was observed under high CO_2 conditions ($P < 0.001$). Additionally a significant 60.3% reduction was observed under water stressed conditions ($P < 0.001$). There was no significant interaction between water and CO_2 treatments despite an increase in canopy assimilation under high CO_2 when the two water stress treatments are compared. There was a significant difference between genotypes in canopy assimilation rates ($P < 0.001$). The highest canopy assimilation rate was observed for ICS 1 (40.78 μmol s^{-1}), whereas SCA 6 had the lowest rate (21.20 μmol s^{-1}). There were significant interactions between carbon dioxide treatment and genotype ($P < 0.05$). A significant increase in assimilation rate under elevated CO_2 was observed for CL19/10, ICS 1 and Pound 7/B ($P < 0.001$, $P < 0.01$ and $P < 0.01$ respectively) (Figure 7.10). There was also an interaction between carbon dioxide treatment, water availability, and genotype ($P < 0.05$). A significant decrease in assimilation rate under water stress was

observed for CL19/10 ($P < 0.05$), ICS 1 ($P < 0.001$), IMC 47 ($P < 0.001$), Pound 7/B ($P < 0.01$) and SPEC 54/1 ($P < 0.01$). Finally, there was a significant interaction between water treatment and carbon dioxide treatment for CL19/10 ($P = 0.01$). For this clone there was a reduction in assimilation rate under water stress, but only when in combination with elevated CO_2.

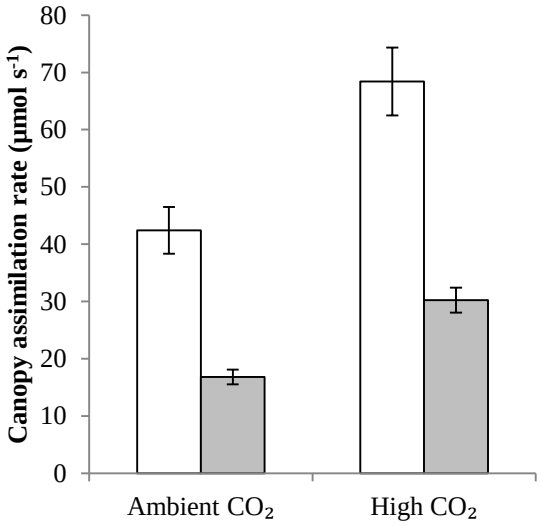

Figure 7.9 Mean estimation of total discrete canopy assimilation rate from trees grown at two concentrations of CO_2 and two watering treatments. Data are combined across six genotypes.
Discrete canopy assimilation rate is estimated using a hypothetical irradiance level of 1556.5 µmol m^{-2} s^{-1}, incident light levels through the canopy, light response data from Lahive (2015), and estimation of leaf area per tree. □ - Well-watered. ▒ - Water-stressed. Error bars show standard error of the mean.

