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Direct and Indirect Impacts of Climate Change on Growth, Productivity and Management of Three Important Tropical Crops: Cassava, Coffee and Cacao.

Submitted by

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In fulfilment of the requirements for the degree of

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Dedication

I dedicate this thesis to, and in loving memory of Pak Tamsil Hidayat who will forever bless our hearts. Tamsil, leader of the pollination team and my lifelong brother and friend - I couldn't have achieved this without you, and I share this achievement equally with you. May this thesis exist as an eternal tribute to your memory and an example of the selfless, joyful, dedicated, passionate, responsible, energetic and positive reality that you brought every day to the lives of your friends, family, colleagues and community.

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Statement of the Contribution of Others

The thesis was initially conceived and drafted by me, Samantha Jay Forbes (SJF) and was improved and developed over time under the supervision of Lucas Cernusak (LC), Tobin Northfield (TN), Darren Crayn (DC) and Alexander Cheesman (AC). Editorial assistance throughout the thesis chapters was provided by LC, TN and AC. SJF initially consulted Juan Carlos Motamayor (JCM) of Mars Incorporated, as an industry supervisor, to develop the initial scope of research for this thesis. SJF collaborated closely with Smilja Lambert (SL) of Mars Incorporated throughout the duration of this thesis, as the industry supervisor in absence of JCM, to develop, plan, implement and manage the pollination field research trials in Tarengge, Sulawesi, Indonesia. SJF consulted with LC, TN and AC to redefine the scope of research for this thesis after an extended leave of absence and in response to changes in the industry supervisor representation. SJF consulted LC and AC to design and implement the research experiments for Chapter 2 and 3, with additional consultation from Roslyn Gleadow (RG) in Chapter 2. Chapter 4 was developed in consultation between SJF and JCM, which was based on company-derived data from Mars Incorporated. TN assisted SJF with the experimental design of Chapter 5. Beau Mangano assisted with cassava cutting propagation in Chapter 2. Krystal Kinnane and AC of James Cook University (JCU) assisted with experimental plant measurement, harvesting and post-harvest processing and evaluation in Chapter 2 and 3. Luke Hoffman and James Milner of JCU assisted with leaf sample preparation for phytochemical analysis in Chapter 2 and 3, respectively. Rose Baulch and Akane Uesugi from the Plant Ecophysiology Research Group Laboratory of Monash University assisted with cassava leaf and tuber cyanide assays in Chapter 2, and leaf phenolic analysis in Chapter 3. Dr Jen Whan and Dr Yi Hu of the Advanced Analytical Centre of JCU assisted with leaf nutrient analysis for Chapter 3. Tamsil Hidayat, Nirwana, Dira, Salbia, Sri Astuti, Suriani Ugin, Marlina, Supratman Leman, Masrijal, Yuliadi, Risal and Aldi from ‘Tim Pollinasi’ at the Mars Cocoa Research station (MCRS), Tarengge, Sulawesi, Indonesia, assisted with the field work and data collection activities in Chapter 4 and 5. Statistical support was provided by TN in Chapter 2, 3 and 4, from Guiliana Mustiga and Alberto Romero in Chapter 4, and Robert Clark and Alex Blake in Chapter 5. Field sites, laboratories and other relevant equipment & infrastructure at the MCRS were provided by Mars Incorporated. Equipment and consumables in each, The Plant Ecophysiology Research Group Laboratory (JCU), The Advanced Analytical Centre (JCU) and the Plant Ecophysiology Research Group Laboratory (Monash University) were utilized to develop this thesis. All journal publications published thus far are co-authored by members of my supervisory panel who were my supervisors at the time of publication, and I have included external collaborators where their level of contribution justified co-authorship. I received an Australian Postgraduate Award (APA) scholarship, replaced by the Research Training Program (RTP) stipend scholarship, with a stipend top-up from Mars Incorporated. Financial support for this thesis and the research it comprises was provided by Mars Incorporated including all travel for field work, conferences and associated expenses, experimental equipment and consumables, field technicians, analytical fees and journal publication costs. Generative AI technology was not used in the preparation of any part of this thesis.

Chapter number	**Title and status of publication**	**Author contributions and statement of support**
1	General Introduction	Samantha J. Forbes (SJF) wrote the initial draft of this chapter with reviews and editorial assistance provided by Lucas Cernusak (LC), Tobin Northfield (TN) and Alexander Cheesman (AC).
2	Forbes, S. J., Cernusak, L. A., Northfield, T. D., Gleadow, R. M., Lambert, S., & Cheesman, A. W. (2020). Elevated temperature and carbon dioxide alter resource allocation to growth, storage and defence in cassava (*Manihot esculenta*). *Global Change Biology*. 173: 103997.	SJF conceived the chapter and developed the research question and the research methodology with assistance from LC, AC and RG. SJF collected the data with assistance from AC. SJF performed the analysis of data with assistance from TN. SJF developed the figures and the tables. SJF wrote the first draft of the paper with subsequent revisions from LC, TN, AC, SL and RG.
3	Forbes, S. J., Northfield, T. D., Lambert, S., Cheesman, A. W., & Cernusak, L. A. (2024). Elevated temperature and carbon dioxide alters growth and leaf-chemical composition in two important neotropical crops, coffee (*Coffea arabica*) and cacao (*Theobroma cacao*).	SJF conceived the chapter and developed the research question and the research methodology with assistance from LC, AC. SJF collected the data with assistance from AC. SJF performed the analysis of data with assistance from TN. SJF developed the figures and the tables. SJF wrote the first draft of the paper with subsequent revisions from LC, TN, AC, and SL.
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	increase yield in cacao. *HortScience*. 54(10): 1718-1727.	the data analysis and figure creation with assistance from TN and SJF. SJF wrote the initial draft of the chapter with reviews from JCM and TN.
5	Forbes, S. J., Northfield, T. D., Cheesman, A. W., Lambert, S., Hidayat, T., Clark, R. E., & Cernusak, L. A. (2024). Artificial pollination may impact fruit size, seed quality and production sustainability in cacao.	SJF and TN conceived the chapter. Team Pollination ('Tim Pollinasi') of the Mars Cocoa Research Station conducted the field work with leadership and assistance by SJF. SJF conducted the data formatting and preparation. TN, Robert Clarke and Alex Blake provided advice on data analysis. SJF conducted the data analysis and figure creation with assistance from TN. SJF wrote the initial draft of the chapter with reviews from TN, LC and AC.
6	General Discussion and Concluding Remarks	SJF wrote this chapter with reviews and edits by all supervisors.

List of Publications Included in this Thesis

Chapter 2

Forbes, S. J., Northfield, T. D., Cernusak, L., Gleadow, R. M., Lambert, S., & A. W. Cheesman. (2019). Elevated temperature and carbon dioxide alter resource allocation to growth, storage and defence in cassava *(Manihot esculenta). Environmental and Experimental Botany,* 173, pp 103997. DOI: 10.1016/j.envexpbot.2020.103997.

Chapter 4

Forbes, S. J., Mustiga, G., Romero, A., Northfield, T., Lambert, S., & J. C. Motamayor. (2019). Supplemental and synchronized pollination may increase yield in cacao. *HortScience*, 54(10), pp 1718-1727. DOI: 10.21273/HORTSCI12852-18.

List of Outputs Related to Work not Included in this Thesis

Apgaua, D. M. G., Tng, D., Forbes, S. J., Ishida, Y. F., Vogado, N. O., Cernusak, L., & S. G. W. Lawrence. (2018). Elevated temperature and CO_2 cause differential growth and drought survival responses in Eucalypt species from contrasting habitats. *Tree Physiology*, 39(11), pp 1806-1820. DOI: 10.1093/treephys/tpz095.

Arnold, S. E. J., Forbes, S. J., Hall, D. R., Farman, D. I., Bridgemohan. P., Spinelli, G. R., Bray, D. P., Perry, G. B., Grey, L., Belmain, S. R., & P. Stevenson. (2019). Floral odors and the interaction between pollinating Ceratopogonid midges and cacao. *Journal of Chemical Ecology*, 45(10), pp 869-878. DOI: 10.1007/s10886-019-01118-9.

Schmidt, J.E., Firl, A., Hamran, H., Imaniar, N. I., Crow, T.M., & Forbes, S. J. Impacts of shade trees on the adjacent cacao rhizosphere in a young diversified agroforestry system. (2022). *Agronomy*, 12(1), pp 195. DOI: 10.3390/agronomy12010195.

Mars Sustainable Solutions, Forbes, S. J., Vavrova, D., Frei Films, The AV Lab of The Cairns Institute, & Biopixel. The intricacies of cocoa pollination. (2022). An animation. https://www.youtube.com/watch?v=fSE87jTdJDI.

List of Student Projects Supervised

Special Topic Report. Pollen trap development: designing an effective trap to evaluate the potential for wind pollination of cacao (*Theobroma cacao L.*) pollen. 2020. Formal report submitted by Bianca Ligasacchi (BAdvSc) in fulfilment of the requirements of James Cook University subject SC3003 – Science and Research Internship. Supervisors: Samantha Forbes and Lucas Cernusak.

Special Topic Report. Understanding the diversity of flower visiting insects in cacao: evaluating their importance as potential pollinators. 2020. Formal report submitted by Rei Asai (MSc) in fulfilment of the requirements of James Cook University subject SC5901 - Special Topic 1. Supervisors: Samantha Forbes and Lori Lach.

Special Topic Report. Evaluating wind dispersal of cacao (*Theobroma cacao* L.) pollen and potential additive effects of mechanised wind application. 2021. Formal report submitted by Edward Gladigau (MSc) in fulfilment of the requirements of James Cook University subject SC5901 - Special Topic 1. Supervisors: Samantha Forbes and Lucas Cernusak.

List of Data Publications

Forbes, S. J., Northfield, T. D., Cheesman, A. W., Lambert, S., Hidayat, T., Clark, R. E., & Cernusak, L. A. (2024). Data from: Artificial pollination may impact fruit size, seed quality and production sustainability in cacao. Dryad Data Repository, DOI: 10.5061/dryad.kh18932g2.

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Abstract

Climate change is impacting agricultural systems and communities in every region across the globe, with especially large impacts expected across tropical and subtropical regions. The effects of climate changes on agricultural crops can be direct, such as accelerated plant development under high temperatures or constrained growth and productivity from moisture limitation, or indirect, such as climate-mediated species mismatches between plants and their vital pollinators. Research on the effects of climate change on species often focuses on the direct effects of a singular abiotic factor (e.g., rising air temperature), due to logistical limitations involved in simultaneously considering multiple effects or indirect effects mediated by pests or beneficial species. However, fewer studies have evaluated the combined (interactive) effects of these factors on plant growth, physiology and yield, making the combined impacts of climate change more difficult to predict and mitigate. Furthermore, the indirect effects of climate change on crop production from, for example, altered ecological interactions between plant species and their pollinating insects, are poorly understood even relative to direct effects. Given this, a deeper understanding of the direct, indirect and interactive impacts of future-predicted climate changes on important agricultural crops across the tropics is needed, allowing for the development of effective mitigation strategies. To address this knowledge-gap, I present a series of studies evaluating the simultaneous direct effects of two concomitant environmental changes associated with climate change (increased temperature and elevated [CO_2]) on three globally important tropical crops: cassava (*Manihot esculenta Crantz*), coffee (*Coffea L.*) and cacao (*Theobroma cacao L.*). Furthermore, using cacao as a case-study species, I also experimentally evaluate the ability of an existing adaptive management strategy to mitigate pollination limitation in the short- and long-term.

In my study on the effects of climate change on cassava, I found that elevated temperature initially led to a surge in cassava plant growth (height) and increases in tuber cyanogenic glucoside (cyanide) concentrations. The addition of elevated [CO_2] over time led to an increasing biomass advantage over plants grown at ambient [CO_2] levels. Elevated temperature and [CO_2] also increased the number of tubers initiated and early tuber expansion, but did not significantly increase either the tuber, or leaf, cyanogenic glucoside concentrations. In my study on the effects of climate change on coffee and cacao, I found non-extreme increases in temperature led to an increase in biomass for both species in well-watered seedlings. Increased [CO_2] helped cacao seedlings moderate temperature-mediated effects on stomatal conductance and instantaneous water-use efficiency and led to overall improvements in photosynthesis (A_{sat}) and instantaneous water-use efficiency for both species. Leaf chemistry was altered differentially between the two species, potentially because of reallocation of nutrients towards secondary metabolite production in coffee, or by dilution under enhanced biomass production in cacao.

In my studies evaluating if artificial (hand) pollination strategies can increase yield and ameliorate management- and climate-driven pollination limitation, I found that artificial pollination may indeed increase yield. However, I further identified multiple physiological trade-offs at the individual (fruit/pod) level in the impacts of artificial pollination management in cacao. Firstly, increasing pollination intensity increased the number of fruit produced at a cost to fruit (pod) size and seed (bean) quality. Secondly, narrowing the pollination time window (condensing pollinations) can reduce early fruit abortion to improve fruit survival and efficiencies, but may lead to reduced production in later years from resource depletion. My results suggest that advanced short-term artificial pollination strategies to improve cacao production may have unintended consequences on bean quality and long-term production sustainability.

Taken together, my findings contribute to a more comprehensive global view of the direct and indirect impacts of climate change on plants, food supply and nutrition security. In this context, the results offer important insights into the future performance, productivity and management of three globally important tropical crops and contributes to the progressive accumulation of information needed to develop and revise effective climate change mitigation strategies.

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Chapter 1. General Introduction

1.1 Background

Anthropogenic climate change may represent the greatest threat to natural ecosystems, biodiversity and human societies to date (Malhi et al., 2020). Due to anthropogenic activity, the concentration of atmospheric CO_2 has now reached a level not seen for the past 500,000 years (Jansen et al., 2007). According to the NOAA Global Monitoring Lab (Lan et al., 2024) the annual average $[CO_2]$ level in 2023 was 419.3 ppm, more than 50% higher than pre-industrial concentration levels. Concomitantly, the global mean surface temperature has increased by 1.1°C since pre-industrial times (IPCC, 2023). The sixth report of the IPCC (IPCC, 2021) also suggests that by the end of this century, $[CO_2]$ may rise to values over 1100 ppm in parallel with temperature increases of up to 5.7 °C, when considering the Fossil-Fuelled Development Shared Socioeconomic Pathway (SSP) 5-8.5. These projected environmental changes are likely to threaten the availability of food, water and shelter, leading to shifts in the range and prevalence of pests and disease, alterations in the habitat ranges for both plants and animals, species extinctions and the destabilisation of the ecosystems on which we all depend (Dunlap and Brulle, 2015).

Plants are expected to face greater abiotic stresses under future climates (IPCC, 2021). Indeed, the cultivation of plants in agricultural systems for food production is one of the sectors most vulnerable to changes in the climate (IPCC, 2021). Climate change thus represents a significant challenge for global agricultural production, impacting food availability, quality and diversity (DaMatta et al., 2010; Leisner, 2020). The effects of climate change on agricultural crops can be both direct and indirect. Climate change commonly affects agricultural crops by altering *i.* the geographic ranges that are biophysically suitable for species cultivation (Porter and Semenov, 2005), *ii.* the physiology, productivity, growth and quality of plants and their products (Schmidhuber and Tubiello, 2007; DaMatta et al., 2010; Springmann et al., 2016; Myers et al., 2017) and *iii.* the ecological interactions occurring within agroecosystems (Fontúrbel et al., 2021).

Though an important foundation to our current understanding, research to date on the impacts of climate change on species has largely focused on the direct effects of alterations in singular abiotic conditions rather than indirect and/or interactive effects. For example, while elevated temperature and increasing atmospheric carbon dioxide concentrations ($[CO_2]$) are expected to have combined effects on agronomic productivity, few studies evaluate the concomitant effects of these factors on plant growth, physiology and yield. Even fewer are studies that focus on the impacts of climate change on between-species interactions (Fontúrbel et al., 2021). By considering only the direct impacts of abiotic factors in singularity, and on single species independently, the combined impacts on species occurring within ecosystems become more difficult to predict and mitigate. Thus, a deeper understanding of the extent of the interactive and indirect climate-driven impacts on important agricultural crops and crucial species interactions is needed.

One important indirect effect of climate change on agricultural crops arises from climate-mediated alterations to ecological interactions between species (Ockendon et al., 2014). For example, in agricultural production systems, the pollination of plants by animals is a crucial mutualistic ecological interaction that is dominated by insects (Ollerton et al., 2011). This globally important mutualism is likely to be disrupted by climate change, as global warming can cause mismatches (e.g., altered timing) between both halves of this interaction (Gérard et al., 2020), with subsequent consequences on crop production (Potts et al., 2010). Species mismatches can put both plants and their pollinating insect species at risk of extinction (Scheffers et al., 2016), decreasing crop production and seriously threatening agricultural production systems that rely on such interactions.

Whether direct or indirect, the harmful impacts of climate change are projected to disproportionately affect the developing countries of the global south (Hertel and Rosch, 2010; Springmann et al., 2016). In general, tropical regions appear to be particularly vulnerable to climate change owing to several biophysical factors (e.g., limited change in water use efficiency of typical C4 crops under increasing [CO_2], tropical crops being closer to their high temperature optima, pre-existing increased prevalence of pests and disease) and socioeconomic constraints (e.g., greater economic and individual livelihood dependence on agriculture, widespread poverty, inadequate technologies) (Rosenzweig and Liverman, 1992). For example, recent studies using regional and global simulation models indicate that even moderate increases in temperatures will drive negative impacts on three major tropical cereal crops such as rice, maize and wheat (Morton, 2007). To make matters worse, the existing threats to tropical agricultural production such as pest and disease outbreak, extreme weather events, and market volatility, are all expected to be exacerbated by climate change (Harvey et al., 2014). Farmers in tropical regions are typically lower-income smallholder farmers that directly depend on agricultural production for their livelihoods (Morton, 2007). With limited resources and capacity to deal with unpredictable shocks or to implement effective adaptive strategies (Morton, 2007), climate change mediated declines in agricultural productivity presents a significant threat to the food security, nutrition, income and overall well-being of smallholder farmers in tropical regions (Morton, 2007; Harvey et al., 2014).

1.2 Thesis Rationale and Objectives

1.2.1 *Rationale*

We can no longer wait passively for these disasters to come. Climate change-mediated declines in agricultural productivity for major tropical crops are already being reported (Porter et al., 2014). While knowledge gaps on the potential impacts of climate change across tropical agriculture exist, it is especially large for smallholder tropical crops (Harvey et al., 2014). This includes both perennial staple crops such as cassava (*Manihot esculenta* Crantz, Euphorbiaceae), as well as commodity crops such as (*Coffea* L., Rubiaceae) and cacao (*Theobroma cacao* L., Malvaceae). Taken together, these three globally transformative crops play a crucial role in ensuring adequate economic livelihoods and food security in many of the world's most poor and food insecure regions.

Cassava is a perennial woody shrub originating in the Amazon basin (Olsen and Schaal, 1999) extensively cultivated by low-income smallholder farmers as an annual crop for its edible starchy tuberous roots. Cassava is the third most important food in the tropics, after rice and maize (Burns et al., 2010) as it provides a vital source of calories for more than a billion people in the global south (Burns et al., 2010; Chetty et al., 2013). In the face of climate change, cassava may represent the future of food security in the food-insecure regions of Africa and other developing nations due to its efficient production of tubers, year-round availability, tolerance to drought and other extreme conditions (Burns et al., 2010). However, the performance of this crop under interactive conditions of elevated temperature and [CO_2] remains largely unknown. The safety of cassava in the human diet depends on cyanogenic glucoside concentrations (Gleadow et al., 2009) which may be altered under future climate change. Despite this, large knowledge gaps exist on the cyanide concentrations of cassava's edible plant tissues under future predicted climate scenarios.