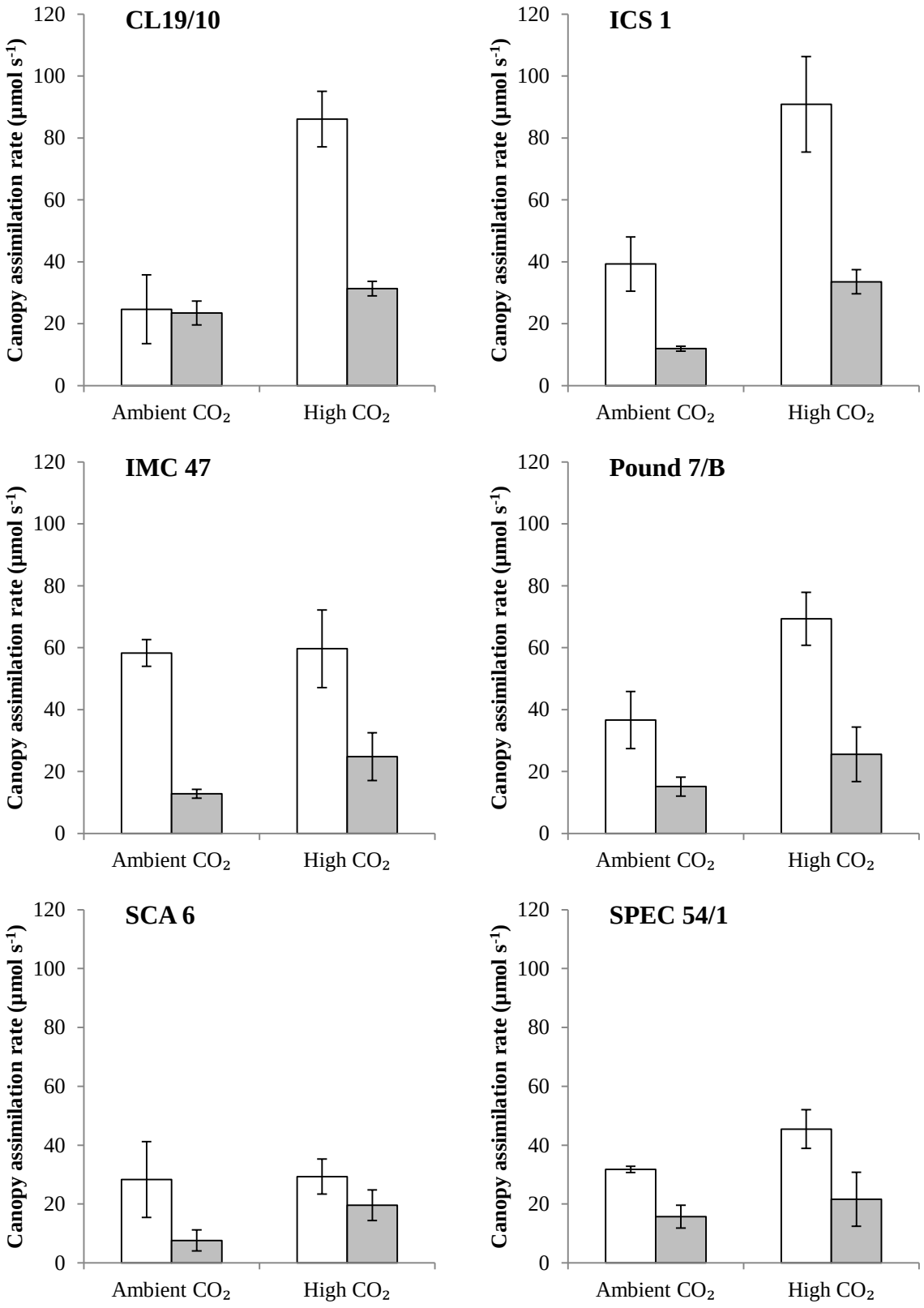

Figure 7.10 Mean estimation of total discrete canopy assimilation rate of six cocoa genotypes grown at ambient and elevated CO_2 and under two watering regimes.
Discrete canopy assimilation rate is estimated using a hypothetical irradiance level of 1556.5 µmol m^{-2} s^{-1}, incident light levels through the canopy, light response data from Lahive (2015), and estimation of leaf area per tree. □ - Well-watered. ▒ - Water-stressed. Error bars show standard error of the mean.

7.4 Discussion

Elevated CO_2 resulted in significant increases in total biomass, woody material and the proportion of woody material in the tree. The increases in photosynthetic rates observed by Lahive (2015) in addition to the net canopy photosynthetic rates reported here, provide explanation for this response as increased assimilation will have direct consequences on tree growth. However, the hard leaf and soft leaf weights, hard leaf total area, and specific leaf area were unaffected. These results again reflect the findings of Lahive in which no differences were found in leaf number, fresh and dry weight, leaf area and flush interval of adult trees; however stem diameter was seen to increase under elevated CO_2. Previous work identifying increases in leaf growth in cocoa under elevated CO_2 has been shown in young trees (Baligar et al., 2005). However, the assimilation investment of trees into leaf growth decreases as trees mature (Poorter et al., 2012). As the trees in this study were older and were reproductively active, assimilate partitioning may have moved towards wood and reproductive growth, thus explaining why no change in leaf growth is observed here. Sour orange trees grown under elevated CO_2 conditions did show increases in final biomass of leaves of around 20% (Kimball et al., 2007). However this is lower than the 55% observed for biomass of large branches, trunks and stumps, again demonstrating a reduced investment in growth of leaves. These citrus trees were studied for a total of 17 years under elevated CO_2. It may be that longer exposure to elevated CO_2 is required before long term effects on leaf biomass are observed. Naturally, with increased wood investment, the branch area may increase, expanding the space available for leaf growth. The 2 year experimental period of this study may not have been long enough to allow for a significant increase in branch area; however space restrictions and pruning may have also limited the extent to which canopy size could grow. Finally, flush interval and leaf longevity on the tree were not monitored in this study and may provide further insight into the investment of trees into leaf growth under high

CO_2 conditions. Flush interval was monitored on these trees previously by Lahive (2015) however no evidence was found to suggest a response to high CO_2 conditions.

The water stress treatment resulted in a significant reduction on total biomass, total hardened leaf weight and area, total woody material weight, and woody material percentage of biomass. Again reductions in photosynthetic rate due to water stress (Lahive, 2015) would have reduced assimilate availability for growth of the trees. Furthermore, Lahive (2015) also observed a reduction in leaf production with the addition of longer intervals between flushing under water stress. In the present study, no significant differences were found in the soft (flush) leaf fresh and dry weights, however, the flushing events were not studied in this experiment. The final harvest of the trees may not have occurred at a time when all trees were flushing. Furthermore it is likely that trees from different experimental treatments were flushing at different times to each other. Large standard errors were observed in the data and may be representative of variation due to the lack of flushing synchrony. The strong links between the flushing cycles of cocoa and water availability (Alvim and Alvim, 1977; Sale, 1970b) suggest that the water stress treatment followed by a re-water event, as was the case in this study, may stimulate flushing and leaf expansion. It may be that the 'vigorous' flushes which occurred on the arrival of irrigation after a dry treatment period, as described by Sale (1970b), were also present in this study, but were not significant due to the misalignment of flushes with the destructive harvest. The total fresh and dry weights of soft leaves from water stressed trees were consistently greater than the well-watered trees in this study. This may be indicative of more 'vigorous' flushing under water stress. However, new flush leaves have been observed to wilt or abort if the water stress conditions continue after bud break (Sale, 1970b; Alvim, 1977). The trend of increased soft leaf weight and the contrasting significant reduction in hard leaf weight suggests that flushing was stimulated by water stress yet the continuation of the water stress treatment caused subsequent wilt of soft leaves as described

by Sale (1970b). Specific leaf area (SLA) did not change in response to water stress despite plants (e.g. *Populus* x *canadensis* (Moench) (Marron et al., 2003), and vegetable amaranth (*Amaranthus*) (Liu and Stützel, 2004)) often reducing their specific leaf area under droughted conditions. A reduction in SLA has the advantage of reducing transpirational water loss (Lambers et al., 2008b; Taiz and Zeiger, 2010) yet no evidence of SLA plasticity is observed here. Finally, changes in the root growth were not measured here. Increased allocation of photosynthate to roots during drought stress is possible (Cannell, 1985), however, this was not testable here.

The net canopy productivity calculation in this study provided a basic assessment of total tree assimilation using a combination of canopy size, photosynthetic light response data, and light penetration through the canopy. The results confirmed the trends observed in tree biomass as trees under elevated CO_2 demonstrated a significant increase in net assimilation rates. Additionally, net assimilation was reduced under water stress and significant variation was seen between genotypes in their productivity. CL19/10, ICS 1 and Pound 7/B demonstrated increases in net photosynthetic rates under high CO_2 and decreases under water stress treatments. IMC 47 and SPEC 54/1 demonstrated a reduction in net photosynthetic rates under the water tress treatment but did not show significant responses to high CO_2. No significant responses to treatment were observed for the genotype SCA 6. Overall, a broad array of responses was apparent, which holds positive scope for future breeding of cocoa to suit varied climates. It should be noted that other factors will affect the net canopy productivity which were not accounted for in this study. The leaf age has bearing on the photosynthetic rate of cocoa as assimilation declines with leaf age to a varying degree depending on irradiance exposure (Miyaji et al., 1997). This was not accounted for in this study as mean photosynthetic rates were calculated using light response data from one of the youngest fully hardened leaves on each tree (Lahive, 2015) and applied across the canopy.

Additionally, light response data were recorded in November 2013 and applied to the same trees in July 2015. The photosynthetic behaviour of the trees may have changed over this time. Position of the leaf in the canopy affects the photosynthetic rate due to the variation in irradiance penetration through the canopy (Miyaji et al., 1997). This was accounted for to an extent as the canopies were split into layers to allow for variation in light moving vertically down the canopy. The ceptometer records multiple light levels across a meter length and generates an average. This would have allowed for sun flecks as well as light entering the canopy from the sides. Finally, the angle of the leaf to the direction of light plays a role in light absorption with maximum absorption at a perpendicular angle (Pury and Farquhar, 1997). This variable was not factored into this study of net canopy productivity. Overall, the results further support findings of Daymond et al. (2002b) and Lahive (2015) in demonstrating the variability in canopy structure and photosynthetic rates across genotypes in cocoa.

7.5 Conclusions

The destructive harvest and calculations of net canopy productivity reveal increases in total biomass, total wood biomass, and percentage of wood in the total biomass in response to elevated CO_2. This is likely to be a direct result of the significant increase in net canopy assimilation under elevated CO_2. Total hard leaf weight and leaf area was unaffected. The water stress treatment significantly reduced total biomass, total hard leaf weight, total wood weight, percentage of wood in total biomass, and total hard leaf area. It is likely that this is a direct response to a significant reduction in net canopy assimilation under the water stress treatment. Specific leaf area and total soft leaf weight were unaffected by all treatments. There was no overall treatment interaction between elevated CO_2 and water stress. Genotypic

variation was significant in all parameters suggesting strong variability in tree responses and highlighting scope for breeding cocoa suited to future climates.