Coffee, grown in approximately 80 tropical countries by an estimated 25 million smallholder farmers (Waller et al., 2007), is the world's most economically important perennial fruit tree crop (Davis et al., 2012). *Coffea arabica* (hereafter referred to as 'coffee'), is one of almost 125 *Coffea* species (Krishnan et al., 2015) that originates in the Afromontane rainforests of the Ethiopian uplands (Anthony et al., 2002) and is categorized as highly sensitive to climate change (DaMatta et al., 2019). Coffee trees produce berries which when roasted, produce the world's favourite beverage and the second-most valuable export commodity (after oil) for developing countries (Pendergrast, 2009). Coffee yield is known to be strongly determined by climatic conditions (Tavares et al., 2018) and climate can both directly, and indirectly, influence the incidence of serious pests and diseases such as coffee berry borer (Jaramillo et al., 2011). Since the biochemical composition of coffee leaves and berries underpins its value and resistance to pests and disease (Ahmed et al., 2021), with significant implications for farmers and consumers alike, more studies are needed that evaluate any climate-mediated alterations to this important aspect of the coffee plant.

Cacao is a tropical tree species native to the lowland rainforests of South America (Motamayor et al., 2002). Cacao is cauliflorous, producing large fruits (pods) on its trunk and main branches that each contain between 30 - 40 seeds (beans) (Lima et al., 2011). These beans constitute the raw product cocoa, upon which the multibillion-dollar chocolate industry is based (Lima et al., 2011). The cacao tree is an important tropical cash crop for approximately 6 million farmers across 61 countries, most of which are smallholders (Dand, 2011; Clay, 2013). However, productivity stagnation and declines have been reported since the beginning of this century (Vaast and Somarriba, 2014; Aremu-Dele et al., 2022), owing to a complex set of physical and socioeconomic factors (Adeniyi and Ogunsola, 2014; Moriarty et al., 2014), as well as climate change (Lahive et al., 2019). Pollination limitation is as a major yield-reducing factor in cacao (Falque et al., 1995; Groeneveld et al., 2010;

Toledo-Hernández et al., 2017; Wanger et al., 2021). While the ultimate causes of pollination limitation in cacao remain largely unexplored, the artificial (hand) pollination of cacao flowers is increasingly being evaluated as an agronomical intervention to overcome pollination limitation (Vera-Chang et al., 2016; Forbes and Northfield, 2017; Toledo-Hernández et al., 2020; Toledo-Hernández et al., 2023). However, revisions of this management practice are needed, for optimization and to ensure long-term efficiency.

Most existing studies on agricultural crops focus on plant productivity and yield in the face of climate change. However, climate-mediated alterations in the biochemical composition of specific plant organs (i.e., the seeds of coffee and cacao, and the tubers and leaves of cassava) can have major consequences for crop value, consumption choices, human health and nutrition, and plant defence against pests and disease (Gleadow et al., 2009; Vaast and Somarriba, 2014; Jay-Allemand et al., 2015; Kundu and Vadassery, 2019; Ahmed et al., 2021). To develop a more comprehensive view of the true impact of climate change on our global food supply and nutrition security, we must expand our focus and adopt integrated approaches that combine evaluations of crop growth, physiology and production with quality, chemical / nutritional composition measures. Lastly, adaptive approaches that generate resilience and sustainability in agricultural systems require longer-term perspectives to improve our understanding of the effects of our management responses to both environmental and agricultural system change. Thus, studies evaluating the long-term sustainability of existing adaptation strategies, are also needed.

1.2.2 *Objectives*

In this thesis, I address some of these knowledge-gaps for three globally important perennial crops – cassava, coffee and cacao – by evaluating: a) if the early growth, physiology and biochemistry of each of these species is altered under future-predicted, interactive, climate conditions, and b) if artificial pollination is an effective strategy to reduce pollination limitation and sustainably enhance yields in cacao production systems. To achieve this, I identified the following two overarching objectives:

Objective 1: to evaluate the direct and interactive effects of future-predicted elevated air temperature and elevated [CO_2] on the physiology, tissue biochemistry and resource partitioning of cassava, coffee and cacao. I experimentally addressed this objective in the climate-controlled glasshouse studies of **Chapters 2 and 3**.

Objective 2: to evaluate the ability of adaptive artificial pollination strategies, and identify the pollination conditions (i.e., pollen genotype, pollination intensity, and pollination timing) that are needed, to ameliorate pollination limitation and drive sustainable yield enhancements in cacao, without compromising pod size or bean quality. I addressed this objective in the Indonesian field studies of **Chapters 3 and 4**.

Adaptation is a pivotal factor that will determine the severity of future climate change impacts on the agriculture (Lobell et al., 2008). In addressing these objectives, I endeavoured to better understand some of the future impacts of climate change on three globally important agricultural crops. The information obtained by the projects contained within this thesis may contribute to the development of current and future crop management and adaptation strategies and help to improve the long-term productivity and profitability of smallholder farms, farmers and their livelihoods all over the world.

Chapter 2. Elevated Temperature and Carbon Dioxide alter Resource Allocation to Growth, Storage and Defence in Cassava (*Manihot esculenta*).

Note: References for this published chapter are self-contained.

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Elevated temperature and carbon dioxide alter resource allocation to growth, storage and defence in cassava *(Manihot esculenta)*

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ABSTRACT

Rising atmospheric CO_2 concentrations and global warming can alter how plants partition their resources. This is important for food crops through changes in resource allocation to edible tissues and toxic defence compounds. While research suggests elevated temperature and [CO_2] independently drive changes in plant metabolism and stress levels, and photosynthetic rates, respectively, it is less clear how these environmental changes impact plants when combined. Cassava is an important dietary staple for many developing nations. However, the safety of cassava depends on cyanogenic glucoside concentrations. In a climate-controlled greenhouse, the effects of elevated temperature in the presence and absence of elevated [CO_2] on the growth, physiology and chemical defence of cassava at two growth stages were examined. Growth in cassava was initially increased by elevated temperature. However, across time, simultaneous elevated [CO_2] led to an increasing biomass advantage over plants grown at ambient [CO_2] and temperature. Elevated temperature and [CO_2] also significantly increased tuber initiation and early tuber expansion. Tuber and leaf cyanide concentrations were significantly reduced under elevated temperature, while elevated temperature and [CO_2] produced tuber cyanide concentrations similar to the higher levels found in plants grown at ambient conditions. The findings highlight how future climate change may impact both cassava production and quality.

1. Introduction

Increasing temperatures and atmospheric CO_2 are two major environmental factors associated with global climate change (Schmidhuber and Tubiello, 2007). At the current rate, global air temperatures are likely to reach 1.5 °C above pre-industrial levels between 2030 and 2052 (IPCC, 2018) and [CO_2], which have recently exceeded 410 ppm in 2017, are predicted to continue to climb by approximately 2 ppm per year (Peters et al., 2007, with updates documented at http://carbontracker.noaa.gov). These changes are likely altering plant growth, function and productivity, with direct consequences on agriculture, global food security and wider human health (Schmidhuber and Tubiello, 2007; Springmann et al., 2016).

Tropical and subtropical countries are projected to experience the largest impacts of climate change on food security, particularly in lower income countries of tropical Asia and Africa (Springmann et al., 2016). While elevated temperatures may lead to positive yield-determining impacts for crops grown in mid to high latitude regions (Supit et al., 2010), even slight temperature increases in the range of 1–2 °C, and considering other concomitant climate variables, are predicted to decrease major staple crop yields in tropical regions by the mid-to-late 21st century (e.g., Lobell et al., 2008; Tatsumi et al., 2011; Knox et al., 2012; Rosenzweig et al., 2014). Indeed, for each degree increase in global mean surface temperature there will likely be significant reductions in the global yields of wheat (6.0 %), rice (3.2 %), maize (7.4 %), and soybean (3.1 %) (Zhao et al., 2017). However, the predicted negative effects of future rises in temperature on crop growth and yield (e.g., Lobell and Field, 2007; Lobell et al., 2008) may be partially offset by the positive stimulatory effects of increasing [CO_2] on crop physiology and morphology (e.g., Drake et al., 1997; Kimball et al., 2002;

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Long et al., 2004; Bannayan et al., 2009; Rosenthal et al., 2012). Despite this, relatively few studies evaluate the combined effects of elevated temperature and concomitant increasing [CO_2] on plant growth and yield. There is a need, therefore, for further studies examining interactive impacts of rising temperature and [CO_2], particularly on major staple tropical crops that are relatively understudied, to help predict impacts of future climate on future food security and livelihoods.

Cassava, *Manihot esculenta* Crantz (Euphorbiaceae) is a perennial woody shrub native to the Amazon basin (Olsen and Schaal, 1999) that is now cultivated extensively throughout the tropics and subtropics, for its edible starchy storage roots (El-Sharkawy, 2006). Because of its high productivity in conditions of unpredictable rainfall and low-fertility soils (Burns et al., 2012a; Okogbenin et al., 2013) cassava is considered one of the most productive tropical crops on marginal lands (Zhang et al., 2008). It shows high resistance to drought (El-Sharkawy, 2004) and a remarkable ability to recover from environmental stresses (Jarvis et al., 2012; Rosenthal and Ort, 2012; Vandegeer et al., 2013; El-Sharkawy, 2014). Regarded as one the most efficient producers of carbohydrates and energy among all food crops (De Vries et al., 2010), cassava is a vital source of calories for more than a billion people in developing and tropical countries (Burns et al., 2010; Chetty et al., 2013). In sub-Saharan Africa, the most food-insecure region in the world, cassava is the most important food crop (Nhassico et al., 2008; Latif and Müller, 2015), accounting for almost two-thirds of direct human caloric intake (Rosenthal et al., 2012).

Although cassava consumption is widespread, all cassava plant parts contain the cyanogenic glucosides linamarin and lotaustralin (Cook and De La Cruz, 1982; Gleadow and Møller, 2014) which are catalytically hydrolysed to release toxic hydrogen cyanide (hereinafter referred to as 'cyanide' for simplicity) upon tissue disruption (e.g., upon consumption) (Jorgensen et al., 2005; Burns et al., 2012b). Cyanogenic glucosides are considered to play an important role in plant defence against herbivores (Zagrobelny et al., 2004) and can cause cyanide poisoning in humans if consumed without processing to sufficiently hydrolyse the glucosides and prevent cyanide formation (Burns et al., 2010; McKey et al., 2010). Cyanide concentrations can be highly variable between cultivars and growing location (Burns et al., 2012a), but generally range between 0.001–1.55 mg g^{-1} (mg HCN equivalents / kg fresh material) in the root parenchyma (hereinafter referred to as 'tubers' for simplicity), 0.9–2 mg g^{-1} in the root cortex (peel) and $0.02-1.86$ mg g^{-1} in the leaf tissue (Burns et al., 2012b). There is a low correlation between tuber and leaf cyanide concentrations (Burns et al., 2012a).

The total cyanide concentration of fresh tubers is commonly used to categorise cassava cultivars as either 'bitter' (above 0.05 mg g^{-1}) or 'sweet' (below 0.05 mg g^{-1}) (FAO/WHO, 2005). Tubers of 'bitter' cultivars are usually peeled, sometimes grated and boiled before consumption to reduce the cyanide concentrations down to those recommended as safe for human consumption ($<$ 0.01 mg g^{-1}) (FAO/WHO, 2005; Burns et al., 2012a). In contrast, tubers of the less-toxic, 'sweet' cassava cultivars are often consumed without adequate processing, despite often having cyanide concentrations well above the recommended 0.01 mg g^{-1} (Burns et al., 2012b). The highly toxic cassava leaves are also consumed regularly as a vegetable and provide an important source of proteins, vitamins and micronutrients to people living within food-insecure regions (Nzwalo and Cliff, 2011; Latif and Müller, 2015). To reduce their toxicity, cassava leaves are typically pounded and boiled before consumption (Lancaster and Brooks, 1983; Achidi et al., 2003; Ngudi et al., 2011). However, this is not a universal practice and the leaves are still consumed without adequate processing in many regions such as Fiji, Indonesia, occasionally in South and Central America (R.M. Gleadow, *personal communication*) and sub-Saharan Africa (Ngudi et al., 2011).

Given the current role and growing importance of cassava to the health and livelihoods of people throughout the tropics, further exploration of the responses of cassava to predicted climatic changes is vital to evaluate potential future food security risks. Moreover, in regions and situations where cassava leaves and tubers are consumed without adequate processing, any changes in cyanide concentrations could have serious public health consequences. Although independent evaluations of temperature (e.g., Alves, 2002; Brown et al., 2016) and [CO_2] (e.g., Gleadow et al., 2009a,b; Rosenthal et al., 2012) effects on cassava have been conducted, no studies to date have investigated the interactive effects of temperature and [CO_2] on growth, productivity, physiology and chemical defence in cassava. This simultaneous evaluation is important given their potentially contrasting effects on plant growth and on resource partitioning between growth and defence. Here, the results of a controlled greenhouse experiment that examined the effects and possible interactions of temperature and [CO_2] on cassava at two different growth stages are presented. Specifically, this study sought to evaluate 1) whether elevated [CO_2] might mitigate any potentially deleterious effects of elevated temperature on plant growth, 2) whether enhanced plant growth under elevated temperature and [CO_2] alters resource allocation, and 3) if the effects of elevated temperature and [CO_2] are maintained as patterns of internal resource demand and allocation shift throughout plant development.

2. Materials and methods

2.1. Planting material and growing conditions

A single cassava cultivar, cv. MAus7, was used in this study. This cultivar is considered a high yielding, 'sweet' cultivar and is a standard cultivar for cassava research within Australia (e.g., Keating and Evenson, 1979; Fukai and Hammer, 1987; Gleadow et al., 2016). A sweet cultivar was selected to better evaluate the potential health implications associated with changing cyanide levels in cassava cultivars that are more likely to be consumed without adequate processing. The study was conducted in a 9 × 7 m walk-in, climate controlled greenhouse facility, divided into three independently regulated 5(L) × 3(W) × 4(H) m chambers. Parallel chambers positioned in a N-S orientation allowed for independent temperature and [CO_2] regulation and received natural sunlight at about 50 % full sun from SOLARO 5220 D O FB climate screen (Ludvig Svensson Inc. Kinna, Sweden). Freshly cut cassava stem cuttings (10 cm in length, $2-3$ cm in diameter, each with three nodes) were planted into free-draining black plastic pots (30 cm diameter, 50 cm depth), each filled with a 10: 1 (v/v) soil and perlite mix. The soil was a potting mix obtained from a local landscaping company. A 50 mm layer of river stones was placed in the pot bases to assist drainage. The pots were maintained in open-air, full-sun conditions until translocation into the greenhouse. Seven days after planting, the stem cuttings had sprouted and generated new plantlets. Twenty days after planting, fifty-four similarly sized cassava plants were moved into the greenhouse facility and randomly allocated to one of the three greenhouse chambers (i.e., eighteen cassava plants per chamber). Each cassava plant, along with their associated treatments, were rotated between chambers monthly to reduce possible chamber effects unrelated to the imposed treatments. Plants were watered daily to field capacity for the duration of the experiment to remove potential effects of water limitation. To reduce the potential impact of nitrogen limitation on cyanogenic glucoside concentrations (Gleadow et al., 1998), each plant was given 20 g of slow-release granular fertilizer (Scotts Osmocote® Plus Trace Elements: Native Gardens) every month after translocation into the greenhouse.

2.2. Treatments

To quantify the effects and potential interactive effects of elevated temperature and [CO_2] on the early developmental stages of cassava, the cassava plants (grouped by chamber) were grown under one of three temperature - [CO_2] treatments. For treatment one ($Temp_{amb}$ $[CO_2]_{amb}$), air temperatures were set to track the ambient conditions of

Cairns, North Queensland, Australia and [CO_2] was set to 400 ppm. For treatment two (Temp$_{elev}$ [CO_2]$_{amb}$), air temperatures were set +4 °C above the ambient treatment and [CO_2] was set to 400 ppm. This treatment evaluated the effects of elevated temperature alone. For treatment three (Temp$_{elev}$ [CO_2]$_{elev}$), air temperatures were set to +4 °C, and the chamber was enriched by pure CO_2 injection to an elevated [CO_2] of 800 ppm. Treatment three evaluated the interactive effects of elevated temperature and elevated [CO_2]. Conditions were monitored with sensors (Temp/ RH: QFM2160, Siemens, Bayswater, VIC, Australia; [CO_2]: GMP222, Vaisala, Helsinki, Finland), and regulated through a feedback control system implemented in the building management software. Air temperature (°C), [CO_2] (ppm) and relative humidity (%) were recorded every 5 min during the experiment. The elevated temperature treatments maintained average daytime temperature of 3.6 °C above ambient, while the elevated [CO_2] treatment was on average 789.7 ppm during the daytime (A.1).

2.3. *Growth measurements, harvest procedure and tissue sampling*

Six cassava plants were destructively harvested prior to experimental treatment (i.e., 20 days after planting) to determine initial plant biomass and leaf area. Nine plants per treatment were destructively harvested on two harvest dates at 36 and 74 days after the treatments had begun (Fig. 1). This ensured plants were well spaced. Measurements describing plant growth were made every week until, and upon the first harvest and again on final harvest. Growth measurements included stem height (root collar to apical meristem, mm), average root collar stem diameter (mm), average mid stem diameter (at mid-way point between root collar and apical meristem, mm) and the number of fully expanded leaves per plant. The ratio of stem mass to height (g cm^{-1}), here after referred to as stem 'stoutness', was calculated by dividing the total plant stem dry mass by the total stem height.

Plants harvested on each of the two harvest dates were separated into leaves, petioles, stems (above-ground biomass), roots, tubers and stem cutting (below-ground biomass). First, the above-ground biomass of plants was removed by cutting the stem at the root collar. The leaves were then detached and classified as either fully expanded, immature or senescent. Expanded leaves were defined as leaves that were fully developed and contributing to photosynthesis whilst immature leaves were young, soft leaves that were typically purple in colour and unexpanded. Senescent leaves were defined by yellow/brown lamina colouration on > 50 % of the leaf surface. Fresh weight was determined for all tissue types before oven drying at 60 °C for at least 72 h.

Leaf area of fresh target leaves (see gas exchange below) was measured by scanning the target leaves from each harvest date, and all leaves from the initial harvest (i.e., 20 days after planting) using a flatbed scanner (Canon CanoScan Flatbed Scanner LiDE120) and calculating lamina area against a scaled calibration using Image-J 1.52a Software (Schneider et al., 2012). Fresh weight (g) and leaf thickness (μm) of the target leaves was also determined before drying and determining dry mass. At both harvest dates, the target leaves were dried separately from the remaining expanded leaves for nitrogen and phenolics analysis (see plant composition below). Upon the 74-day harvest date only, ten leaf discs (5 mm diameter) were sampled from the fresh target leaf of each plant for determination of cyanogenic glucoside concentration (see plant composition below). Leaf discs were excised from the middle of the centre lobe (avoiding the mid-rib) and the fresh weight determined before being snap-frozen in liquid nitrogen and freeze-dried at -55 °C for 48 h until dry. The dried leaf disc samples were sealed in Eppendorf tubes and stored in a dark, air-conditioned laboratory until further chemical compositional analysis.