Chapter 8. General Discussion

8.1 Introduction

The change in climate in response to anthropogenic greenhouse gas emissions will have direct implications for agriculture around the world. Atmospheric carbon dioxide levels have been increasing since pre-industrial times and are expected to continue increasing in-line with human activity (IPCC, 2014). A clear relationship between increases in greenhouse gases and the increases in mean global surface temperature has been observed with an increase of 0.85°C between the years 1880-2012 (IPCC, 2014). Future climate projections predict a range of further increases in temperature depending on the scale of future anthropogenic emissions. Precipitation levels are expected to change with increases in mid-latitude wet regions and decreases in mid-latitude and subtropical dry regions (IPCC, 2014). Around 75% of the world's cocoa is grown within the 8° boundary of the equator, however significant farming is found up to 20° south in South America, and up to 19° north in the Dominican Republic (Wood, 1985a). The range of locations used for cacao farming across the tropics and the degree of variation expected in precipitation patterns, suggests that some cacao growing regions will see increases in rainfall and others decreases.

The smallholder nature of individual cacao farms places great importance on annual yields, as losses pose a threat to entire livelihoods, especially in Africa (Challinor et al., 2007). Their tropical location and limited capacity to change, makes these farms particularly vulnerable under climate change (Morton, 2007). To enable a sustainable future for smallholder cacao farming, a deeper understanding of tree responses to climatic variables will be essential to enable new varieties to be bred and farming practices to be adjusted. A key factor in ensuring adaptability to climate change will be access to this information in cooperation with government support (Challinor et al., 2007).

The impact of elevated CO_2 has been investigated in cacao using seedlings (Baligar et al., 2005; Baligar et al., 2008); however there has been no work using adult trees and no investigation as to the effects on reproductive development. Additionally, although the responses of cacao to water stress have been previously documented (Alvim, 1977; Sale, 1970b; Araque et al., 2012; De Almeida et al., 2016; Moser et al., 2010a), the interactions between elevated CO_2 and water availability have not previously been investigated. This project has examined the responses to elevated CO_2 and water deficit in cacao through the use of a semi-controlled environment glasshouse facility. The experiment used adult trees of six contrasting genotypes of *T. cacao.* (CL19/10, ICS 1, IMC 47, Pound 7/b, SCA 6 and SPEC 54/1) and, in addition to identifying treatment effects on tree development, the study aimed to identify any genetic variation in response to treatments. The focus here has been on the reproductive development of cacao, from flowers through to beans. This work is complimentary to the research carried out by Lahive (2015) which focused on the vegetative and photosynthetic responses of the same trees under the same treatment conditions.

8.2 Responses to elevated CO_2

The effects of elevated CO_2 on the growth of C3 plants are well established. Generally, photosynthetic rate increases, whilst stomatal conductance decreases, overall improving water use efficiency (Drake et al., 1997; Long et al., 2004). An increase in photosynthetic rate was observed for trees in this study under elevated CO_2 accompanied by an increase in water use efficiency. However, there were no changes in stomatal conductance (Lahive, 2015). With these photosynthetic changes in mind, various aspects of reproductive development were analysed.

The proportion of germinated pollen grains did not change in response to elevated CO_2; however pollen tube length was significantly shorter, suggesting impairment to pollen grain development. Enhanced vegetative growth in response to high CO_2 may have outcompeted pollen development, as is the case for vegetative flushes outcompeting developing pods for assimilates and inducing cherelle wilt (Alvim, 1954).

Increases in elevated CO_2 did not alter the overall numbers of flowers produced, despite enhancement of photosynthetic activity. However, the variation in floral emergence was significantly greater in elevated CO_2 trees. This is similar to the oscillation in flowering patterns observed between young and old cacao trees (Alvim, 1977) and may be indicative of stronger vegetative sink strength. Like the older trees in Alvim (1977), the elevated CO_2 trees had a greater proportion of vegetative growth. During periods of vegetative growth, assimilate availability would be more strongly reduced in trees with increased vegetative mass which would be acting as a sink in direct competition with floral development. In periods of reduced vegetative growth, the enhanced assimilation rates under elevated CO_2 could then potentially boost flowering intensity above levels observed in ambient CO_2-grown trees, overall increasing fluctuations in flower emergence.

In the first year of pod development, fruit set increased under elevated CO_2 indicating that the enhanced photosynthetic rates were enabling a larger pod carrying capacity. Higher fruit set has also been observed in trees of Sour Orange (*Citrus aurantium* L.) grown in elevated CO_2 growth chambers (Kimball et al., 2007). The enhanced assimilate production at high CO_2 is likely to increase the allocation of assimilates to fruit growth resulting in improved fruit set. The links between assimilate availability and fruit setting have been established using stem-injected sucrose supplementation and defoliation studies in Satsuma mandarins (*Citrus unshiu* (Mak.). Defoliation increased fruit abscission whereas sucrose supplementation increased fruit set by 10% compared to control trees (Iglesias et al., 2003). In the present

study, the second year of pod growth did not show any effect of elevated CO_2, implying the initial increase in fruiting capacity was no longer available after a prolonged exposure to treatment. There were no significant changes in incidence of cherelle wilt under high CO_2 which is in contrast to studies showing higher fruit retention in Valencia Orange (*Citrus sinensis* (L.) Osbeck) when grown under elevated CO_2 (Downton et al., 1987). Cacao cherelle wilt occurs as a result of pod competition for available assimilates (Mckelvie, 1956) and therefore incidence of wilt will alter depending on the tree's capacity to maintain a high fruit load. High CO_2 concentrations did not alter the incidence of wilt and thus fruit retention despite enhancements in photosynthetic rates. Instead, regulation of pod capacity may have been through initial fruit-set, measured here through pollination success.