Roots and tubers were washed free from all soil with water and the number of tubers initiated per plant counted. Initiated tubers were defined as roots that appeared visually to be functioning as tubers and that were typically > 5 mm diameter in their middle section, following Vandegeer et al. (2013). Upon the 74-day harvest date only, two tuber core samples (20 mm long, 5 mm diameter) were taken from the two largest tubers per plant (> 10 mm diameter in their middle section) for cyanogenic glucoside analysis following Brown et al. (2016). Each tuber core was sampled from the tuber's middle section, longitudinally and directly underneath (and excluding) the tuber peel to avoid potential confounding effects of intra-tuber variation (Bradbury et al., 1991). The fresh weights of the tuber core samples were determined before they were snap-frozen, freeze-dried, re-weighed for dry mass and stored as described for the leaf disc tissue samples. The mass of tissues subsampled for composition analysis was accounted for in the determination of final dry mass of respective organs.

2.4. *Growth indices*

Leaf mass per unit area (LMA, g m^{-2}) was calculated by dividing the projected leaf area of the target leaf used in gas flux measurements by its dry mass. Harvest index was calculated by dividing the total tuber dry mass by the total plant dry mass. As per Keating et al. (1982) and Gleadow et al. (2016) under Australian harvesting conditions, the swollen (lignified callous) stem cutting was included into the

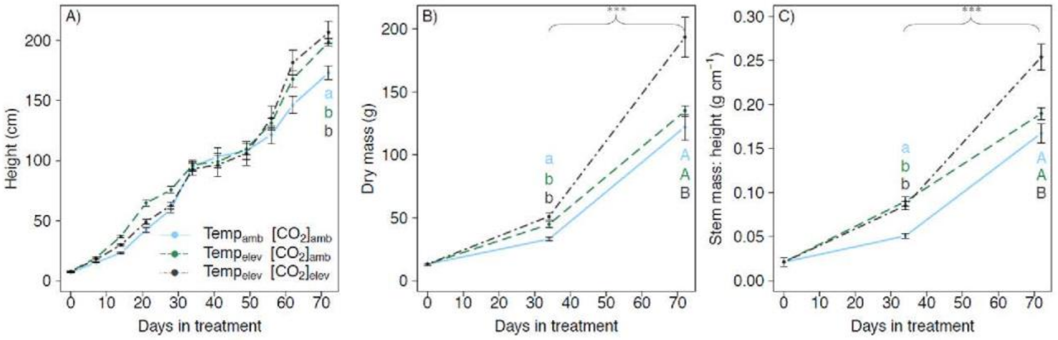

Fig. 1. Mean (± SE) plant growth measurements for young cassava plants grown in three experimental treatments for up to 74 days. Initial values were estimated at 0 days in treatment by destructively harvesting six representative cassava plants, followed by $n = 18$ plants per treatment with $n = 9$ plants destructively harvested at each 36 and 74 days in treatment, respectively. Height (A) was measured each week (approximate) from the treatment start date (0 days in treatment). Dry mass (B) and ratio stem mass: height (C) were measured using tissues obtained upon harvest, oven dried at 60 °C for 14 days to constant dry weight. Pairwise comparisons were conducted among means within, but not between harvest dates. Therefore, means with different letters of the same case represent significant differences between treatments at an alpha level of $P < 0.05$ (Tukey's HSD tests). Significant differences between harvest dates (time) indicated by asterisks. All plants were watered to field capacity daily and provided mineral nutrition once per month.

measurement of total plant mass. The relative growth rate (RGR, g g^{-1} d^{-1}) of plants across time was calculated (Eq. 1), where m_2 and m_1 represent the final and initial total plant dry mass, respectively and where t represents the number of days in treatment (Hoffmann and Poorter, 2002).

$$\text{RGR}=\frac{\ln(m_2)-\ln(m_1)}{t} \tag{1}$$

2.5. Gas exchange

Leaf level gas exchange measurements were conducted prior to each destructive harvest. For the 36-day harvest, gas exchange was conducted once, while at the 74-day harvest, gas exchange was measured on two consecutive days (plants were measured twice) with results averaged. Measurements were obtained from the third newest fully expanded leaf (target leaves) of each plant. Gas exchange was measured between 08:00 and 12:00 using a portable photosynthesis system (Li-Cor 6400, Li-Cor Inc. Lincoln, NE, USA) with Licor-cuvette conditions set to mimic typical midday greenhouse chamber temperature and [CO_2]. Photosynthesis (A), stomatal conductance, intercellular CO_2 concentration (Ci), leaf transpiration (E), leaf temperature, vapour pressure deficit calculated from measured leaf temperature, the ratio of internal to atmospheric CO_2 concentration (Ci/Ca) and instantaneous water use efficiency (WUE, calculated as A/E) were measured using the target leaves. Measurements were made at a photon flux of 1500 μmol m^{-2} s^{-1}, supplied by an artificial LED light source (6400-02B, Li-Cor Inc. Lincoln, NE, USA), with leaf vapour pressure deficit maintained between 1.19 and 1.76 kPa (A.2). Values of leaf **Δ** temperature (°C) were determined by subtracting the air temperature (within the cuvette) from the target leaf temperature ($Tleaf - Tair$). Leaf temperatures during the gas exchange measurements were between 32.8 °C and 33.3 °C for the Temp_{amb} treatment and between 36.5 °C and 38.8 °C for the $\text{Temp}_{\text{elev}}$ treatment.

2.6. Plant composition: cyanogenic glucosides, nitrogen and phenolics

All dried tissue samples were ground using a Qiagen TissueLyser II for leaf material and a Rocklabs Ring Mill for the tuber material and re-dried at 60 °C for 48 h prior to weighing-out sub-samples. Cyanogenic glucoside concentrations were evaluated in both leaf and tuber tissues obtained at the 74-day harvest. Cyanogenic glucosides were measured as the total amount of cyanide (mg g^{-1}) evolved from 10 mg (± 1 mg) dried leaf or tuber tissue, as per Vandegeer et al. (2013) and quantification of evolved cyanideconducted using colorimetric assays as per Gleadow et al. (2016). Nitrogen concentration (i.e., the total concentration of elemental nitrogen, % dry weight) was determined using 40 mg (± 1 mg) of oven-dried target leaf tissue obtained at the 74-day harvest using a Costech 4010 Elemental Analyser and following the Dumas method (Buckee, 1994). Leaf phenolic diversity (i.e., the number of different phenolic compounds) and relative phenolic concentration (mAU) were determined using target leaf tissue obtained at the 74-day harvest. Phenols were extracted and analysed following Uesugi and Kessler (2016). For this, 10 mg (± 1 mg) of leaf tissue per sample was extracted in 1 ml of 80 % methanol, with the samples vortexed and sonicated for 15 min. Five microlitres of this supernatant liquid was analysed for secondary metabolites by high-performance liquid chromatography (HPLC) on an Agilent® 1260 series HPLC equipped with a Poroshell 120, EC-C18 reverse-phase column (2.7 μm, 3 × 150 mm, Agilent® Technologies Australia) using a standard method targeted at phenolic compounds (Keinanen et al., 2001). The elution system consisted of solvents A) 0.25 % H_3PO_4 in water (pH 2.2) and B) acetonitrile that was 0–2 min, 0–12 % of B; 2–3.3 min, 12–18 % of B and 3.3 – 10 min, 18–58 % of B, and 10 – 11 min, 58 % of B, with the flow rate of 0.5 ml/min. Peaks of phenolics were identified to compound classes using UV spectra information and quantified according to the specific compound. For each compound class, a standard curve for a representative compound was created and expressed sampled compounds as the equivalents of the respective standard.

2.7. Statistical analysis

To evaluate the effects of elevated temperature and [CO_2] on growth, storage, gas exchange and defence in cassava, generalized linear models or generalized linear mixed models using the glm and glmer functions, respectively, were conducted as necessary in the lme4 package (Bates et al., 2015) in R (R Core Team, 2015). As measurements for the description of plant growth, storage and gas exchange were conducted at each of two harvest dates, the models describing the response variables within these groups included fixed effects of *i.* treatment (Temp_{amb} $[CO_2]_{\text{amb}}$; $\text{Temp}_{\text{elev}}$ $[CO_2]_{\text{amb}}$; $\text{Temp}_{\text{elev}}$ $[CO_2]_{\text{elev}}$) and *ii.* time (harvest at 36 and 74 days), as well as a treatment × time interaction. As the measurements describing plant defense were only conducted using leaves obtained at the 74-day harvest, the models describing these response variables included a fixed effect of treatment only.

For the models describing each, plant height, total plant dry mass, LMA, stem 'stoutness', photosynthesis, instantaneous WUE, leaf cyanide, tuber cyanide and leaf nitrogen, a gamma distribution was used to account for the positive relationship between the mean and variance in plant growth, with an identity-link function to maintain the additive interaction terms when applicable (i.e., so that the interaction was not additive on a log-scale as is the case for a log-link function). The model for plant height included a random effect of plant to account for similarities between plants across measurement dates. The model describing tuber dry mass also assumed a gamma distribution, with a log-link function to account for the large difference in scales for tuber dry mass between the two harvest dates that led to negative predictions for tuber mass at 36 days when an identity-link function was used. For the models describing each, tuber number, harvest index, stomatal conductance, leaf **Δ** temperature and leaf phenolics (diversity and concentration), a normal distribution with an identity-link function fit the data best.

For all statistical models, sequential likelihood ratio tests for each fixed effect and their interactions were performed using the lrtest function in the lmtest package (Zeileis and Hothorn, 2002) in R (R Core Team, 2015). Where a fixed effect was found to have a significant effect on the response variable, Tukey's HSD tests were used using the multcomp package (Hothorn et al., 2008) in R (R Core Team, 2015) to determine where these differences occurred. All result graphics were created using the plotrix package (Lemon, 2006) in R (R Core Team, 2015).

3. Results

3.1. Growth

3.1.1. Plant height and growth indices

Across all treatments, substantial plant growth was observed with plants reaching an average of 98.7 cm at the 36-day harvest and 192.7 cm at the 74-day harvest (χ^2 = 1207.9, df = 1, $P < 0.0001$; Table 1; Table A3). No cassava plants branched during the experiment. There are three points to note about changes in height. Firstly, stem elongation generally increased linearly across time but was punctuated by short periods where stem elongation appeared to plateau, consistent with a possible dynamic growth pattern between stem elongation and alternate (non-stem) tissue development (e.g., leaf flushing, tuber initiation) (Fig. 1A). Secondly, plants grown at elevated temperature (i.e., $\text{Temp}_{\text{elev}}$) were on average taller at the end of the experiment than plants grown in the treatment with ambient temperature (χ^2 = 11.2, df = 2, P = 0.0037; Fig. 1A; Table A3). Thirdly, there was a significant [CO_2] effect but this varied across time, as shown by the significant

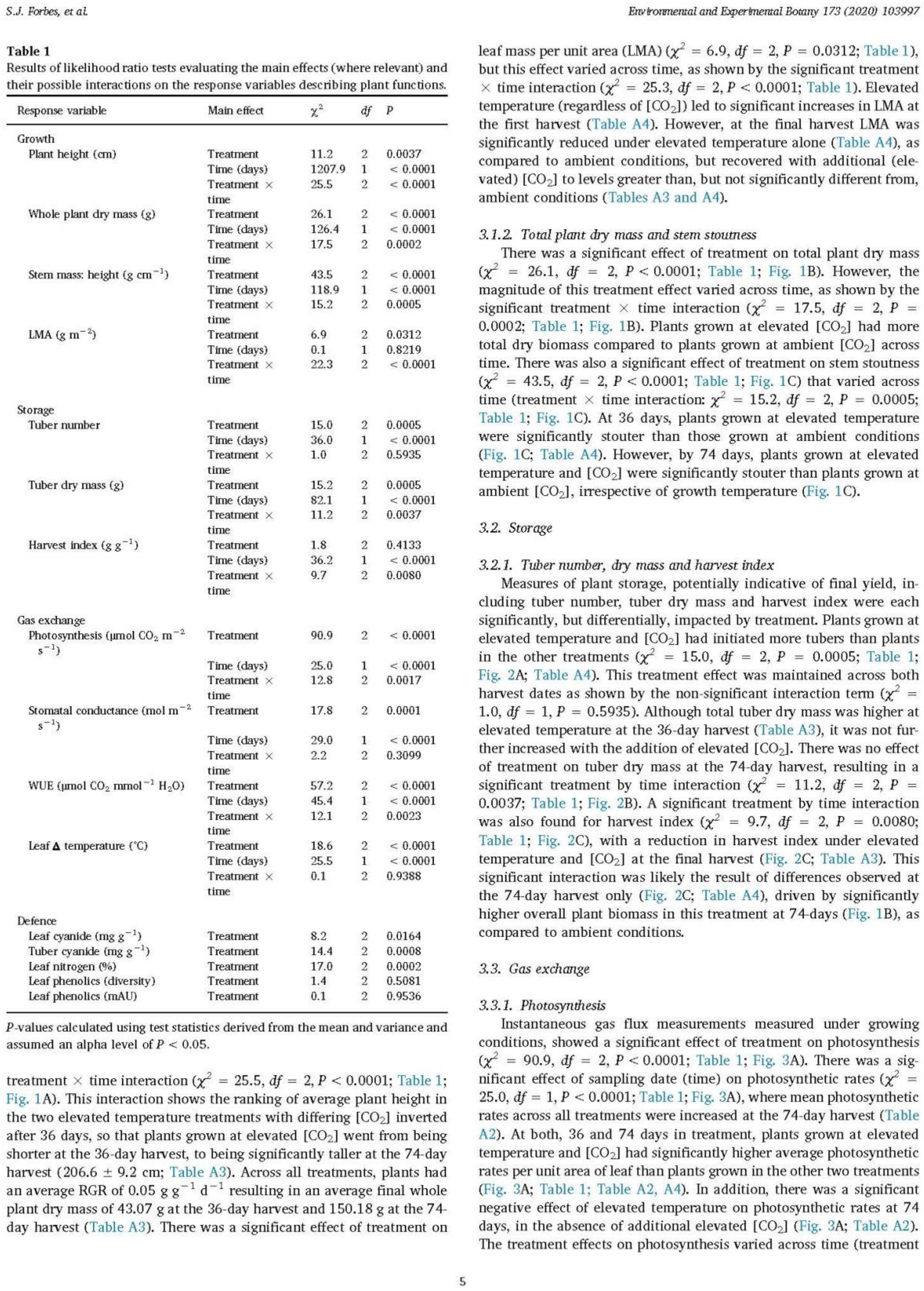

Table 1
Results of likelihood ratio tests evaluating the main effects (where relevant) and their possible interactions on the response variables describing plant functions.

Response variable	Main effect	χ^2	*df*	*P*
Growth				
Plant height (cm)	Treatment	11.2	2	0.0037
	Time (days)	1207.9	1	< 0.0001
	Treatment × time	25.5	2	< 0.0001
Whole plant dry mass (g)	Treatment	26.1	2	< 0.0001
	Time (days)	126.4	1	< 0.0001
	Treatment × time	17.5	2	0.0002
Stem mass: height (g cm^{-1})	Treatment	43.5	2	< 0.0001
	Time (days)	118.9	1	< 0.0001
	Treatment × time	15.2	2	0.0005
LMA (g m^{-2})	Treatment	6.9	2	0.0312
	Time (days)	0.1	1	0.8219
	Treatment × time	22.3	2	< 0.0001
Storage				
Tuber number	Treatment	15.0	2	0.0005
	Time (days)	36.0	1	< 0.0001
	Treatment × time	1.0	2	0.5935
Tuber dry mass (g)	Treatment	15.2	2	0.0005
	Time (days)	82.1	1	< 0.0001
	Treatment × time	11.2	2	0.0037
Harvest index (g g^{-1})	Treatment	1.8	2	0.4133
	Time (days)	36.2	1	< 0.0001
	Treatment × time	9.7	2	0.0080
Gas exchange				
Photosynthesis (μmol CO_2 m^{-2} s^{-1})	Treatment	90.9	2	< 0.0001
	Time (days)	25.0	1	< 0.0001
	Treatment × time	12.8	2	0.0017
Stomatal conductance (mol m^{-2} s^{-1})	Treatment	17.8	2	0.0001
	Time (days)	29.0	1	< 0.0001
	Treatment × time	2.2	2	0.3099
WUE (μmol CO_2 $mmol^{-1}$ H_2O)	Treatment	57.2	2	< 0.0001
	Time (days)	45.4	1	< 0.0001
	Treatment × time	12.1	2	0.0023
Leaf **Δ** temperature (°C)	Treatment	18.6	2	< 0.0001
	Time (days)	25.5	1	< 0.0001
	Treatment × time	0.1	2	0.9388
Defence				
Leaf cyanide (mg g^{-1})	Treatment	8.2	2	0.0164
Tuber cyanide (mg g^{-1})	Treatment	14.4	2	0.0008
Leaf nitrogen (%)	Treatment	17.0	2	0.0002
Leaf phenolics (diversity)	Treatment	1.4	2	0.5081
Leaf phenolics (mAU)	Treatment	0.1	2	0.9536

P-values calculated using test statistics derived from the mean and variance and assumed an alpha level of $P < 0.05$.

treatment × time interaction ($\chi^2 = 25.5$, $df = 2$, $P < 0.0001$; Table 1; Fig. 1A). This interaction shows the ranking of average plant height in the two elevated temperature treatments with differing [CO_2] inverted after 36 days, so that plants grown at elevated [CO_2] went from being shorter at the 36-day harvest, to being significantly taller at the 74-day harvest (206.6 ± 9.2 cm; Table A3). Across all treatments, plants had an average RGR of 0.05 g g^{-1} d^{-1} resulting in an average final whole plant dry mass of 43.07 g at the 36-day harvest and 150.18 g at the 74-day harvest (Table A3). There was a significant effect of treatment on leaf mass per unit area (LMA) ($\chi^2 = 6.9$, $df = 2$, $P = 0.0312$; Table 1), but this effect varied across time, as shown by the significant treatment × time interaction ($\chi^2 = 25.3$, $df = 2$, $P < 0.0001$; Table 1). Elevated temperature (regardless of [CO_2]) led to significant increases in LMA at the first harvest (Table A4). However, at the final harvest LMA was significantly reduced under elevated temperature alone (Table A4), as compared to ambient conditions, but recovered with additional (elevated) [CO_2] to levels greater than, but not significantly different from, ambient conditions (Tables A3 and A4).

3.1.2. *Total plant dry mass and stem stoutness*

There was a significant effect of treatment on total plant dry mass ($\chi^2 = 26.1$, $df = 2$, $P < 0.0001$; Table 1; Fig. 1B). However, the magnitude of this treatment effect varied across time, as shown by the significant treatment × time interaction ($\chi^2 = 17.5$, $df = 2$, $P = 0.0002$; Table 1; Fig. 1B). Plants grown at elevated [CO_2] had more total dry biomass compared to plants grown at ambient [CO_2] across time. There was also a significant effect of treatment on stem stoutness ($\chi^2 = 43.5$, $df = 2$, $P < 0.0001$; Table 1; Fig. 1C) that varied across time (treatment × time interaction: $\chi^2 = 15.2$, $df = 2$, $P = 0.0005$; Table 1; Fig. 1C). At 36 days, plants grown at elevated temperature were significantly stouter than those grown at ambient conditions (Fig. 1C; Table A4). However, by 74 days, plants grown at elevated temperature and [CO_2] were significantly stouter than plants grown at ambient [CO_2], irrespective of growth temperature (Fig. 1C).

3.2. *Storage*

3.2.1. *Tuber number, dry mass and harvest index*

Measures of plant storage, potentially indicative of final yield, including tuber number, tuber dry mass and harvest index were each significantly, but differentially, impacted by treatment. Plants grown at elevated temperature and [CO_2] had initiated more tubers than plants in the other treatments ($\chi^2 = 15.0$, $df = 2$, $P = 0.0005$; Table 1; Fig. 2A; Table A4). This treatment effect was maintained across both harvest dates as shown by the non-significant interaction term ($\chi^2 = 1.0$, $df = 1$, $P = 0.5935$). Although total tuber dry mass was higher at elevated temperature at the 36-day harvest (Table A3), it was not further increased with the addition of elevated [CO_2]. There was no effect of treatment on tuber dry mass at the 74-day harvest, resulting in a significant treatment by time interaction ($\chi^2 = 11.2$, $df = 2$, $P = 0.0037$; Table 1; Fig. 2B). A significant treatment by time interaction was also found for harvest index ($\chi^2 = 9.7$, $df = 2$, $P = 0.0080$; Table 1; Fig. 2C), with a reduction in harvest index under elevated temperature and [CO_2] at the final harvest (Fig. 2C; Table A3). This significant interaction was likely the result of differences observed at the 74-day harvest only (Fig. 2C; Table A4), driven by significantly higher overall plant biomass in this treatment at 74-days (Fig. 1B), as compared to ambient conditions.

3.3. *Gas exchange*

3.3.1. *Photosynthesis*

Instantaneous gas flux measurements measured under growing conditions, showed a significant effect of treatment on photosynthesis ($\chi^2 = 90.9$, $df = 2$, $P < 0.0001$; Table 1; Fig. 3A). There was a significant effect of sampling date (time) on photosynthetic rates ($\chi^2 = 25.0$, $df = 1$, $P < 0.0001$; Table 1; Fig. 3A), where mean photosynthetic rates across all treatments were increased at the 74-day harvest (Table A2). At both, 36 and 74 days in treatment, plants grown at elevated temperature and [CO_2] had significantly higher average photosynthetic rates per unit area of leaf than plants grown in the other two treatments (Fig. 3A; Table 1; Table A2, A4). In addition, there was a significant negative effect of elevated temperature on photosynthetic rates at 74 days, in the absence of additional elevated [CO_2] (Fig. 3A; Table A2). The treatment effects on photosynthesis varied across time (treatment

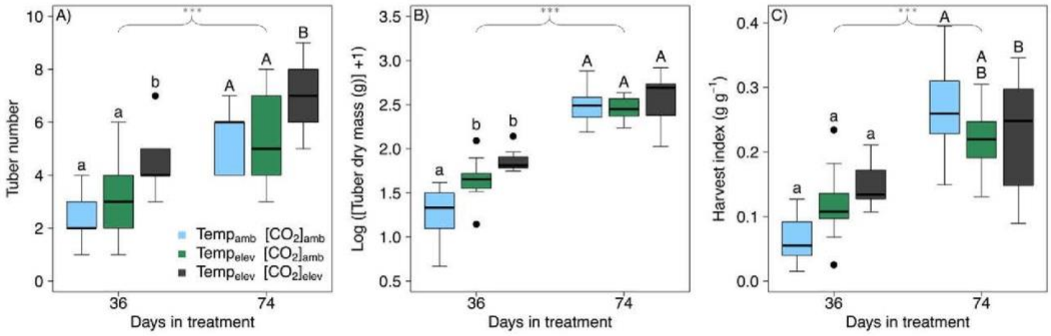

Fig. 2. Mean (± SE) plant storage measurements for young cassava plants grown in three experimental treatments for up to 74 days. Measurements were made on $n = 18$ plants per treatment with $n = 9$ plants destructively harvested at each 36 and 74 days in treatment, respectively. A) Tuber number, B) tuber dry mass (g) and C) harvest index (g g^{-1}) were evaluated using tissues obtained upon harvest, oven dried at 60 °C for 14 days to constant dry weight. Presented mean values for B) tuber dry mass were log(x + 1)-transformed to match the statistical analysis. Pairwise comparisons were conducted among means within, but not between harvest dates. Therefore, means with different letters of the same case represent significant differences between treatments at an alpha level of $P < 0.05$ (Tukey's HSD tests). Significant differences between harvest dates (time) indicated by asterisks. All plants were watered to field capacity daily and provided mineral nutrition once per month.

× time interaction: $\chi^2 = 12.8$, $df = 2$, $P = 0.0017$; Table 1; Fig. 3A) and were likely driven by the increase in average photosynthetic rates between the 36- and 74-day measurements, for plants grown at ambient [CO$_2$] (Fig. 3A; Table A2).

3.3.2. Stomatal conductance

Stomatal conductance was observed to be generally very high with an overall average of 0.41 mol m^{-2} s^{-1} and ranging between 0.26 – 0.53 mol m^{-2} s^{-1} (Fig. 3B; Table A2). There was a significant effect of treatment on stomatal conductance ($\chi^2 = 17.8$, $df = 2$, $P = 0.0001$;

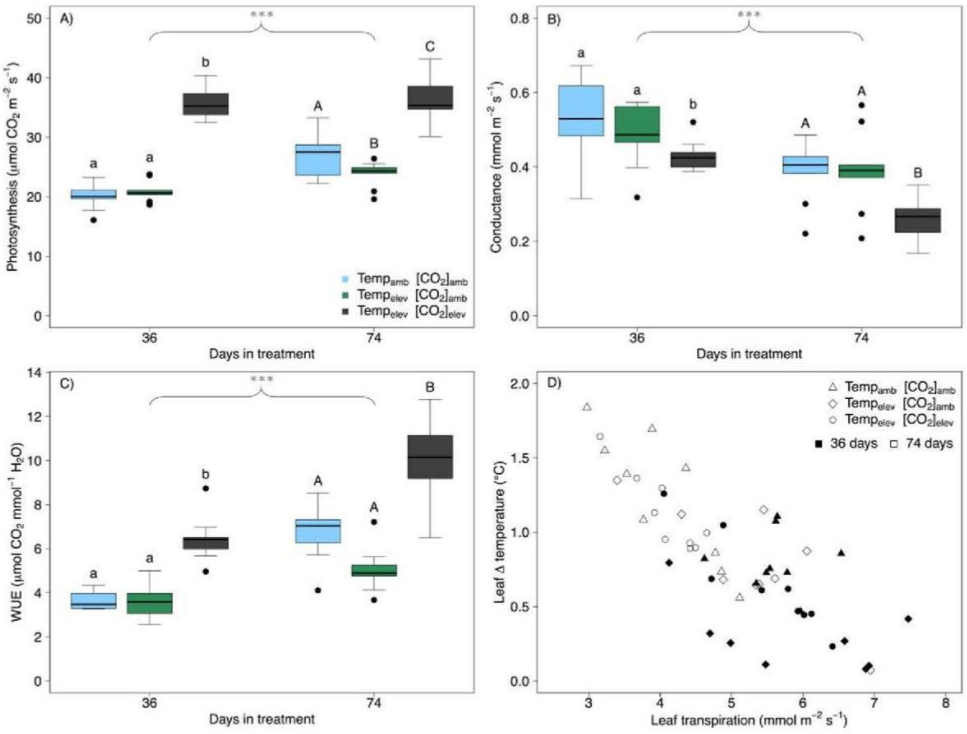

Fig. 3. Mean (± SE) A) Photosynthesis, B) stomatal conductance, C) instantaneous water use efficiency and D) correlation between leaf **Δ** temperature and leaf transpiration for young cassava plants grown in the three experimental treatments for up to 74 days. Measurements were made on $n = 18$ plants per treatment with $n = 9$ plants destructively harvested at each 36 and 74 days in treatment, respectively. Pairwise comparisons were conducted among means within, but not between harvest dates. Therefore, means with different letters of the same case represent significant differences between treatments at an alpha level of $P < 0.05$ (Tukey's HSD tests). Gas exchange measurements were made at a photon flux of 1500 µmol m^{-2} s^{-1}, supplied by an artificial LED light source (Li-Cor Inc. Lincoln, NE, USA) and with the temperature and CO$_2$ environment within the leaf cuvette set to reflect the typical midday greenhouse chamber (treatment) conditions. Significant differences between harvest dates (time) indicated by asterisks. All plants were watered to field capacity daily and provided mineral nutrition once per month.

Table 1; Fig. 3B), with plants grown and measured at elevated [CO_2] having significantly lower stomatal conductance, as compared to the plants grown and measured at ambient [CO_2] (Fig. 3B). There was also a significant effect of sampling date (time) on stomatal conductance ($\chi^2 = 29.05$, $df = 1$, $P < 0.0001$; Table 1; Fig. 3B), whereby mean stomatal conductance declined at the 74-day harvest (Table A2). However, the effects of sampling date were consistent within treatments, i.e., the treatment × time interaction was not significant ($\chi^2 = 2.3$, $df = 2$, $P = 0.3099$; Table 1; Fig. 3B).

3.3.3. *Instantaneous water use efficiency (WUE)*

There was a significant effect of treatment on instantaneous WUE ($\chi^2 = 57.2$, $df = 2$, $P < 0.0001$; Table 1; Fig. 3C) at both sampling dates. Plants grown and measured at elevated [CO_2] had significantly higher WUE compared to plants grown and measured at ambient [CO_2] (Fig. 3C; Table A4), driven by the higher photosynthetic rates and the lower conductance observed in plants grown at elevated [CO_2] (Fig. 3B; Tables A2 and A4). A significant effect of sampling date (time) on plant WUE ($\chi^2 = 45.4$, $df = 1$, $P < 0.0001$; Table 1; Fig. 3C) was also detected, where mean WUE across all treatments increased at the 74-day harvest (Table A2). Moreover, the effects of treatment on plant WUE were found to vary across time, as shown by the significant treatment × time interaction ($\chi^2 = 12.1$, $df = 2$, $P = 0.0023$; Table 1; Fig. 3C), likely driven by the increase in average WUE between the 36- and 74-day measurements for plants grown at ambient conditions (Fig. 3C; Table A2).

3.3.4. *Leaf* **Δ** *temperature and transpiration*

Elevated temperature alone led to significantly lower leaf **Δ** temperature values ($\chi^2 = 18.6$, $df = 2$, $P < 0.0001$; Table 1; Fig. 3D; Table A2). Sampling date (time) also had a significant effect of on leaf **Δ** temperature ($\chi^2 = 25.5$, $df = 1$, $P < 0.0001$; Table 1; Fig. 3D), whereby mean leaf **Δ** temperature increased at the 74-day harvest (Table A2), consistent with a generally lower conductance on this date. The effects of sampling date were consistent within treatments, as shown by the non-significant treatment × time interaction ($\chi^2 = 0.1$, $df = 2$, $P = 0.9388$; Table 1; Fig. 3D). When leaf **Δ** temperature values were plotted against leaf transpiration rates (Fig. 3D), it was observed that while leaf temperature was always higher than the cuvette air temperature, there was a strong negative correlation between leaf **Δ** temperature and leaf transpiration, as shown by a Pearson correlation coefficient value of −0.8 ($t = -9.6$, $df = 51$, $P < 0.0001$; Fig. 3D). This correlation shows reductions in leaf **Δ** temperature values with increasing transpiration rates (Fig. 3D), or inversely an increase in leaf **Δ** temperature with decreased transpiration rates as a result of elevated [CO_2] and altered stomatal conductance (Fig. 3B).

3.4. *Defence*

3.4.1. *Leaf and tuber cyanogenic glucosides*

There was an overall significant effect of treatment on both leaf ($\chi^2 = 8.2$, $df = 2$, $P = 0.0164$; Table 1; Fig. 4A) and tuber ($\chi^2 = 14.4$, $df = 2$, $P = 0.0008$; Table 1; Fig. 4B) cyanogenic glucoside concentration. Plants grown at elevated temperature had significantly lower leaf cyanogenic glucoside concentrations than the plants grown at ambient temperature (Fig. 4A; Table A4) with no significant differences in leaf cyanogenic glucoside concentration detected as result of elevated [CO_2] (Table A4). Although tuber cyanogenic glucoside concentration was similarly and significantly lower at elevated temperature as compared to ambient temperature, this was found only at ambient [CO_2] (Tables A3 and A4).

3.4.2. *Leaf nitrogen and phenolics*

Leaf nitrogen concentrations were detected to be significantly lower in plants grown at elevated [CO_2] than in plants grown at ambient [CO_2] ($\chi^2 = 17.0$, $df = 2$, $P = 0.0002$; Fig. 4C, Table A4). In contrast, relative phenolic concentration in leaf tissue was on average higher in plants grown under elevated temperature and further increased under elevated [CO_2] (Table A3), although these treatment effects were not statistically significant ($\chi^2 = 0.1$, $df = 2$, $P = 0.9536$; Table 1; Fig. 4D). Moreover, there was no significant effect of treatment on leaf phenolic diversity ($\chi^2 = 1.4$, $df = 2$, $P = 0.5081$; Table 1).

4. Discussion

Here, the response of young cassava plants to the interactive effects of elevated temperature and [CO_2] is reported for the first time. Unlike the initial hypothesis, there was no significant negative effect of elevated temperature on cassava growth and biomass accumulation. However, although elevated temperature alone led to significant increases in plant height, it was only with the simultaneous addition of elevated [CO_2] that an increase in whole-plant dry mass was observed. This increase in biomass likely resulted from the enhanced photosynthesis observed at elevated [CO_2].

For the cultivar used here (cv. MAus7) under typical field conditions, tubers are generally initiated within 45–90 days of planting (Fukai et al., 1984) with the main period of tuber expansion beginning between 90–120 days after planting (Wholey and Cock, 1974; Boerboom, 1978). Thus, at both harvests tuber initiation and their expansion were likely in the initial stages. Despite this, all plants had initiated at least one tuber at the 36-day harvest, with plants grown at elevated [CO_2] having significantly more tubers at both 36 and 74 days than plants grown at ambient [CO_2].

Regarding tuber expansion, a significant increase in tuber dry mass at 36 days for plants grown at elevated temperature, as compared to ambient conditions, was detected that was further increased (although not significantly) when supplemented with elevated [CO_2]. Although these trends had dissipated at the 74-day harvest, tuber dry mass was still, on average, highest at elevated temperature and [CO_2]. These results support Cruz et al. (2016), who showed a significant increase in tuber mass from elevated [CO_2]. The fact that the lowest average tuber mass in this study was observed at elevated temperature at the final harvest could suggest possible negative effects of elevated temperature on tuber expansion that may be ameliorated with simultaneous elevated [CO_2].

Despite the early harvest and limited tuber growth, the observed initiation of additional tuberous roots at elevated temperature and [CO_2] may lead to higher final yields (Pellet and El-Sharkawy, 1993; Rosenthal et al., 2012; Cruz et al., 2016). Cassava genotypes that initiate fewer than nine tuberous roots per plant are 'sink' limited (Cock et al., 1979). Therefore, increases in sink capacity by increased tuber initiation at elevated temperature and [CO_2] may drive photosynthetic enhancements that lead to positive effects on yield (Pellet and El-Sharkawy, 1993; Rosenthal et al., 2012). It is possible that more rapid tuber initiation was being observed and thereby increasing yield potential, with elevated temperature and [CO_2]. However, given the probable role of plant ontogenetic shifts in resource investment towards tubers, further work examining tuber yield over a full harvest cycle would be required to confirm this.

The cyanogenic glucoside concentration in tubers, as well as leaf material, was significantly impacted by growing conditions. On a dry mass basis, cassava tubers are generally reported to contain cyanide levels in the range of 0.01–0.5 mg g^{-1} (Wobeto et al., 2007). Cassava leaves often contain higher cyanide concentrations than tubers, in the range of 0.05–1.3 mg g^{-1} (Wobeto et al., 2007; Latif and Müller, 2015), with variation dependent upon the cultivar, soil composition, environmental conditions and plant age (Burns et al., 2012b; Panghal et al., 2019). In the current research, significantly reduced tuber cyanide concentrations at elevated temperature alone were detected, as compared to that of plants grown at ambient conditions. When at elevated temperatures and [CO_2], the average tuber cyanide concentration increased to levels similar to, but not significantly different from,

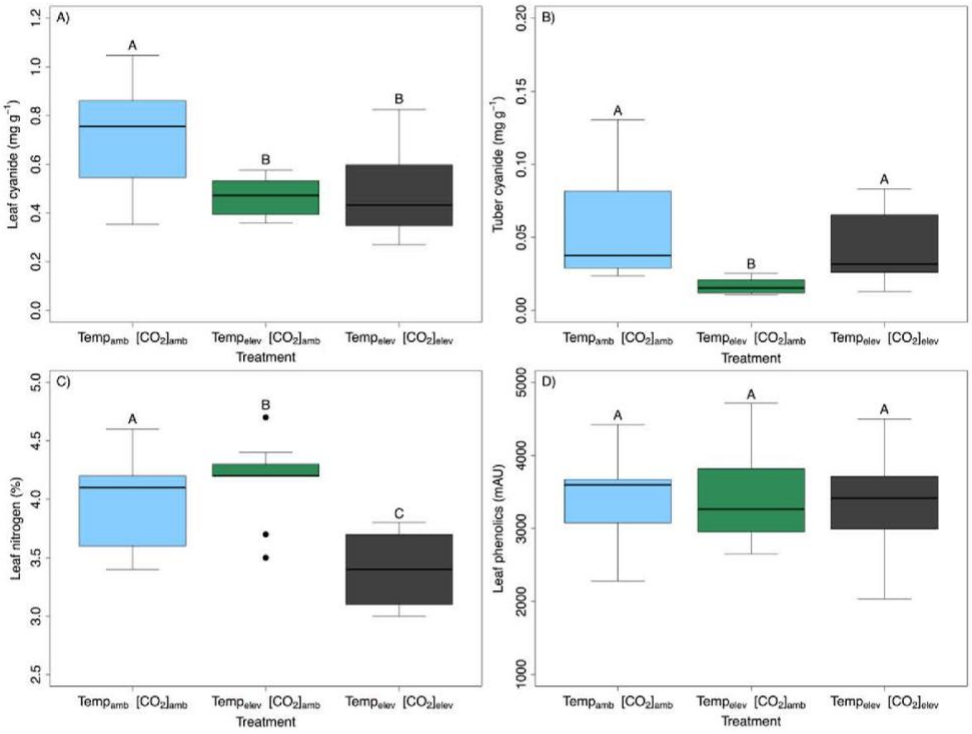

Fig. 4. Mean (± SE) plant defence measurements for young cassava plants grown in three experimental treatments for 74 days. Measurements were made on $n = 9$ plants per treatment. A) leaf cyanide (representing cyanogenic glucoside concentration, mg g^{-1}), B) tuber cyanide (representing cyanogenic glucoside concentration), C) leaf nitrogen and D) leaf phenolic concentration were measured using tissues obtained upon harvest, oven dried at 60 °C for 14 days to constant dry weight. Means with different letters and the same case represent significant differences between treatments at an alpha level of $P < 0.05$ (Tukey's HSD tests). All plants were watered to field capacity daily and provided mineral nutrition once per month.

concentrations from plants grown at ambient conditions. Similar results were observed in leaf tissues, where average leaf cyanide concentrations were significantly reduced in plants grown at elevated temperature. However, unlike tuber cyanide concentrations, cyanide concentrations of leaves at elevated temperatures did not reach levels similar to those of ambient conditions when supplemented with elevated [CO_2]. These results further support the previous findings of cyanide concentration varitation with environmental conditions.

Considering the role of cyanogenic glucosides in plant defence against herbivores and pathogens (Gleadow and Woodrow, 2002; Zagrobelny et al., 2004; Blagbrough et al., 2010), as well as the toxicity of these compounds to humans (Cliff et al., 1985; Burns et al., 2010; McKey et al., 2010), reductions in cyanogenic glucoside concentrations may be viewed as both positive (increased food quality) or negative (increased pest pressure and associated yield reductions, decreased food security) responses to climate change. In future climate scenarios where increases in air temperature are expected to be accompanied by concomitant increases in [CO_2], the results suggest an increase in the quality of cassava leaves for human (and perhaps livestock) consumption by decreased cyanide concentrations and possible reduced dependence upon processing before human consumption. This is important as cassava leaves are increasingly an important source of proteins, vitamins and micronutrients, especially within food-insecure regions (Nzwalo and Cliff, 2011; Latif and Müller, 2015). However, as with tubers, increased pest damage on above-ground biomass may also result.