Very similar responses to elevated CO_2 were observed for maximum pod size and rate of growth. No effect of CO_2 was observed in the first year; however, in the second year high CO_2 resulted in increases in pod size and rate of growth. The delay in response of pod growth to CO_2 may indicate the additional assimilates generated from the higher rates of photosynthesis (Lahive, 2015) are not immediately partitioned to pod development. This is in contrast to the findings of Downton et al. (1987) in which Valencia Orange (*Citrus sinensis* (L.) Osbeck) trees showed increases in yield of up to 70% within the first 12 months of growth under 800ppm elevated CO_2. Duration of cacao pod growth and time of maximum rate of growth post-pollination did not change in response to high CO_2.

In year 1, bean development showed a particular sensitivity to elevated CO_2 with a reduction in bean dry weight per pod, single bean weight, and bean to husk ratio, whilst all other yield components measured showed no response to treatment. Although increases in photosynthetic rate have been observed in cacao under elevated CO_2 (Lahive, 2015; Baligar et al., 2008), no additional assimilates appeared to be partitioned towards reproductive growth. The observed reductions in bean investment may have been a result of sink

competition with vegetative growth, as is demonstrated through the increase in cherelle wilt when flushing occurs (Alvim, 1954). By the second year of study, assimilate partitioning appeared to have shifted in favour of reproductive growth as pod weight, husk weight, husk thickness, total bean weight, and single bean weight all increased in response to high CO_2. However, a reduction in bean to husk ratio was also present indicating an increased investment in husk over bean. A similar shift towards reproductive growth after a prolonged exposure to elevated CO_2 was also observed in sour orange (Kimball et al., 2007), a study in which an investment in fruiting over wood was not observed until the 5^{th} year of study. However, in the Kimball study the shift is likely to represent the point at which the trees develop to reproductive maturity. The trees in this study were grafted onto rootstock, therefore eliminating the juvenile stages of development. Overall, an enhancement of reproductive growth was observed in response to elevated CO_2 however this did not become apparent until the second year of study.

Lipid analysis of beans from the genotype SCA 6 showed reduced total fat, stearic acid, oleic acid and linoleic acid under elevated CO_2 conditions in year 1. Despite these changes, the overall proportion of unsaturated fatty acids did not change. In year 2 no treatment responses were observed. Initial vegetative growth in response to elevated CO_2 may have increased sink competition early in the study resulting in the detrimental effects observed on fat content. The lack of response in the second year may represent a shift in assimilate partitioning towards reproductive growth with increasing tree age and size, as is suggested by the pod yield parameters. A significant interaction between water treatment and CO_2 treatment in the analysis of the beans from genotype Pound 7/B in year 2, appear to show an increase in total fat content from ambient CO_2 to high CO_2 under well-watered conditions. Furthermore a decrease in unsaturated fatty acid content suggests that harder cocoa butters were also formed

under elevated CO2 with higher melting points which can be more desirable for processing depending on the purpose (Wood, 1985c).

Trees grown in high CO_2 had significantly increased total biomass, attributable to increases in woody material. Overall there were no significant increases in leaf weight or area at the point of the tree harvest. These results are in line with the findings of Lahive (2015) in which no significant changes in leaf number, weight or area were observed in the same adult trees used in this experiment. Increases in leaf growth under high CO_2 in cacao seedlings has been identified by Lahive (2015) and Baligar et al. (2005) suggesting a change in response to elevated CO_2 dependent on the developmental stage of the tree. Prolonged exposure to elevated CO_2 may result in increases in leaf investment if increased woody development results in an increased surface area for leaf growth. A seventeen year CO_2 enrichment study with Sour Oranges trees did show an increase in leaf biomass, however the investment into woody biomass was far greater (Kimball et al., 2007). The increases in vegetative growth under high CO_2 observed here are likely to be a response to the increased photosynthetic rates observed by Lahive (2015) under these conditions. Further to these observations, calculations of net canopy assimilation rate in this study also suggest significant increases in canopy photosynthetic capacity in the high CO_2 treatment. These calculations have provided a whole-tree picture as to the combined factors of photosynthetic rate, leaf area, and canopy light interception.

Overall, enhancement of pod growth and size, along with increased investment in yield parameters, and net canopy assimilation rates, was observed in the second year of study in response to the high CO_2 treatment. The absence of enhanced reproductive growth in year 1 despite enhanced photosynthetic activity may be explained through competition for assimilates between vegetative and reproductive sinks.