Cyanogenic glucosides are nitrogen-containing secondary metabolites (Blagbrough et al., 2010; Gleadow and Møller, 2014) and can represent between 1–25 % of total leaf nitrogen content, depending on the leaf age (Gleadow et al., 1998; Miller et al., 2004). When under elevated temperatures, there was an apparent recovery of cyanogenic glucosides in tubers, and to a degree in leaves, when plants were also grown at elevated [CO_2]. This may be associated with changes in nitrogen allocation in leaves due to the partial down-regulation of photosynthetic potential (Saralabai et al., 1997; Gleadow et al., 1998) under elevated [CO_2]. Interestingly, although there was a reduction in the total leaf nitrogen at elevated [CO_2]; the significant reduction in LMA as a result of elevated temperature alone at the final harvest (as compared to ambient) and increase with additional [CO_2] (as compared to high temperature alone), led to there being no significant differences in leaf nitrogen content per unit leaf area. Reductions in leaf cyanide concentration for plants grown at elevated temperature may also have been due to dilution effects from increased leaf biomass in these treatments. Despite the high photosynthetic rates, and presumably higher availability of carbon substrates in plants grown at elevated temperature and [CO_2], there was no alteration in the quantity or diversity of leaf phenolic compounds. Further evaluations of cassava's phytochemical responses to climate changes are needed, particularly considering the relationship between leaf nutritional constituents and insect success (Lindroth et al., 1997; Cannon, 1998) and the direct losses to cassava production from pests and diseases (Herren and Neuenschwander, 1991; De Souza and Long, 2018).

The translation of results from controlled studies such as presented here, to real-world projections of crop yields at future climate change scenarios is challenging and requires consideration of other environmental factors. For example, although elevated temperature appeared to have a stimulatory effect on plant meristematic activity; this manifested itself as an 'overinvestment' in plant height and a decrease in plant structural integrity, with plants appearing slim and with an apparent reduction in stem lignification in comparison to plants grown at ambient conditions or elevated [CO_2] (*personal observation*). In natural or field situations, this insubstantial plant architecture may render plants non-competitive and more susceptible to physical damage. This may be particularly so because stems of cultivated cassava are much more brittle than those of its wild relatives (Menard et al., 2013). The dynamic interplay between higher carbon gain at elevated [CO_2] and higher carbon demand at elevated temperatures may have unforeseen and important implications when translated to the field. Plants in this study were grown under favourable, well-watered and fertilized conditions and despite the general perception that cassava is tolerant to poor soil fertility and drought; independent and interactive effects of water stress and fertilization can also influence cassava yields during early growth stages (Connor et al., 1981; De Tafur et al., 1997). The translation of our results to field conditions may help identify emerging concerns under conditions of significant climate change. For example, the physiological alterations observed in leaf function found here, such as increased WUE at elevated [CO_2], might be considered favourable to plant growth; however, these changes may cause significant heat-stress damage during extreme weather events (e.g., during acute heat waves or drought), by reducing evaporative cooling, driving higher and detrimental leaf temperatures (Ball et al., 1988; Leuzinger and Korner, 2007).

Here, the interactive effects of elevated temperature and [CO_2] on cassava are demonstrated, ranging from modified plant architecture to modified yield quantity and quality. As cassava becomes an increasingly vital crop for ensuring food security in the rapidly changing tropics (Jarvis et al., 2012), the current findings highlight the importance of evaluating both independent and interactive factors on cassava production. Future evaluations of cassava should incorporate additional factors that seek to describe the impact of extreme weather events, water and nutrient availability, and include the implications of plant chemical compounds on cassava pests and diseases (Trujillo et al., 2004; Campo et al., 2011).

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Author contributions

SJF, LAC, TDN and AWC contributed to project conception, experimental design, conducting experiments and data collection and interpretation; TDN provided statistical expertise and SJF performed the data analysis; SJF, LAC, TDN, RMG, SL and AWC were involved in drafting of the article with LAC and AWC providing final article approval and technical & logistical support; SJF and SL obtained project funding.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A

See Table A1.

Table A1
Day and night time chamber conditions.

	Day			Night		
Treatment	Temp °C	RH %	[CO_2] ppm	Temp °C	RH %	[CO_2] ppm
$Temp_{amb}$ [CO_2]$_{amb}$	29.6 ± 1.5	81.2 ± 3.6	435.5 ± 20.3	24.3 ± 1.0	88.5 ± 1.5	510.5 ± 31.5
$Temp_{elev}$ [CO_2]$_{amb}$	33.2 ± 1.5	83.5 ± 4.58	429.5 ± 15.2	27.7 ± 1.0	90.1 ± 2.4	505.9 ± 23.5
$Temp_{elev}$ [CO_2]$_{elev}$	33.2 ± 1.6	83.7 ± 4.9	789.7 ± 15.8	27.7 ± 1.0	91.2 ± 3.0	800.0 ± 17.0

Values are given as means (± SD) of records taken every five minutes across the 74-day experiment. Values are averaged by Day, (07:00–17:00) and night (17:00–07:00) time periods.
Temp, temperature; RH, relative humidity and [CO_2], atmospheric carbon dioxide concentration inside the chamber. All plants were watered to field capacity daily.

Table A2
Gas exchange measurements for cassava plants at two harvest dates.

	Ambient temperature		Elevated temperature			
	Ambient [CO_2]		Ambient [CO_2]		Elevated [CO_2]	
Measurement	36 days	74 days	36 days	74 days	36 days	74 days
Photosynthesis (*A*)	19.92 ± 0.69	27.22 ± 1.12	20.99 ± 0.58	23.85 ± 0.78	35.60 ± 0.91	36.18 ± 1.33
Stomatal conductance (mol m^{-2} s^{-1})	0.53 ± 0.04	0.39 ± 0.03	0.48 ± 0.03	0.39 ± 0.04	0.43 ± 0.01	0.26 ± 0.02
Intracellular CO_2 (*Ci*)	296.0 ± 2.8	212.5 ± 5.9	283.1 ± 4.6	232.9 ± 11.8	586.2 ± 4.1	436.8 ± 24.2
Leaf transpiration (*E*)	5.48 ± 0.26	4.09 ± 0.16	5.96 ± 0.39	5.26 ± 0.36	5.62 ± 0.17	4.21 ± 0.25
Leaf temperature (*Tleaf*)	32.79 ± 0.12	33.28 ± 0.10	36.52 ± 0.10	37.52 ± 0.15	37.14 ± 0.09	38.77 ± 0.16
Air temperature (*Tair*)	32.79 ± 0.12	32.16 ± 0.02	36.42 ± 0.09	36.71 ± 0.13	37.14 ± 0.09	37.54 ± 0.08
Vapour pressure deficit (kPa)	1.19 ± 0.03	1.24 ± 0.04	1.37 ± 0.06	1.52 ± 0.05	1.43 ± 0.05	1.76 ± 0.06
Ci/Ca	0.79 ± 0.01	0.58 ± 0.02	0.76 ± 0.01	0.63 ± 0.03	0.78 ± 0.01	0.58 ± 0.03

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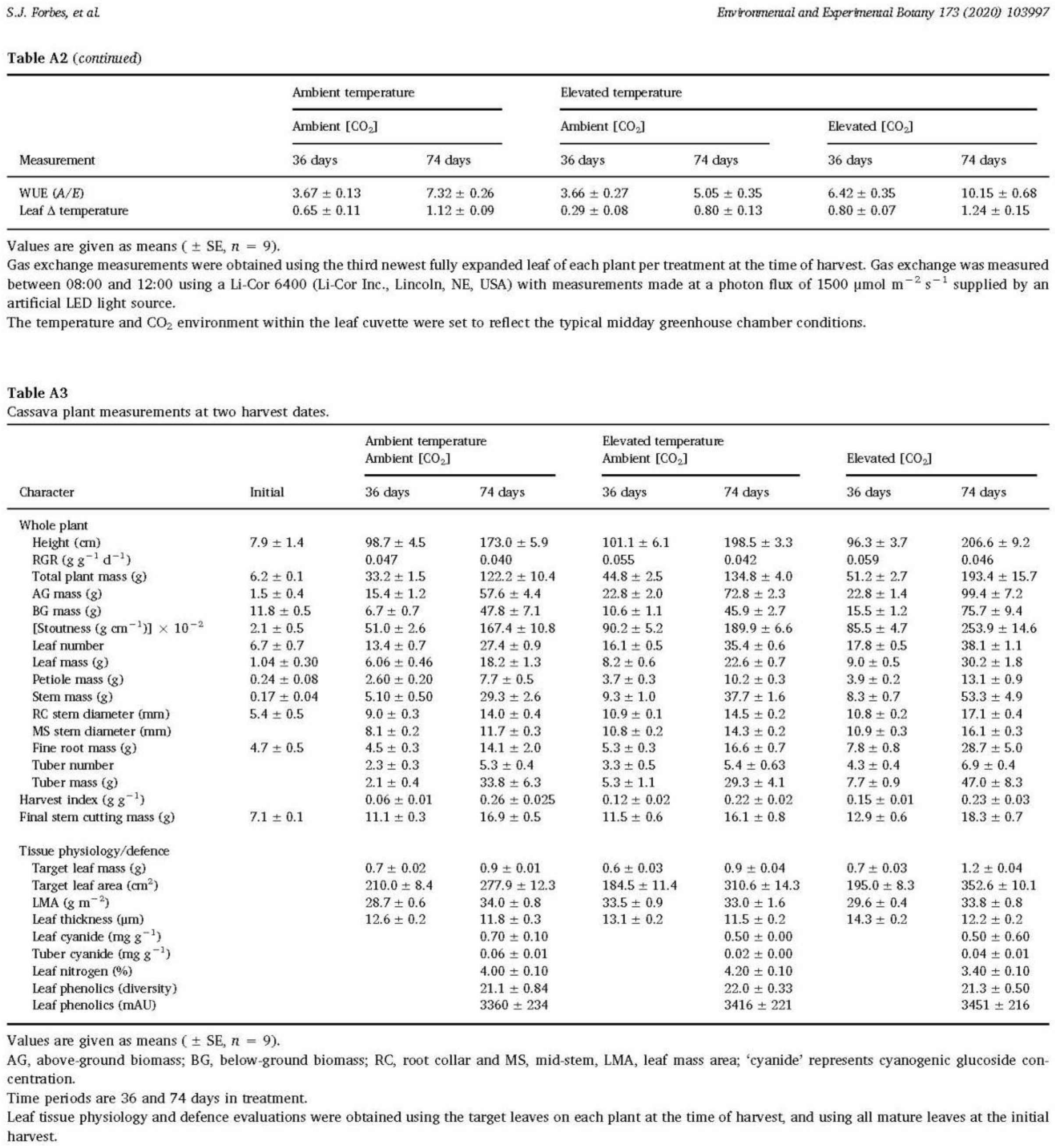

Table A2 (*continued*)

	Ambient temperature		Elevated temperature			
	Ambient [CO_2]		Ambient [CO_2]		Elevated [CO_2]	
Measurement	36 days	74 days	36 days	74 days	36 days	74 days
WUE (*A*/*E*)	3.67 ± 0.13	7.32 ± 0.26	3.66 ± 0.27	5.05 ± 0.35	6.42 ± 0.35	10.15 ± 0.68
Leaf Δ temperature	0.65 ± 0.11	1.12 ± 0.09	0.29 ± 0.08	0.80 ± 0.13	0.80 ± 0.07	1.24 ± 0.15

Values are given as means (± SE, $n = 9$).

Gas exchange measurements were obtained using the third newest fully expanded leaf of each plant per treatment at the time of harvest. Gas exchange was measured between 08:00 and 12:00 using a Li-Cor 6400 (Li-Cor Inc., Lincoln, NE, USA) with measurements made at a photon flux of 1500 μmol m^{-2} s^{-1} supplied by an artificial LED light source.

The temperature and CO_2 environment within the leaf cuvette were set to reflect the typical midday greenhouse chamber conditions.

Table A3

Cassava plant measurements at two harvest dates.

		Ambient temperature		Elevated temperature			
		Ambient [CO_2]		Ambient [CO_2]		Elevated [CO_2]	
Character	Initial	36 days	74 days	36 days	74 days	36 days	74 days
Whole plant							
Height (cm)	7.9 ± 1.4	98.7 ± 4.5	173.0 ± 5.9	101.1 ± 6.1	198.5 ± 3.3	96.3 ± 3.7	206.6 ± 9.2
RGR (g g^{-1} d^{-1})		0.047	0.040	0.055	0.042	0.059	0.046
Total plant mass (g)	6.2 ± 0.1	33.2 ± 1.5	122.2 ± 10.4	44.8 ± 2.5	134.8 ± 4.0	51.2 ± 2.7	193.4 ± 15.7
AG mass (g)	1.5 ± 0.4	15.4 ± 1.2	57.6 ± 4.4	22.8 ± 2.0	72.8 ± 2.3	22.8 ± 1.4	99.4 ± 7.2
BG mass (g)	11.8 ± 0.5	6.7 ± 0.7	47.8 ± 7.1	10.6 ± 1.1	45.9 ± 2.7	15.5 ± 1.2	75.7 ± 9.4
[Stoutness (g cm^{-1})] × 10^{-2}	2.1 ± 0.5	51.0 ± 2.6	167.4 ± 10.8	90.2 ± 5.2	189.9 ± 6.6	85.5 ± 4.7	253.9 ± 14.6
Leaf number	6.7 ± 0.7	13.4 ± 0.7	27.4 ± 0.9	16.1 ± 0.5	35.4 ± 0.6	17.8 ± 0.5	38.1 ± 1.1
Leaf mass (g)	1.04 ± 0.30	6.06 ± 0.46	18.2 ± 1.3	8.2 ± 0.6	22.6 ± 0.7	9.0 ± 0.5	30.2 ± 1.8
Petiole mass (g)	0.24 ± 0.08	2.60 ± 0.20	7.7 ± 0.5	3.7 ± 0.3	10.2 ± 0.3	3.9 ± 0.2	13.1 ± 0.9
Stem mass (g)	0.17 ± 0.04	5.10 ± 0.50	29.3 ± 2.6	9.3 ± 1.0	37.7 ± 1.6	8.3 ± 0.7	53.3 ± 4.9
RC stem diameter (mm)	5.4 ± 0.5	9.0 ± 0.3	14.0 ± 0.4	10.9 ± 0.1	14.5 ± 0.2	10.8 ± 0.2	17.1 ± 0.4
MS stem diameter (mm)		8.1 ± 0.2	11.7 ± 0.3	10.8 ± 0.2	14.3 ± 0.2	10.9 ± 0.3	16.1 ± 0.3
Fine root mass (g)	4.7 ± 0.5	4.5 ± 0.3	14.1 ± 2.0	5.3 ± 0.3	16.6 ± 0.7	7.8 ± 0.8	28.7 ± 5.0
Tuber number		2.3 ± 0.3	5.3 ± 0.4	3.3 ± 0.5	5.4 ± 0.63	4.3 ± 0.4	6.9 ± 0.4
Tuber mass (g)		2.1 ± 0.4	33.8 ± 6.3	5.3 ± 1.1	29.3 ± 4.1	7.7 ± 0.9	47.0 ± 8.3
Harvest index (g g^{-1})		0.06 ± 0.01	0.26 ± 0.025	0.12 ± 0.02	0.22 ± 0.02	0.15 ± 0.01	0.23 ± 0.03
Final stem cutting mass (g)	7.1 ± 0.1	11.1 ± 0.3	16.9 ± 0.5	11.5 ± 0.6	16.1 ± 0.8	12.9 ± 0.6	18.3 ± 0.7
Tissue physiology/defence							
Target leaf mass (g)		0.7 ± 0.02	0.9 ± 0.01	0.6 ± 0.03	0.9 ± 0.04	0.7 ± 0.03	1.2 ± 0.04
Target leaf area (cm^2)		210.0 ± 8.4	277.9 ± 12.3	184.5 ± 11.4	310.6 ± 14.3	195.0 ± 8.3	352.6 ± 10.1
LMA (g m^{-2})		28.7 ± 0.6	34.0 ± 0.8	33.5 ± 0.9	33.0 ± 1.6	29.6 ± 0.4	33.8 ± 0.8
Leaf thickness (μm)		12.6 ± 0.2	11.8 ± 0.3	13.1 ± 0.2	11.5 ± 0.2	14.3 ± 0.2	12.2 ± 0.2
Leaf cyanide (mg g^{-1})			0.70 ± 0.10		0.50 ± 0.00		0.50 ± 0.60
Tuber cyanide (mg g^{-1})			0.06 ± 0.01		0.02 ± 0.00		0.04 ± 0.01
Leaf nitrogen (%)			4.00 ± 0.10		4.20 ± 0.10		3.40 ± 0.10
Leaf phenolics (diversity)			21.1 ± 0.84		22.0 ± 0.33		21.3 ± 0.50
Leaf phenolics (mAU)			3360 ± 234		3416 ± 221		3451 ± 216

Values are given as means (± SE, $n = 9$).

AG, above-ground biomass; BG, below-ground biomass; RC, root collar and MS, mid-stem, LMA, leaf mass area; 'cyanide' represents cyanogenic glucoside concentration.

Time periods are 36 and 74 days in treatment.

Leaf tissue physiology and defence evaluations were obtained using the target leaves on each plant at the time of harvest, and using all mature leaves at the initial harvest.

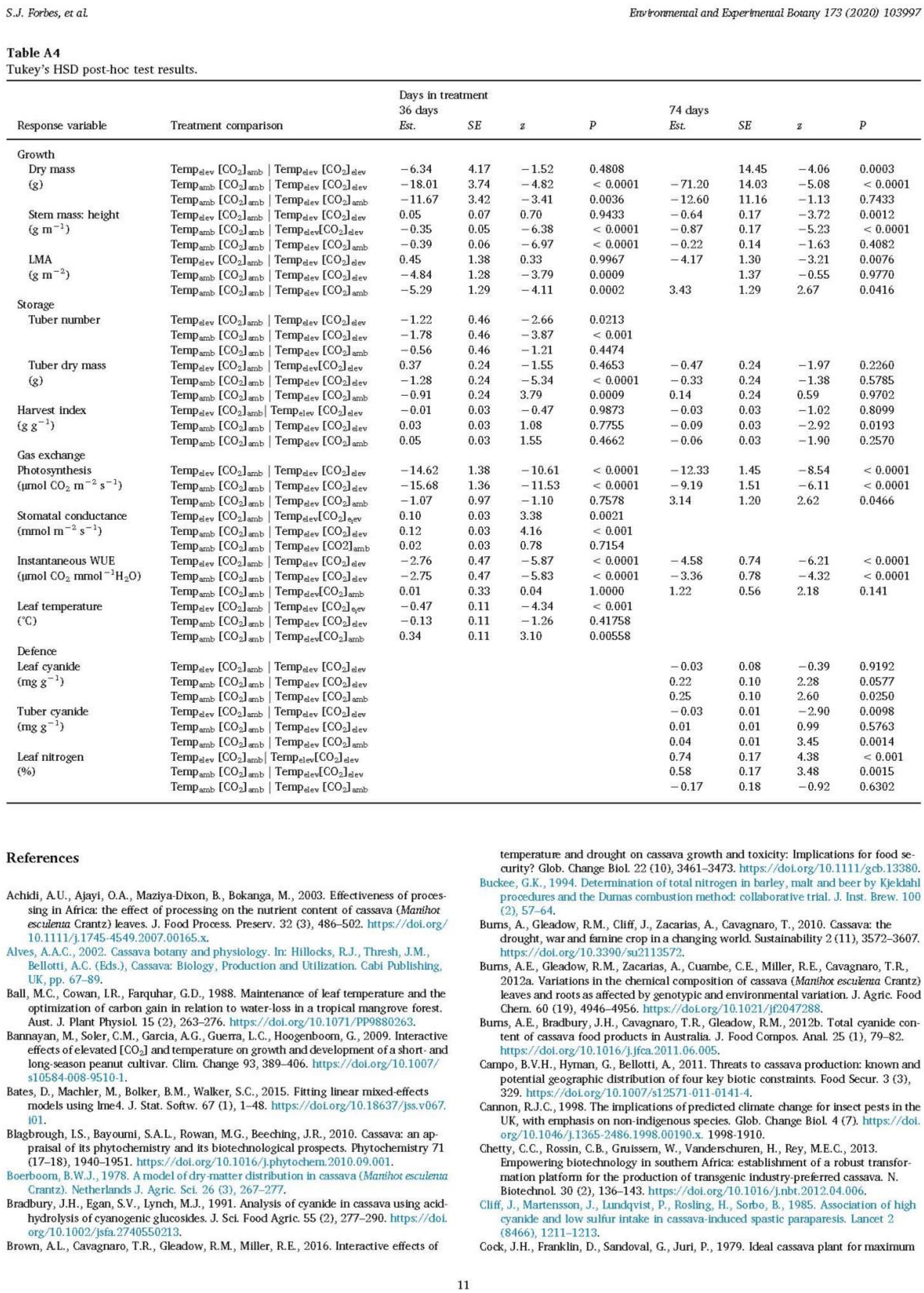

Table A4
Tukey's HSD post-hoc test results.