8.3 Responses to water Stress

The detrimental effects of water stress on the photosynthetic rates of plants are well documented. However, water-stress can also play a regulatory role in reproductive behaviour. An example of this would be the floral stimulation of coffee which is enhanced when a return of water follows a period of drought (Alvim, 1960). Water deficit regulation in cacao has also been identified in studies identifying leaf flushing and flower emergence in close connection with the return of irrigation or rain after a period of drought (Alvim, 1977; Sale, 1970b; Alvim and Alvim, 1977). This study aimed to explore further the impacts of water deficit stress on the reproductive development of cacao.

There was no effect of water stress on pollen germination; however there was an increase in the length of pollen tubes for the genotypes IMC 47 and Pound 7/B. Water deficit has been identified as a key factor in increasing pollen sterility (Saini, 1997; Salter and Goode, 1967); however no such effect was observed here. A potential explanation of these findings may be that a reduction in vegetative growth under water stress conditions decreased sink competition within the trees, freeing assimilates for pollen grain development. Furthermore, the timing of pollen sampling was not coordinated in association with the re-watering schedule of the trees. This may have resulted in the miss-timing of water stress with the crucial meiosis stages of pollen grain development. Future study would require closer links between the studies of pollen performance, the developmental stages of the pollen grain, and environmental variables.

Flower emergence in cacao has been strongly associated with water availability (Alvim and Alvim, 1977; Sale, 1970b) yet the results of this study do not reflect this. Water stress did not appear to alter the pattern of floral emergence despite strong evidence suggesting a transition from water stress to adequate soil moisture stimulates flowering (Alvim and Alvim, 1977).

Furthermore, the total number of flowers did not increase in response to the water stress treatment despite research demonstrating water stress increasing the induction of cacao flowers (Sale, 1970b). It is proposed that the suppression of flower emergence under water stress, as seen by (Sale, 1970b), requires longer-term moisture deficit than was simulated in this study. In the hydroponic irrigation system used in this study, the moisture retention of the soil substrate is low and therefore dries more rapidly than the soils used in other studies. This, in addition to the pot size, limited the time irrigation could be withheld from a tree before severe leaf drop and damage was caused. Additionally, the transition of trees from a 'dry' to a 'wet' treatment did not occur here as, despite frequent re-watering events, the trees were maintained in a permanent water stress treatment for the duration of the study. Overall it is suggested that the findings here are not comparable to those of Sale (1970b).

Water stress had no statistically significant effect on fruit set in the first year of study; however, despite no statistically significant data, pollination success was generally lower under water stress conditions. A reduction in fruit set may have been expected under water stress through detrimental impacts observed on photosynthetic capacity in cacao (Lahive, 2015). Furthermore, reduced pod set has been observed by Sale (1970b) in a study in which trees were maintained in continuous dry soil moisture conditions. The results from harvest 2 contradict these findings with significant increases in fruit set under the water stress treatment. It is likely that the decrease in vegetative growth observed under water stressed trees in the destructive harvest, reduced the sink competition for reproductive development, resulting in the enhanced fruit set observed. The sink strength of vegetative growth over early fruit development is demonstrated in cacao through the observed increases in cherelle wilt during periods of leaf flushing (Alvim, 1954). The contrasting effect of water stress on fruit set in this study compared to the effects observed by Sale (1970b), may be explained by the differing methods of maintaining water stress and the duration between watering.

No significant effects of water stress were observed on the incidence of cherelle wilt despite prior research identifying increased incident of wilt in such conditions (Sale, 1970b). It is generally accepted that availability of photosynthates to developing pods is a key factor in cherelle wilt in cacao (Alvim, 1977), and that reduced photosynthetic activity/capacity will reduce such assimilate availability. Although water stress was observed to decrease photosynthetic rate (Lahive, 2015) and reduce vegetative biomass in the trees in this study, no impact of water stress was seen in the wilting response.

Pod size and rate of growth was reduced in response to water stress in the first year of pod growth study, however no effect of water stress was observed in the following year. The lack of continuation in this treatment effect suggests some form of adaptation to water stress over time. Adaptation to water stress can take many forms including increased root-shoot ratio, osmotic adjustment and molecular changes (Kozlowski and Pallardy, 2002). Improvements in WUE under water stress were observed in these trees early in the experiment (Lahive, 2015). Pod growth analysis in the first year coincided with a more erratic control over soil water levels. Improvements in the water stress treatment methods meant tighter control was achieved in the second year of study. This, in combination with potential adaptation to water stress and improvements in WUE, may have lessened the impacts of the water stress treatment in year 2, providing an explanation as to why no effect was observed. No effect of water stress was observed on duration of pod development and time to maximum rate of growth.