		Days in treatment							
		36 days				74 days			
Response variable	Treatment comparison	*Est.*	*SE*	*z*	*P*	*Est.*	*SE*	*z*	*P*
Growth									
Dry mass	$Temp_{elev}\ [CO_2]_{amb} \mid Temp_{elev}\ [CO_2]_{elev}$	−6.34	4.17	−1.52	0.4808		14.45	−4.06	0.0003
(g)	$Temp_{amb}\ [CO_2]_{amb} \mid Temp_{elev}\ [CO_2]_{elev}$	−18.01	3.74	−4.82	< 0.0001	−71.20	14.03	−5.08	< 0.0001
	$Temp_{amb}\ [CO_2]_{amb} \mid Temp_{elev}\ [CO_2]_{amb}$	−11.67	3.42	−3.41	0.0036	−12.60	11.16	−1.13	0.7433
Stem mass: height	$Temp_{elev}\ [CO_2]_{amb} \mid Temp_{elev}\ [CO_2]_{elev}$	0.05	0.07	0.70	0.9433	−0.64	0.17	−3.72	0.0012
(g m^{-1})	$Temp_{amb}\ [CO_2]_{amb} \mid Temp_{elev}[CO_2]_{elev}$	−0.35	0.05	−6.38	< 0.0001	−0.87	0.17	−5.23	< 0.0001
	$Temp_{amb}\ [CO_2]_{amb} \mid Temp_{elev}\ [CO_2]_{amb}$	−0.39	0.06	−6.97	< 0.0001	−0.22	0.14	−1.63	0.4082
LMA	$Temp_{elev}\ [CO_2]_{amb} \mid Temp_{elev}\ [CO_2]_{elev}$	0.45	1.38	0.33	0.9967	−4.17	1.30	−3.21	0.0076
(g m^{-2})	$Temp_{amb}\ [CO_2]_{amb} \mid Temp_{elev}\ [CO_2]_{elev}$	−4.84	1.28	−3.79	0.0009		1.37	−0.55	0.9770
	$Temp_{amb}\ [CO_2]_{amb} \mid Temp_{elev}\ [CO_2]_{amb}$	−5.29	1.29	−4.11	0.0002	3.43	1.29	2.67	0.0416
Storage									
Tuber number	$Temp_{elev}\ [CO_2]_{amb} \mid Temp_{elev}\ [CO_2]_{elev}$	−1.22	0.46	−2.66	0.0213				
	$Temp_{amb}\ [CO_2]_{amb} \mid Temp_{elev}\ [CO_2]_{elev}$	−1.78	0.46	−3.87	< 0.001				
	$Temp_{amb}\ [CO_2]_{amb} \mid Temp_{elev}\ [CO_2]_{amb}$	−0.56	0.46	−1.21	0.4474				
Tuber dry mass	$Temp_{elev}\ [CO_2]_{amb} \mid Temp_{elev}[CO_2]_{elev}$	0.37	0.24	−1.55	0.4653	−0.47	0.24	−1.97	0.2260
(g)	$Temp_{amb}\ [CO_2]_{amb} \mid Temp_{elev}\ [CO_2]_{elev}$	−1.28	0.24	−5.34	< 0.0001	−0.33	0.24	−1.38	0.5785
	$Temp_{amb}\ [CO_2]_{amb} \mid Temp_{elev}\ [CO_2]_{amb}$	−0.91	0.24	3.79	0.0009	0.14	0.24	0.59	0.9702
Harvest index	$Temp_{elev}\ [CO_2]_{amb} \mid Temp_{elev}\ [CO_2]_{elev}$	−0.01	0.03	−0.47	0.9873	−0.03	0.03	−1.02	0.8099
(g g^{-1})	$Temp_{amb}\ [CO_2]_{amb} \mid Temp_{elev}\ [CO_2]_{elev}$	0.03	0.03	1.08	0.7755	−0.09	0.03	−2.92	0.0193
	$Temp_{amb}\ [CO_2]_{amb} \mid Temp_{elev}\ [CO_2]_{amb}$	0.05	0.03	1.55	0.4662	−0.06	0.03	−1.90	0.2570
Gas exchange									
Photosynthesis	$Temp_{elev}\ [CO_2]_{amb} \mid Temp_{elev}\ [CO_2]_{elev}$	−14.62	1.38	−10.61	< 0.0001	−12.33	1.45	−8.54	< 0.0001
(μmol CO_2 m^{-2} s^{-1})	$Temp_{amb}\ [CO_2]_{amb} \mid Temp_{elev}\ [CO_2]_{elev}$	−15.68	1.36	−11.53	< 0.0001	−9.19	1.51	−6.11	< 0.0001
	$Temp_{amb}\ [CO_2]_{amb} \mid Temp_{elev}\ [CO_2]_{amb}$	−1.07	0.97	−1.10	0.7578	3.14	1.20	2.62	0.0466
Stomatal conductance	$Temp_{elev}\ [CO_2]_{amb} \mid Temp_{elev}[CO_2]_{e,ev}$	0.10	0.03	3.38	0.0021				
(mmol m^{-2} s^{-1})	$Temp_{amb}\ [CO_2]_{amb} \mid Temp_{elev}\ [CO_2]_{elev}$	0.12	0.03	4.16	< 0.001				
	$Temp_{amb}\ [CO_2]_{amb} \mid Temp_{elev}\ [CO2]_{amb}$	0.02	0.03	0.78	0.7154				
Instantaneous WUE	$Temp_{elev}\ [CO_2]_{amb} \mid Temp_{elev}\ [CO_2]_{elev}$	−2.76	0.47	−5.87	< 0.0001	−4.58	0.74	−6.21	< 0.0001
(μmol CO_2 $mmol^{-1}H_2O$)	$Temp_{amb}\ [CO_2]_{amb} \mid Temp_{elev}\ [CO_2]_{elev}$	−2.75	0.47	−5.83	< 0.0001	−3.36	0.78	−4.32	< 0.0001
	$Temp_{amb}\ [CO_2]_{amb} \mid Temp_{elev}[CO_2]_{amb}$	0.01	0.33	0.04	1.0000	1.22	0.56	2.18	0.141
Leaf temperature	$Temp_{elev}\ [CO_2]_{amb} \mid Temp_{elev}\ [CO_2]_{e,ev}$	−0.47	0.11	−4.34	< 0.001				
(°C)	$Temp_{amb}\ [CO_2]_{amb} \mid Temp_{elev}\ [CO_2]_{elev}$	−0.13	0.11	−1.26	0.41758				
	$Temp_{amb}\ [CO_2]_{amb} \mid Temp_{elev}[CO_2]_{amb}$	0.34	0.11	3.10	0.00558				
Defence									
Leaf cyanide	$Temp_{elev}\ [CO_2]_{amb} \mid Temp_{elev}\ [CO_2]_{elev}$					−0.03	0.08	−0.39	0.9192
(mg g^{-1})	$Temp_{amb}\ [CO_2]_{amb} \mid Temp_{elev}\ [CO_2]_{elev}$					0.22	0.10	2.28	0.0577
	$Temp_{amb}\ [CO_2]_{amb} \mid Temp_{elev}\ [CO_2]_{amb}$					0.25	0.10	2.60	0.0250
Tuber cyanide	$Temp_{elev}\ [CO_2]_{amb} \mid Temp_{elev}\ [CO_2]_{elev}$					−0.03	0.01	−2.90	0.0098
(mg g^{-1})	$Temp_{amb}\ [CO_2]_{amb} \mid Temp_{elev}\ [CO_2]_{elev}$					0.01	0.01	0.99	0.5763
	$Temp_{amb}\ [CO_2]_{amb} \mid Temp_{elev}\ [CO_2]_{amb}$					0.04	0.01	3.45	0.0014
Leaf nitrogen	$Temp_{elev}\ [CO_2]_{amb} \mid Temp_{elev}[CO_2]_{elev}$					0.74	0.17	4.38	< 0.001
(%)	$Temp_{amb}\ [CO_2]_{amb} \mid Temp_{elev}[CO_2]_{elev}$					0.58	0.17	3.48	0.0015
	$Temp_{amb}\ [CO_2]_{amb} \mid Temp_{elev}\ [CO_2]_{amb}$					−0.17	0.18	−0.92	0.6302

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Chapter 3. Elevated Temperature and Carbon Dioxide alters Growth and Leaf Phytochemistry in Two Important Tropical Crops, Coffee (*Coffea arabica*) and Cacao (*Theobroma cacao*)

3.1 Abstract

Contemporary climate change is likely to affect agronomic productivity across the globe. In the tropics this will include both coffee (*Coffea* L. spp.) and cacao (*Theobroma cacao* L.) production, impacting the millions of vulnerable farmers whose main economic and social livelihoods depend on proceeds from the production of these crops. While research has demonstrated the influence of elevated temperature on plant metabolism and stress, as well as the effects of increasing CO_2 concentrations ([CO_2]) on photosynthetic rates, interactive effects of these two important drivers are less explored. This simultaneous evaluation is important given their potentially contrasting effects on plant growth, physiology, and biochemistry. In a climate-controlled glasshouse, I investigated the combined effects of increased temperature and [CO_2] on the growth, physiology and phytochemistry of coffee and cacao seedlings. For coffee, increasing temperature led to an increase in biomass when considering non-extreme temperatures and non-limiting soil moisture. An increase in biomass was also found for cacao under the same experimental conditions. Increased [CO_2] helped cacao seedlings moderate temperature-mediated effects on stomatal conductance and instantaneous water-use efficiency (WUE). Overall improvements in photosynthesis (A_{sat}) and instantaneous WUE were observed for both species. Leaf chemistry was altered differentially for the two species, potentially because of reallocation of nutrients towards secondary metabolite production in coffee, or by dilution under enhanced biomass production in cacao.

3.2 Introduction

Climate change is impacting agricultural systems and communities in every region across the globe with the largest impacts projected to be experienced in tropical and subtropical regions, particularly the countries of tropical Africa and Asia (Springmann et al., 2016). Elevated temperature and increasing atmospheric carbon dioxide concentrations ([CO_2]) are two major factors of climate change that directly affect global agricultural crop production (Taub, 2010; Wheeler and Von Braun, 2013; DaMatta et al., 2019; IPCC, 2023). For example, the biophysically suitable geographic ranges for species cultivation (Porter and Semenov, 2005), the trophic interactions occurring within agroecosystems (Ali et al., 2014), and plant growth, physiology, productivity and quality (Schmidhuber and Tubiello, 2007; DaMatta et al., 2010; Myers et al., 2017) are some of many important agricultural facets that can be altered by such climate factors. While moderate warming has been shown to have positive impacts on crop yields in more temperate regions (Supit et al., 2010), higher temperatures in tropical systems can cause negative physiological, biochemical, and molecular impacts, affecting key biological processes, such as photosynthesis and yield (Salvucci and Crafts-Brandner, 2004; Hikosaka et al., 2006; Sage and Kubien, 2007). Even slight global surface air temperature increases in the range of 1–2°C are predicted to severely decrease major staple crop yields in tropical regions by the mid-to-late 21st century (Lobell et al., 2008; Asseng et al., 2011; Tatsumi et al., 2011; Rosenzweig et al., 2014; Zhao et al., 2017). This could be potentially exacerbated by concomitant and increasingly variable climate factors (e.g., heat waves, drought, intensified rainfall) (Collier et al., 2008; Powell and Reinhard, 2016; Beillouin et al., 2020). On the other hand, positive stimulatory effects of increasing [CO_2] on leaf photosynthesis with subsequent benefits to crop physiology, morphology and adaptation to climate change are widely reported (Drake et al., 1997; Kimball et al., 2002; Long et al., 2004; Bannayan et al., 2009; Rosenthal et al., 2012; Kirkham, 2016). Increases in [CO_2] can lead to improved water use efficiency (Liu et al., 2019), reallocation of carbon and nitrogen resources among plant organs, and changes to the secondary metabolite concentrations of plant tissues (Salazar-Parra et al., 2015). Short-term stimulatory effects of increasing [CO_2] on plant primary productivity may, at least partially, offset the predicted negative effects of future rises in temperature on crop growth and yield (Lobell and Field, 2007; Lobell et al., 2008). It is unclear if this stimulatory effect is consistently maintained over time or if leaf-level acclimation and mechanisms such as progressive nitrogen limitation may moderate the positive influence of elevated [CO_2] under future climate change scenarios (Tausz-Posch et al., 2020).

Coffee (*Coffea* L. spp.) and cacao (*Theobroma cacao* L.) are two of the world's most economically important perennial fruit tree crops (Edwin and Masters, 2005; Davis et al., 2012) . Together, they provide the economic basis of livelihoods for over 30 million smallholder farmers, predominantly in developing tropical countries that are recognized as more vulnerable to the effects of climate change (DaMatta and Ramalho, 2006; Davis et al., 2012; Ovalle-Rivera et al., 2015; Lahive et al., 2019). *Coffea arabica* L. (hereafter referred to as 'coffee') is an upland adapted species, originating in the Afromontane rainforests of the Ethiopian uplands (Anthony et al., 2002), and which thrives in annual mean temperatures between 18 and 23°C (DaMatta and Ramalho, 2006). Cacao is a lowland species, native to the Amazon basin (Motamayor et al., 2002) and prefers a slightly broader annual mean temperature range between 18 and 34°C (Lahive et al., 2019). Despite their differing origins and native elevational ranges, both species are now cultivated and bred throughout the humid tropical, subtropical, and arid-subtropical regions of the globe. Consequently, the climates under which cultivated plants are grown may exceed the range of climates experienced by plants in the native range and therefore climate change may pose a threat to future growth and production. This even in the context of advancements in selective breeding for broader environmental tolerance, as barriers to adoption of such advanced planting materials often exist in both coffee (Akenroye et al., 2021) and cacao (Alemagi et al., 2014).

It is also important to consider changes in plant chemical (minerals and metabolites) composition under a changing climate. Alterations to plant chemical composition can have important implications for factors such as plant developmental progression (De-la-Cruz Chacón et al., 2013), reproduction (Endara et al., 2022), herbivory (Iason, 2005), responses to environmental changes (Jay-

Allemand et al., 2015), and both animal and human nutrition (Acamovic and Brooker, 2005; Blank et al., 2011). For example, increased temperature may alter concentrations of Chlorogenic acid (CGA) in coffee leaves and fruits (Ahmed et al., 2021). CGA is known to modulate the feeding behaviour of herbivorous insects (Kundu and Vadassery, 2019) and contribute to beverage sensory attributes (dos Santos Scholz et al., 2018), therefore alterations in the concentrations of this compound may impact herbivory and coffee beverage quality (Barbosa et al., 2019).

Here, I present the results of a controlled glasshouse experiment that examined the effects and possible interactions of elevated temperature and increased [CO_2] on seedlings of coffee and cacao. I hypothesised deleterious effects of elevated temperature on plant growth and gas exchange in both species, with potential benefits of increased [CO_2] to growth and leaf chemistry. Specifically, I sought to answer the following questions: can elevated [CO_2] can mitigate any potentially deleterious effects of elevated temperature on plant growth and leaf-level gas exchange? and does enhanced plant growth under elevated temperature and [CO_2] alter leaf chemistry.

3.3 Materials and Methods

3.3.1 *Planting Material and Growing Conditions*

Coffee (*Coffea arabica* L., Rubiaceae, cv. Bourbon) saplings (*n* = 100, approximately 3 months old), grown in plastic planting tubes (50 x 50 mm at top, 120 mm tall, 250 ml volume) were obtained from the Skybury nursery in the Atherton Tablelands. The saplings were grown from seeds planted in late August 2016 and had received only water during their development. Sixty coffee saplings, selected for physical homogeneity, were transplanted into 25 L pots on 3 December 2016, and each provided with 500 mL of liquid seaweed fertilizer (Seasol®) to reduce possible transplanting shock. Cacao (*Theobroma cacao* L., Malvaceae) seeds were obtained from the Goodman Cacao Estate in Killaloe, North Queensland, Australia. Ten ripe cacao pods were harvested from a single cacao tree (SG2 hybrid variety, Papua New Guinea origin), the seeds were extracted, de-pulped, and primed in water for 24 hours. Large, round and healthy seeds were planted into 25 L pots (*n* = 60) on 6 December 2016, with five seeds, spaced evenly, per pot. All the planted cacao seeds had germinated 10 days after planting (DAP). At 14 DAP, four of the five cacao saplings per pot were removed by harvesting at the root collar, producing a homogeneous subset of saplings (*n* = 60) for experimental trial. A low level of genetic variation in both species was used to maintain some control over genetic variation between plants and their responses under the experimental comparisons. The pots used for both coffee and cacao saplings were free-draining black plastic pots (30 cm diameter, 50 cm depth), each filled with a 10:1 (w/w) soil and perlite mix. The soil was a potting mix obtained from a local landscaping company and a 25mm layer of river stones was placed in the base of each pot to assist drainage. Coffee and cacao saplings were maintained in a shade house under 75% shade and ambient [CO_2] until being moved into the glasshouse to initiate the experiment.

3.3.2 *Glasshouse Facility and Chamber Treatments*

This study was conducted in a climate-controlled glasshouse facility, located at the James Cook University's Environmental Research Complex, Cairns, Australia. The glasshouse is divided into three independently regulated and parallel growth chambers each 5(L) × 3(W) × 4(H) m. The chambers are arranged in a N-S orientation, with independent temperature and [CO_2] regulation, receiving natural sunlight at about 50 % full sunlight, due to SOLARO 5220 D O FB climate screening (Ludvig Svensson Inc.). Fifty-four seedlings per species were translocated into the glasshouse on 8 December 2016 for coffee, and on 12 December 2016 for cacao. For each species, the seedlings were randomly allocated to one of three chambers (i.e., eighteen seedlings per species per chamber).

To quantify the effects and potential interactive effects of elevated temperature and [CO_2] on the seedlings of coffee and cacao, I subjected seedlings to one of three treatments. In the first treatment (i.e., $Temp_{amb}$ $[CO_2]_{amb}$), air temperatures were set to track the ambient temperature conditions of Cairns, North Queensland and [CO_2] was set to 400 ppm. In the second treatment, air temperatures were set to track ambient + 4°C and [CO_2] was set to 400 ppm (i.e., $Temp_{elev}$ $[CO_2]_{amb}$). In the third treatment, air temperatures were set to + 4°C above the ambient, and [CO_2] was set to 800 ppm (i.e., $Temp_{elev}$ $[CO_2]_{elev}$). Seedlings and their associated treatments were rotated between chambers monthly to reduce possible chamber effects unrelated to the imposed treatments. All seedlings were watered daily to field capacity for the duration of the experiment and given 20 g of slow-release granular fertilizer (Scotts Osmocote® Plus Trace Elements: Native Gardens) once per month. To improve micronutrient availability, a one-off application of micronutrient foliar spray (Manutec Trace Elements) was applied to all cacao seedlings.

Chamber conditions were monitored with sensors (Temp/ RH: QFM2160, Siemens, Bayswater, VIC, Australia; [CO_2]: GMP222, Vaisala, Helsinki, Finland), and regulated through a feedback control system implemented in the building management software. Air temperature (°C), [CO_2] (ppm) and relative humidity (%) were recorded every 5 minutes during the experiment (Supplementary Table 1).

3.3.3 *Seedling Growth Measurements, Harvesting Procedure and Tissue Sampling*

For coffee and cacao, six seedlings were destructively harvested prior to the initiation of the experimental treatments (initial harvest, 8 and 12 December 2016, respectively) to determine initial plant biomass and leaf area. After growth in treatment conditions, nine seedlings per species and per treatment were destructively harvested on two harvesting events on 07 – 08 March (first harvest) and 24 – 25 April 2017 (final harvest). During the experiment, stem height (root collar to apical meristem, mm) and the number of branches (coffee only) were evaluated weekly until the first harvest. Upon destructive harvest, each seedling was separated into cotyledons (cacao only), leaves, petioles (cacao only, coffee leaves had petioles included), main stems, branches, and roots. The harvesting procedure was to first remove the above-ground biomass by cutting the stem at the root collar. The leaves (and their petioles) were then detached and classified as either fully expanded, immature or senescent, before petiole removal (cacao only). Expanded leaves were defined as leaves that were fully developed and contributing to photosynthesis whilst immature leaves were young, small, soft leaves that were typically light green (coffee) or translucent pink/light green (cacao) and still expanding. After leaf and petiole removal, the remainder of the above-ground biomass was separated into branch and stem tissues. The below ground root systems were obtained by gently washing the roots free from all soil with water before processing for fresh and dry mass. All tissue types (cotyledons, leaves, petioles, main stems, branches and roots) were oven dried at 60°C for 14 days before being re-weighed for dry mass (g).

Leaves used for gas exchange measurements (see gas exchange below) were processed separately to determine leaf morphological and biophysical characteristics. Leaf thickness (µm) was measured on whole leaves before determination of total lamina area (cm^2) using a scanner (Canon CanoScan Flatbed Scanner LiDE120) and image processing in Image-J 1.52a Software (Schneider et al., 2012). Leaf mass per unit area (LMA, $g\ m^{-2}$) was calculated after weighing the dried leaf tissue, with this oven-dried material also used for subsequent nutrient and phenolic content analysis.

3.3.4 *Seedling Growth Indices*

The total dry biomass (g) of seedlings was calculated as the combined dry mass (g) of all harvested tissues (leaves, petioles, main stems, branches and roots but excluding senesced leaves and cotyledons). The relative growth rate (RGR, $g\ g^{-1}\ d^{-1}$) of seedlings across time was calculated (See Eq. 3.1) (Hoffmann and Poorter, 2002), where m_2 and m_1 represent the population average dry biomass at the final and initial harvests, respectively, and where t represents the number of days between harvests (Supplementary Table 2, 3).

Eq. 3.1.

$$\mathrm{RGR} = \frac{\overline{\ln(m_2)} - \overline{\ln\,(m_1)}}{t}$$

3.3.5 *Gas Exchange Measurements*

Leaf gas exchange measurements were conducted prior to the final harvest, using the third newest fully expanded leaf of each seedling (n = 9), hereafter referred to as the ‘target’ leaves. Gas exchange was measured between 08:00 and 12:00 using a portable photosynthesis system (Li-Cor 6400, Li-Cor Inc., Lincoln, NE, USA) with Licor-cuvette conditions set to mimic typical midday glasshouse chamber temperature and [CO_2] of each treatment (i.e., $Temp_{amb}$ 32 °C and $Temp_{elev}$ 36 °C). Measurements were made at a photon flux of 1500 µmol $m^{-2}\ s^{-1}$, supplied by an artificial LED light source (6400-02B, Li-Cor Inc.), with leaf vapour pressure deficit maintained between 1.46 and

2.36 kPa. Leaf temperatures during the gas exchange measurements for both species were between 33.4°C and 34.7°C for the Temp_{amb} treatment and between 35.4°C and 41.0°C for the $\text{Temp}_{\text{elev}}$ treatments.

3.3.6 *Leaf Phytochemistry*

I evaluated the nutrient and phenolic composition of leaf tissues of both species at the final harvest using the dried target leaves. Dried leaf tissue was ground using a Qiagen TissueLyser II, digested using a nitric acid and peroxide microwave digest (Bergof SW-4) and analysed for macronutrients (i.e., P, K, Ca, Mg) and micronutrients (i.e., Fe, Zn; see Supplementary Table 2, 3) via Inductively Coupled Plasma Optical Emission Spectrometer ICP-OES spectroscopy (Agilent 5100, Agilent Technologies Inc.).

Nitrogen (N) and Carbon (C) concentration were determined using a Costech 4010 Elemental Analyser and following the Dumas method (Buckee, 1994). Elemental concentrations were used to calculate C:N and N:P mass ratios. Phenolic extraction and analysis of leaf phenolic diversity (i.e., the number of different phenolic compounds) and the total phenolic concentration (mAU) were conducted following procedures described by Uesugi and Kessler (2016) and with modifications as per Forbes et al., (2020) using HPLC analysis. Furthermore, two microlitres for coffee samples, and five microlitres for cacao samples, of the supernatant liquid were injected into the HPLC for analysis. The different injection volumes were standardized by dividing the peak area by the volume injected for each species and converted to absorption units per microlitre (AU μl^{-1}). Standard curves for compound quantification were unavailable therefore I interpreted absorbance (the area under the curve of each compound peak) as equivalent to concentration. Given the differing HPLC injection amounts for each species, any differences in phenolic composition between species should be interpreted with caution. The major phenolic component of the coffee phenolic profile was also identified and measured separately.

3.3.7 *Statistical Analysis*

As there were no substantial differences in analysis results and data interpretation between the first and final harvest dates, I present data obtained from the final harvest only. To evaluate the effects of elevated temperature and $[CO_2]$ on growth, gas exchange and leaf phytochemistry in establishing coffee and cacao seedlings, I applied generalized linear models using the *glm* function in the lme4 package (Bates et al., 2015) in R (Team, 2021). The models included fixed effects of each, treatment (i.e., Temp_{amb} $[CO_2]_{\text{amb}}$; $\text{Temp}_{\text{elev}}$ $[CO_2]_{\text{amb}}$; $\text{Temp}_{\text{elev}}$ $[CO_2]_{\text{elev}}$) and species (i.e., coffee, cacao), as well as a treatment × species interaction term.

The models describing stomatal conductance (g_s), LMA, phenolic concentration, and chlorogenic acid content used a normal distribution with an identity-link function to maintain the additive interaction terms when applicable (i.e., so that the interaction was not additive on a log-scale as is the case for a log-link function). For the models describing stem height, total dry biomass, branch number, branch mass, photosynthesis (A_{sat}), instantaneous WUE, phenolic diversity, leaf N, leaf P, leaf C:N ratio and leaf N:P ratio, I used a gamma distribution with a log-link function to account for the positive relationship between the mean and variance.

For all statistical models, I performed sequential likelihood ratio tests for each fixed effect and their interactions (where relevant) using the *lrtest* function in the *lmtest* package (Zeileis and Hothorn, 2002) in R (R Core Team, 2021). Where a fixed effect or interaction term was found to have a significant effect on a response variable, Tukey's HSD post-hoc tests were used to determine where these differences occurred, using the *emmeans* package (Lenth, 2016) in R (R Core Team, 2021). I conducted Tukey's HSD post-hoc tests separately for each species whenever a significant treatment × species interaction was found, as Tukey's HSD post-hoc tests on main effects are unreliable in such circumstances. Unless otherwise stated, all error bars and intervals represent standard errors.

3.4 Results

3.4.1 *Seedling Growth and Architecture*

3.4.1.1 Stem Height

For both species and across all treatments, I observed substantial increases in seedling height across time (days in treatment), with stem height generally increasing across time (Figure 3.1A, B; Supplementary Table 3.2, 3.3). Regardless of treatment, at the final harvest, coffee seedlings had grown to an average height of 73.4 ± 3.3 cm (Supplementary Table 3.2), while cacao seedlings reached a taller average height of 96.3 ± 4.8 cm (Supplementary Table 3.3).

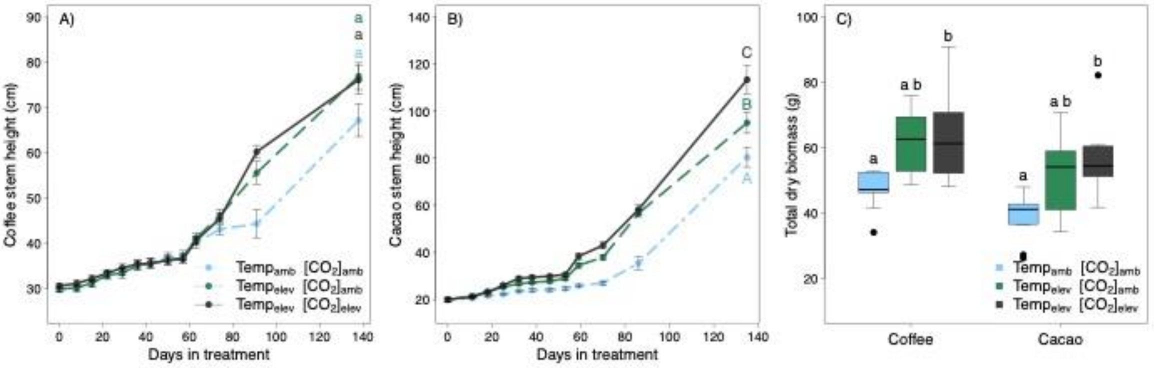


Figure 3.1. Seedling height (A, B) and C) total biomass measurements for coffee and cacao seedlings grown for ~140 days in three experimental treatments (Temp_{amb} $[\text{CO}_2]_{\text{amb}}$; $\text{Temp}_{\text{elev}}$ $[\text{CO}_2]_{\text{amb}}$; $\text{Temp}_{\text{elev}}$ $[\text{CO}_2]_{\text{elev}}$). There were $n = 18$ seedlings per species and treatment at the first harvest (91 and 75 days in treatment for coffee and cacao, respectively) and $n = 9$ seedlings per species and treatment at the final harvest. Statistical analysis was conducted on data obtained from the final harvest only. Means with different letters of the same case (e.g., a, b) represent significant differences between treatments at an

alpha level of $P < 0.05$ (Tukey's HSD tests). All seedlings were watered to field capacity daily and provided mineral nutrition once per month.

I found significant main effects of each, treatment ($\chi^2 = 20.0$, $df = 2$, $P < 0.0001$; Table 3.1) and species ($\chi^2 = 32.6$, $df = 1$, $P < 0.0001$; Table 3.1) on stem height (Figures 3.1A, B), as well as a significant two-way treatment × species interaction ($\chi^2 = 6.3$, $df = 2$, $P = 0.0420$; Table 3.1). Tukey's HSD post-hoc test indicated significant effects of treatment on stem height in cacao seedlings (Figure 3.1B), but not for coffee (Figure 3.1A). Here, while elevated temperature had a significant stimulatory effect on cacao stem height (Figure 3.1B), as compared to ambient conditions, even greater stimulation of stem height was observed when cacao seedlings were supplied with elevated [CO_2] (Figure 3.1B). Cacao seedlings grown under elevated temperature and elevated [CO_2] were, on average, 18 cm taller than those grown under elevated temperature alone, and 33 cm taller than those grown under ambient conditions (Supplementary Table 3.3).

Table 3.1. Results of likelihood ratio tests evaluating main effects and their possible interactions on the response variables describing coffee and cacao seedlings.

Response variable	**Main effect**	χ^2	*df*	***P***
Growth				
Stem height	Treatment	20.0	2	**< 0.0001**
	Species	32.6	1	**< 0.0001**
	Treatment × species	6.3	2	**0.0420**
Total dry biomass	Treatment	27.2	2	**< 0.0001**
	Species	10.9	1	**0.0009**
	Treatment × species	0.5	2	0.7904
LMA	Treatment	1.8	2	0.412
	Species	52.8	1	**< 0.0001**
	Treatment × species	1.4	2	0.4935
Branch number	Treatment	19.6	2	**< 0.0001**
Branch mass	Treatment	21.6	2	**< 0.0001**
Gas exchange				
Photosynthesis (A_{sat})	Treatment	41.3	2	**< 0.0001**
	Species	1.5	1	0.219
	Treatment × species	5.0	2	0.0814
Stomatal conductance (g_s)	Treatment	2.8	2	0.2478
	Species	6.5	1	**0.0108**
	Treatment × species	9.1	2	**0.0105**
WUE	Treatment	39.4	2	**< 0.0001**
	Species	9.6	1	**0.0019**
	Treatment × species	8.2	2	**0.0163**
Phytochemistry				
Nitrogen (N)	Treatment	5.85	2	**0.0537**
	Species	72.8	1	**< 0.0001**
	Treatment × species	6.9	2	**0.0324**
Phosphorus (P)	Treatment	19.6	2	**< 0.0001**
	Species	9.4	1	**0.0022**
	Treatment × species	1.4	2	0.4923

C:N mass ratio	Treatment	5.5	2	0.0642
	Species	65.7	1	**< 0.0001**
	Treatment × species	5.1	2	0.0773
N:P mass ratio	Treatment	7.9	2	**0.0192**
	Species	62.6	1	**< 0.0001**
	Treatment × species	4.1	2	0.1275
Phenolic diversity	Treatment	5.5	2	0.0638
	Species	92.5	1	**< 0.0001**
	Treatment × species	2.8	2	0.251
Phenolic concentration	Treatment	16.5	2	**0.0002**
	Species	127.8	1	**< 0.0001**
	Treatment × species	17.3	2	**0.0002**
Chlorogenic acid Abs.	Treatment	15.1	2	**0.0005**

[z] *P*-values were calculated using test statistics derived from the mean and variance and assumed an alpha level of $P < 0.05$.

[y] LMA, leaf mass (g) per unit area; WUE, instantaneous water use efficiency (A/E); Phenolic diversity, the number of phenolic compounds; Abs., absorbance (AU μl^{-1}).

3.4.1.2 Total Biomass and LMA

Across all treatments, coffee seedlings had an average RGR of 0.017 g g^{-1} d^{-1}, resulting in an average total seedling dry mass of 57.7 ± 3.4 g at the final harvest (Supplementary Table 3.2). For cacao, across all treatments, seedlings had an average RGR of 0.023 g g^{-1} d^{-1}, resulting in an average total whole seedling dry mass of 48.7 ± 3.4 g at the final harvest (Supplementary Table 3.3).

I found significant main effects of treatment ($\chi^2 = 27.2$, $df = 2$, $P < 0.0001$; Table 3.1) and species ($\chi^2 = 32.6$, $df = 1$, $P = 0.0009$; Table 3.1) on total dry biomass (Figure 3.1C), with no significant interaction between these main effects (Table 3.1). Tukey's HSD post-hoc test indicated significant effects of treatment on total seedling dry biomass that were similar for both species. Here, seedlings grown under the elevated temperature and elevated [CO_2] treatment had significantly greater total dry biomass than seedlings grown under ambient temperature conditions, though not significantly greater than seedlings grown under elevated temperature alone (Figure 3.1C). Across all treatments coffee seedlings had an average LMA of 65.2 ± 1.8 g m^{-2} (Supplementary Table 3.2) and cacao had lower average LMA of 50.7 ± 1.7 g m^{-2} (Supplementary Table 3.3). No effects of treatment were observed on LMA for either species (Table 3.1). Treatment differences in coffee total plant biomass, but not height, may be driven by significantly increased branch initiation (number of branches) and total branch mass (g) in coffee plants grown under elevated temperature and [CO_2], as compared to ambient conditions (Supplementary Figure 3.1).

3.4.2 *Gas Exchange*

3.4.2.1 Photosynthesis (A_{sat}), Stomatal Conductance (g_s) and Instantaneous Water-Use Efficiency (A/E)

Overall, the average A_{sat}, regardless of treatment, was found to be similar for the two species, averaging 6.5 ± 1.6 µmol CO_2 m^{-2} s^{-1} in coffee and 7.1 ± 1.6 µmol CO_2 m^{-2} s^{-1} in cacao (Figure 3.2A, B; Supplementary Table 3.4).

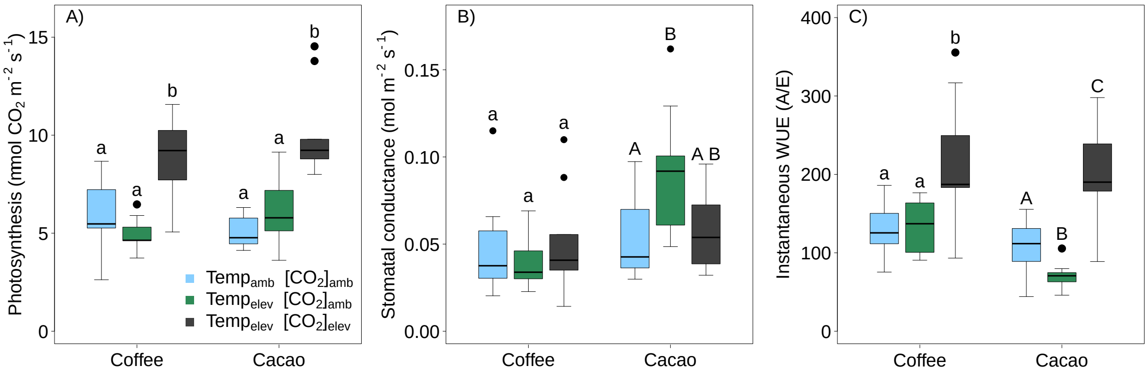


Figure 3.2. Leaf A) photosynthesis (A_{sat}), B) stomatal conductance (g_s), and C) instantaneous water use efficiency (WUE; calculated as photosynthesis (A) / leaf transpiration (E)) for coffee and cacao

seedlings grown for ~140 days in three experimental treatments (Temp$_{amb}$ [CO$_2$]$_{amb}$; Temp$_{elev}$ [CO$_2$]$_{amb}$; Temp$_{elev}$ [CO$_2$]$_{elev}$). There were $n = 9$ seedlings per species and per treatment at the final harvest. Means with different letters of the same case (e.g., a, b) represent significant differences between treatments at an alpha level of $P < 0.05$ (Tukey's HSD tests). Letters of different case (e.g., a, A) in the same panel represent separate Tukey's comparisons for each species. All seedlings were watered to field capacity daily and provided mineral nutrition once per month.