Pod weight, husk weight, total bean dry weight, and single bean weight were all reduced in response to water stress but only in the first year of study. In the second year the detrimental effects of water stress were not observed. The decrease in husk weight was not reflected in the husk thickness suggesting a decrease in the fibrous material which forms the husk wall. A decrease in the carbon investment of the husk could improve the investment efficiency of the

tree with regards to bean yield; however changes in husk density could increase susceptibility to pests such as the cocoa pod borer (Azhar et al., 1995). The dry weight of total beans per pod decreased; however the fresh weights did not, suggesting a compensation of fresh weight with increased water content. This is further supported by a non-significant trend for increased bean moisture content in harvest 1 in response to water stress. The general decrease in pod investment under water stress is likely to be a reflection of the reduced photosynthetic rates observed under the water stress treatment (Lahive, 2015). The response to water stress in the second year of study was limited to an increase in bean to husk ratio and an increase in bean number per pod. These results are in contrast the detrimental effects of water deficit observed in year 1 and suggest some form of tolerance to the water stress treatment had developed. The observed increases in bean number are in contrast to previous studies in the literature which suggests water stress should result in a decrease seed number through an impairment of ovule health (Kokubun et al., 2001; Sun et al., 2004). The reduction in vegetative growth observed in this study in response to water stress may have reduced the sink competition in favour of reproductive growth, despite the decreases in photosynthetic rate (Lahive, 2015). However, to make firmer assumptions regarding effects of water stress on ovule health in cacao, efforts to quantify pollen deposition will also be required in future research as pollen grain number has strong links to final bean number (Falque et al., 1995).

Water stress did not result in any detrimental effects on lipid quantity in either genotype studied. Prior research has identified temperature as the key factor in the fatty acid composition of cocoa butter (End, 1990; Chaiseri and Dimick, 1989; Lehrian et al., 1980) and many other oil crops (Canvin, 1965). However any clear relationship between water stress and lipid composition in cocoa butter was not established despite clear effects of water stress on photosynthesis and vegetative growth. Although no detrimental effects were observed in fat content, a significant interaction between water and CO_2 treatments in the genotype Pound

7/B, suggests that water stress may increase the total fat, palmitic and stearic acid content under ambient CO_2 conditions. It is theorised here that the suppression of vegetative growth observed under water stress, reduced vegetative growth and thus vegetative sink strength for assimilates, thereby freeing assimilates for reproductive growth. The absence of this response under high CO_2 may be due to the higher photosynthetic rate preventing as strong a reduction in vegetative development. Overall, the increases in the proportion of the saturated palmitic and stearic fatty acids would increase hardness and melting point of the butter, which may be favourable to many industry users (Wood, 1985c; End, 1990).

Water stress reduced the final tree biomass, hardened leaf weight, hardened leaf area, and woody biomass. Overall a general reduction in vegetative growth was observed, which is likely to be in response to the reduced photosynthetic rates and increased time periods between leaf flushing observed under water stress (Lahive, 2015). Leaf flushing and flushing interval were not studied here; however the biomass of flush leaves at the point of final harvest was recorded. Strong links between new leaf emergence and reductions in water stress are already established in cacao (Alvim and Alvim, 1977; Sale, 1970b). The arrival of rains or irrigation after a period of water stress acts as a trigger for bud break in cacao (Alvim and Alvim, 1977) which may have suggested that there would be an increase in soft flush leaves detected at the point of harvest in the water stressed trees of this study. No significant response to water stress was observed in soft leaf weight; however there was a trend for soft leaf weight to be consistently greater for water stressed trees. This, along with a significant decrease in hard leaf weight, suggests flushing may have been stimulated by the water stress/re-water cycle but that new leaves were subsequently lost due to the continuation of the water deficit. The growth and any potential loss of flush leaves was not monitored in this study, however wilting after flushing was observed by Sale (1970b) when conditions of water stress persist. Finally, in this study a lack of leaf flush synchrony at the point of harvest may

have masked any effect of water stress on leaf flushing, resulting in the lack of significant treatment effect here.

Calculation of total net canopy assimilation rate revealed significant reductions in photosynthetic capacity in the trees maintained under water stress. To gain this insight into the photosynthetic activity of a tree as a whole, photosynthetic light response data (Lahive, 2015) was used in combination with light filtration through the canopy, and the leaf area of individual trees.

Overall, in this study water stress was detrimental to photosynthetic rate (Lahive, 2015), wood and leaf biomass, hard leaf area, and the proportion of wood in the total tree biomass. Observations revealed reductions in maximum pod size and rate of growth, and most yield components including bean and husk weight in response to water deficit. These effects were predominantly observed in the first year of study and not in the second, suggesting either developmental adaptations to water stress or reduced sensitivity with increased tree maturity. No effect of water stress was observed on the lipid content of beans.

8.4 Interaction between CO_2 and water deficit

The observed reduction in stomatal conductance of many plants in response to elevated CO_2 can have a positive effect on water use efficiency by reducing transpiration rates (Ainsworth and Rogers, 2007; Long et al., 2004). Overall, this can serve to alleviate the effects of water stress. As it is likely that increased atmospheric concentrations of carbon dioxide will coincide with increases in periods of drought in certain areas, it is essential that the interactions between the two factors are understood to help gain a realistic insight into how cacao may perform under future scenarios. Lahive (2015) identified an improvement in water use efficiency of the trees in this study; however this was attributed to the enhancement of

photosynthetic rate, as no change in stomatal conductance was observed under elevated CO_2 in adult trees. Overall, this study revealed little evidence to suggest alleviation of the effects of water stress under elevated CO_2. When data from all genotypes were combined, the detrimental effects of water stress on net canopy assimilation rates were partially offset under high CO_2. Despite the lack of response overall, genotypes did demonstrate variation in the interaction between the two treatments. With specific focus on reproductive development, the genotype which demonstrated the clearest alleviation of water stress was ICS 1. For this genotype, maximum pod size, pod fresh weight, husk fresh weight, bean fresh and dry weight per pod, and mean single bean fresh and dry weight were all reduced in response to water stress. In all cases this effect was less marked when trees were also maintained in high CO_2. No such treatment interaction was observed in any of the other genotypes.

8.5 Genotypic variation

Photosynthetic analysis of the trees in Lahive's study revealed little genotypic difference in response to treatment (Lahive, 2015). However, in this study there was genotypic variation amongst the reproductive parameters measured. This variation serves to highlight the high degree of natural variation amongst genotypes in factors such as flower number, pod size, pod weight, and canopy size. Such variation was previously identified in respect to biomass partitioning by Daymond et al. (2002a). Variation between genotypes in response to treatment was more restricted to pollen tube length; pod size, rate of pod growth, time to maximum growth rate and 95% maximum pod size, and finally net canopy assimilation. Yield parameters such as bean weight, husk thickness, and testa weight did vary in response to treatment but the overall response did not vary significantly between genotypes. One exception to this is the aforementioned interaction between elevated CO_2 and water stress in

the genotype ICS 1, involving the less marked effects of water stress when trees are grown under high CO_2. This interaction was not demonstrated in other genotypes and may provide a useful factor for breeding future cacao genotypes with greater resilience to climate change. The general lack of variation in yield components between genotypes in response to treatment may indicate limitation to the degree of yield improvements which can be achieved for farming in future climates. Future breeding programmes need to utilise the existing genetic variation to increase yields as findings here suggest that genotypes may not differentially respond to climate change.

8.6 Conclusion

This thesis has studied the effects of long term exposure of six genotypes of adult cacao trees to conditions of elevated carbon dioxide and water deficit stress. Little treatment effect was observed upon pollen development and flowering, however water stress generally reduced tree investment into pod growth and elevated CO_2 enhanced tree investment. The effects of treatment on yield components such as husk and bean weight were consistent across genotypes despite genotypes having natural variation in their reproductive characteristics. Breeding cacao for future climate scenarios will need to focus on the variation in the severity of water stress effects observed on the individual genotypes, in combination with the potential benefits provided by elevated CO_2. As an example, ICS 1 generally produced the largest bean weights per pod yet demonstrated a consistent sensitivity to water stress; whereas SPEC 54/1 responded minimally to treatments but also had low vigour and response to CO_2. Water stress is likely to be detrimental to cacao production; whereas increased atmospheric CO_2 concentrations hold potential to improve yields. Alleviation of water stress effects on bean weights under elevated CO_2 will be a trait of great importance in breeding to

safeguard against yield losses in dryer conditions. From this body of work, the data most suitable for publication include data demonstrating the effects of elevated CO_2 and water stress specifically on the growth of pods, yield components, bean fat content, and bean fatty acid profile. This data, whilst being directly relevant to the cocoa industry, showed consistency and defined genotypic variation in response to treatment. The publication of these findings may be useful in building future studies around climate change and cocoa research.

8.7 Future research

Future research will need to further explore the genetic diversity of cacao, particularly with respect to drought tolerance and potential yield improvements under elevated CO_2. The methods of drought simulation may also need to be revised. The benefits of hydroponic systems in water stress research may need to be weighed against conventional soil-based experiments when working with trees of this size. Whilst hydroponics allow for long-term studies without soil nutrient depletion, the length of time water can be withheld from a plant is considerably shorter and therefore the simulation of droughted conditions may be unrealistic in comparison to conditions in the field. High temperature stress should also be researched as this will almost certainly play a key role in cacao agriculture in the future. The genetic diversity in the partitioning of assimilates between yield components should be explored in association with enhanced photosynthetic rates under increased CO_2. These studies focused on the effects of treatments largely within the context of photosynthetic rate and assimilate production. Future research should also consider hormonal control of processes such as flowering in response to environmental conditions. To observe clearer responses between environmental factors and tree development, the use of control

environment studies or free air CO_2 enrichment is recommended. This will allow for the study of treatment interactions, and provide valuable insights into how trees grow at the farm level. Deeper understanding of the genetic diversity in response to future climate variables will enable cacao breeding programmes to tailor new varieties which will maintain and improve yields for farmers in the future.

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