Despite no significant species main effects (Table 3.1), I found a significant main effect of treatment on A_{sat} ($\chi^2 = 41.3$, $df = 2$, $P < 0.0001$; Table 3.1) as well as a moderately significant treatment × species interaction ($\chi^2 = 5.0$, $df = 2$, $P = 0.0814$; Table 3.1), suggesting that the effects of treatment on A_{sat} may vary according to the species. Tukey's HSD post-hoc test indicated significant effects of treatment on A_{sat} that were similar for both species, whereby elevated [CO$_2$] had a positive stimulatory effect on seedling A_{sat}, as compared to photosynthetic rates observed under ambient [CO$_2$] conditions (Figure 3.2A). Interestingly, the results show differing species A_{sat} responses in the elevated temperature but ambient [CO$_2$] treatment (Figure 3.2A). Here, under elevated temperature alone, A_{sat} is slightly reduced in coffee seedlings, but slightly increased in cacao (Figure 3.2A), as compared to ambient temperature conditions.

The average g_s in coffee seedlings (0.04 ± 0.02 mol m^{-2} s^{-1}) was generally lower than those recorded for cacao (0.06 ± 0.02 mol m^{-2} s^{-1}), regardless of treatment. I found a significant main effect of species ($\chi^2 = 6.5$, $df = 1$, $P = 0.0108$; Table 3.1) on g_s, as well as a significant treatment × species interaction ($\chi^2 = 9.1$, $df = 2$, $P = 0.0105$; Table 3.1). Tukey's HSD post-hoc test indicated that differences in g_s between the two species were the result of significant treatment effects within cacao seedlings, but not coffee (Figure 3.2B). In cacao, elevated temperature, at ambient [CO$_2$], had a positive stimulatory effect on g_s rates, as compared to ambient temperature conditions (Figure 3.2B). However, when also supplied with elevated [CO$_2$], g_s was reduced and produced rates similar to those observed under ambient temperature and [CO$_2$] conditions (Figure 3.2B).

I found significant effects of each treatment ($\chi^2 = 39.4$, $df = 2$, $P < 0.0001$; Table 3.1) and species ($\chi^2 = 9.6$, $df = 1$, $P = 0.0019$; Table 3.1) on instantaneous WUE (Figure 3.2C), with a significant interaction between these two main effects ($\chi^2 = 8.2$, $df = 2$, $P = 0.0163$; Table 3.1) suggesting that any effects of treatment on instantaneous WUE may vary according to the species. Tukey's HSD post-hoc test indicated that elevated [CO$_2$] significantly enhanced instantaneous WUE in both species, as compared to ambient conditions (Figure 3.2C). However, I observed differing species responses in WUE to the elevated temperature alone treatment, as compared to ambient temperature conditions (Figure 3.2C). Here, cacao, but not coffee, seedlings exhibited significantly reduced instantaneous WUE when grown under elevated temperature alone, even in the well-watered conditions of this experiment (Figure 3.2C).

3.4.3 *Leaf Phytochemistry*

3.4.3.1 Phenolic Diversity and Concentration

Phenolic diversity (i.e., the number of phenolic compounds detected) was significantly higher in cacao seedlings than in coffee seedlings, regardless of treatment ($\chi^2 = 92.5$, $df = 1$, $P < 0.0001$; Table 3.1; Figure 3.3A).

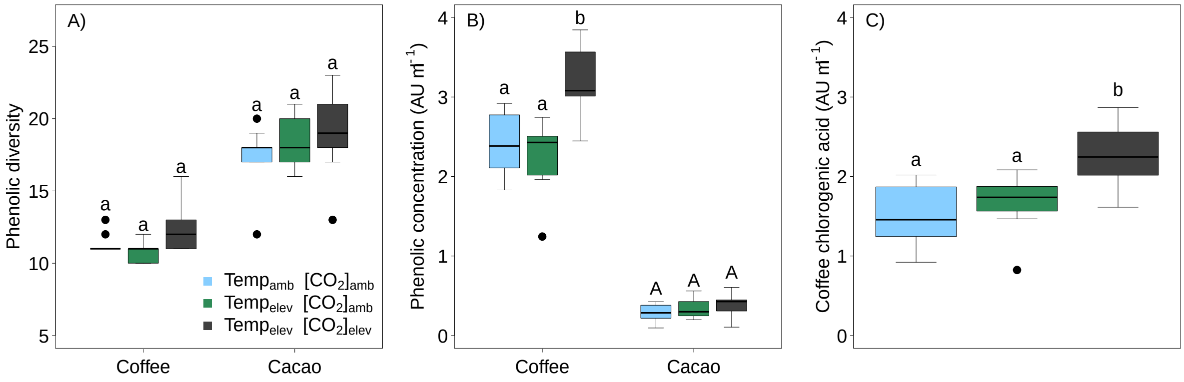


Figure 3.3. Leaf A) phenolic diversity, B) phenolic concentration, and C) coffee chlorogenic acid for coffee and cacao seedlings grown for ~140 days under three experimental treatments (Temp_{amb} $[CO_2]_{\text{amb}}$; $\text{Temp}_{\text{elev}}$ $[CO_2]_{\text{amb}}$; $\text{Temp}_{\text{elev}}$ $[CO_2]_{\text{elev}}$). Means with different letters of the same case (e.g., a, b) represent significant differences between treatments at an alpha level of $P < 0.05$ (Tukey's HSD tests). Letters of different case (e.g., a, A) in the same panel represent separate Tukey's comparisons for each species. All seedlings were watered to field capacity daily and provided mineral nutrition once per month.

On average, I detected 18 ± 0.8 distinct phenolic compounds in cacao leaves (Supplementary Table 3.3) though there were a diversity of phenolic compounds that could not be detected for cacao due to the limitations of the standards used. Only 11 ± 0.2 phenolic compounds were found in coffee leaves (Supplementary Table 3.2). In contrast, the concentration of the detected phenolic compounds was significantly greater in coffee leaves than those measured in cacao ($\chi^2 = 127.8$, $df = 1$, $P < 0.0001$; Table 3.1; Figure 3.3B). I also found a significant effect of treatment ($\chi^2 = 16.5$, $df = 2$, $P = 0.0002$; Table 3.1) on phenolic concentration, that varied between the two species, as shown by the significant treatment × species interaction term ($\chi^2 = 17.3$, $df = 2$, $P = 0.0002$; Table 3.1). Tukey's HSD post-hoc test showed that the differences in phenolic concentration between species were the result of significant treatment effects within coffee seedlings, but not cacao seedlings (Figure 3.3B). In coffee leaves, the concentration of phenolic compounds was significantly higher in seedlings grown under elevated $[CO_2]$, as compared to those grown under ambient $[CO_2]$ conditions and regardless of chamber temperature (Figure 3.3B).

3.4.3.2 Chlorogenic Acid

Chlorogenic acid was identified as the major phenolic component of the phenolic profile present in coffee leaves. I found a significant effect of treatment ($\chi^2 = 15.1$, $df = 2$, $P = 0.0005$; Table 3.1) on the chlorogenic acid concentration (Figure 3.3C). Tukey's HSD post-hoc test indicated that, like the total phenolic concentration in coffee leaves, chlorogenic acid was significantly higher (2.26 ± 0.14 AU μl^{-1}) in seedlings grown under elevated [CO_2], as compared to those grown under ambient [CO_2] conditions (1.53 ± 0.13 AU μl^{-1}) and regardless of chamber temperature (Figure 3.3C).

3.4.3.3 Leaf Nitrogen and Phosphorus

I found a significant main effect of species ($\chi^2 = 72.8$, $df = 1$, $P < 0.0001$; Table 3.1) on leaf N, whereby coffee leaves had significantly higher leaf N content values than cacao (Figure 3.4A).

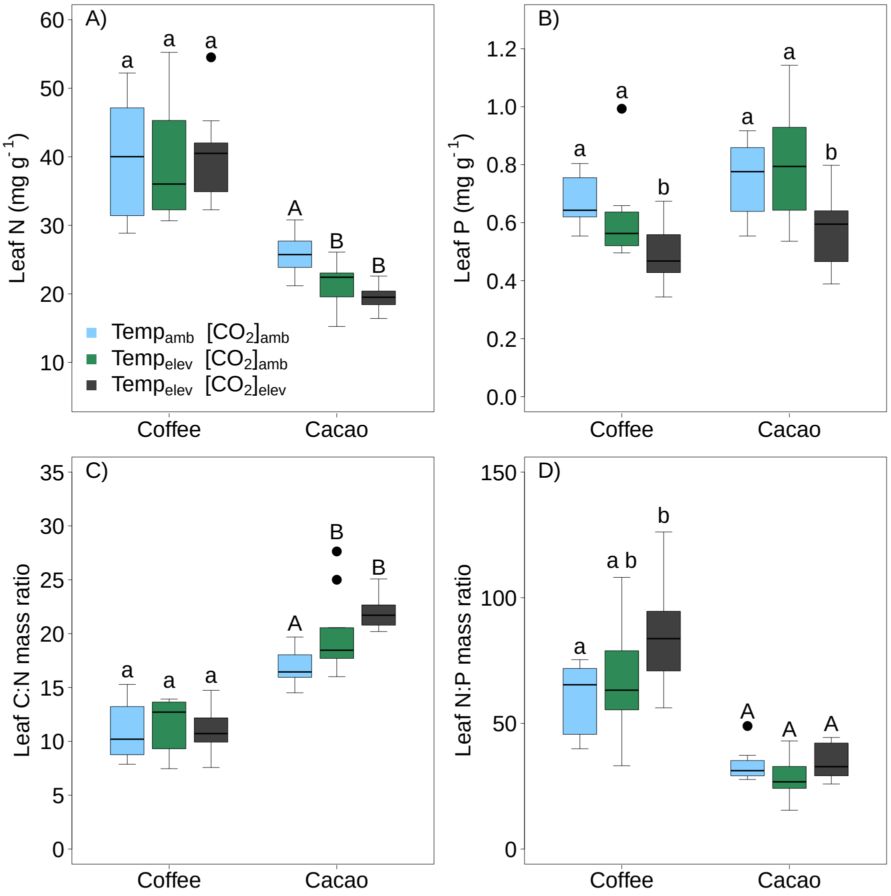


Figure 3.4. Leaf A) N (nitrogen), B) P (phosphorus), C) C:N (carbon to nitrogen) mass ratio, and D) N:P (nitrogen to phosphorus) mass ratio for coffee and cacao seedlings grown for ~140 days under three experimental treatments (Temp$_{amb}$ [CO_2]$_{amb}$; Temp$_{elev}$ [CO_2]$_{amb}$; Temp$_{elev}$ [CO_2]$_{elev}$). Means with different letters of the same case (e.g., a, b) represent significant differences between treatments at an alpha level of $P < 0.05$ (Tukey's HSD tests). Letters of different case (e.g., a, A) in the same panel

represent separate Tukey's comparisons for each species. All seedlings were watered to field capacity daily and provided mineral nutrition once per month.

The average N content in leaves of the coffee seedlings, regardless of treatment, was 39.8 ± 2.7 mg g^{-1}. Cacao leaves had an average N content of 22.2 ± 0.9 mg g^{-1}, regardless of treatment. There was a moderately significant treatment × species interaction ($\chi^2 = 6.9$, *df* = 2, $P = 0.0324$; Table 3.1). Due to this moderate interaction, Tukey's HSD post-hoc test was conducted for each species and indicated that leaf N responses to treatment were observed to differ for each species (Figure 3.4A). While there were no measurable effects of treatment on leaf N for coffee, in cacao, elevated temperature led to significant decreases in leaf N content, with further (but not significantly greater) declines observed in seedlings grown under elevated [CO_2] (Figure 3.4A).

I found significant main effects of each, treatment ($\chi^2 = 19.6$, *df* = 2, $P < 0.0001$; Table 3.1) and species ($\chi^2 = 9.4$, *df* = 1, $P = 0.0022$; Table 3.1) on leaf P (Figure 3.4B), with no significant interaction between these main effects (Table 3.1). Tukey's HSD post-hoc test revealed elevated [CO_2] significantly reduced leaf P levels in both species, as compared to seedlings grown under ambient [CO_2] conditions (Figure 3.4B). Overall and regardless of treatment, in contrast to leaf N content, cacao leaves had higher average leaf P levels (0.71 ± 0.05 mg g^{-1}) than coffee (0.59 ± 0.04 mg g^{-1}) (Supplementary Table 3.2, 3.3).

1.2.2.1 Leaf C:N and N:P Mass Ratio

Overall and regardless of treatment, the average leaf C:N mass ratio was found to be higher in cacao (19.4 ± 1) than coffee (11.1 ± 0.7) (Figure 3.4C; Table 3.1), as described by the significant main effect of species ($\chi^2 = 65.7$, *df* = 1, $P < 0.0001$; Table 3.1). I also found a moderately significant treatment main effect ($\chi^2 = 5.5$, *df* = 2, $P = 0.0642$; Table 3.1) and a moderately significant treatment × species interaction ($\chi^2 = 5.1$, *df* = 2, $P = 0.0773$; Table 3.1). Due to the moderate interaction, Tukey's HSD post-hoc test was conducted for each species and indicated that leaf C:N mass ratio responses to treatment were observed in cacao, but not coffee (Figure 3.4C). Similar to the results for leaf N, there were no measurable effects of treatment on leaf C:N mass ratio for coffee (Figure 3.4C), but elevated temperature led to significant increases in cacao leaf C:N mass ratio, with further (but not significantly greater) increases observed in seedlings grown under elevated [CO_2] (Figure 3.4C).

I found significant effects of each treatment ($\chi^2 = 7.9$, *df* = 2, $P = 0.0192$; Table 3.1) and species ($\chi^2 = 62.6$, *df* = 1, $P < 0.0001$; Table 3.1) on leaf N:P mass ratio (Figure 3.4D). In contrast to leaf C:N mass ratio, the average leaf N:P mass ratio, regardless of treatment, was higher in coffee (70.5 ± 6.6) than cacao (32.2 ± 2.5) (Figure 3.4D; Table 3.1). Tukey's HSD post-hoc test on each species indicated that leaf N:P mass ratios responses to treatment were observed in coffee, but not cacao (Figure 3.4D). Here, leaf N:P mass ratios were slightly, but not significantly, enhanced under elevated temperature alone, but the addition of elevated [CO_2] significantly enhanced the leaf N:P mass ratio, as compared to those observed under ambient [CO_2] conditions (Figure 3.4D).

3.5 Discussion

Similar to other studies (Cheesman and Winter, 2013; Singh Ramesh et al., 2023) I found a positive relationship between temperature and biomass production in each, coffee and cacao. I was unable to detect a significant negative effect of increased temperature on most evaluated parameters. However, the increases in stomatal conductance and reductions in instantaneous WUE for cacao seedlings grown under elevated temperature were ameliorated, and improved, respectively, by also growing under elevated [CO_2]. Leaf chemistry was altered differentially between the two species, potentially by reallocation of nutrients towards secondary metabolite production in coffee, or by dilution under enhanced biomass production in cacao.

Growth and biomass production

My findings on the impacts of elevated [CO_2] on coffee and cacao growth and biomass are consistent with previous research findings documenting the stimulatory effects of elevated temperature (Sale, 1969) and elevated [CO_2] (Lahive et al., 2018; Hebbar et al., 2020) on cacao seedling height and biomass. However, our results contrasted with Hebbar et al., (2020) who found a negative effect of elevated temperature on biomass production in their study on the independent and interactive effects of elevated temperature, elevated [CO_2], and water deficit on 6-month-old cacao seedlings. This disparity is likely due to differences in the average temperatures adopted across studies. While Hebbar et al., (2020) involved elevated temperatures of up to 39°C from a control level of 36°C, my study involved lower temperatures, with the elevated temperature treatment averaging 33°C during the day, as compared to an ambient control treatment averaging 29.5°C, which is coincident with the suggested optimal temperature ranges (i.e., 18 to 32°C) for cacao growth (DaMatta and Ramalho, 2006). Importantly, the observed differences between studies may be partially attributed to high genetic variation between plant materials used, which are often propagated by seed.

For coffee, and in contrast to the results for cacao, I observed no significant differences in coffee seedling height under the differing experimental treatments. However, coffee exhibited increases in biomass production when grown in temperatures above its reported optimal range (DaMatta and Ramalho, 2006), as compared to ambient conditions, with even greater (but not statistically significant) biomass production benefits in seedlings grown under elevated [CO_2].

The difference in response of height and biomass to experimental treatments for the two species studied are likely explained by the differing early growth strategies. For the orthotropic-oriented (upwards) early growth stages of cacao, the effects of increased temperature were expressed as stem elongation and increased leaf mass, leading overall increases in total biomass. However, in the more plagiotropic-oriented (branching) early growth stages of coffee, the effects of increased temperature were expressed as increased branch initiation (number) and branch mass. Thus, for coffee, early plagiotropic growth explains the observed large allocation of biomass to branch growth in addition to stem height. Branching in this study was consistent with Arcila-Pulgarín et al. (2002), with the first pair of branches initiated when approximately 8 pairs of leaves had formed. However, branching in this study was generally initiated earlier for seedlings grown under elevated temperature, with added benefits of elevated [CO_2] to branch initiation observed over time, suggesting an additional benefit of elevated [CO_2] to branch mass in coffee, at least in this well-watered study.

Due to small chamber dimensions and other logistical challenges of continuing my experimentation beyond the 4.5 months, I was unable to evaluate the two species to maturity within a valid experimental design. However, the observed architectural alterations to coffee seedlings at this developmental stage appear favourable. The management of *C. arabica* seedlings for a higher number of orthotropic stems per plant (up to four stems) has been shown to increase the number of plagiotropic branches per plant, which in turn, leads to overall increases in canopy density, available leaf area to sustain fruit development, net assimilation of CO_2, and crop yield (da Silva Angelo et al., 2019). Future studies evaluating the effects of elevated temperature and [CO_2] on mature, yielding plants would be beneficial.

Gas exchange

My results support previous short-and long-term research showing that increased [CO_2] can increase plant photosynthetic performance (Baligar et al., 2008; Ramalho et al., 2013; Ghini et al., 2015; Rodrigues et al., 2016; Lahive et al., 2018), reduce g_s and transpiration, and often improve both instantaneous and intrinsic WUE. Previous studies of *C. arabica* have also shown that increases in photosynthesis, together with almost unchanged g_s, led to significantly increased instantaneous WUE (Ramalho et al., 2013; Ghini et al., 2015; DaMatta et al., 2016; Rodrigues et al., 2016). Photosynthetic stimulation under elevated [CO_2] has also been shown to improve coffee plant performance (Ramalho et al., 2013; Ghini et al., 2015) bean yields (Ghini et al., 2015) and maintained bean quality (Ramalho et al., 2018). A slight decreasing trend in A_{sat} values of coffee seedlings grown under elevated temperature was observed in this study, but this effect was reduced when supplied with elevated [CO_2], to levels significantly higher than at ambient conditions. Similarly, Rodrigues et al. (2016) while showing a decrease in A under elevated temperatures of up to 42 ºC, showed a significantly greater decline in plants growing under ambient [CO_2] as compared to those grown at elevated [CO_2].

The lack of g_s response by coffee saplings observed in this study and previous others contrasts with reports of g_s reductions (stomatal acclimation) under elevated [CO_2] in most plants from all functional groups (Rodrigues et al., 2016). However, g_s values in coffee are typically low and stomatal limitations represent the main constraint to photosynthesis (Martins et al., 2014b). Thus, as observed in our study, greater potential photosynthetic gains associated with elevated [CO_2] may be facilitated in the absence of stomatal acclimation. Maintenance of higher A_{sat} values with simultaneous maintained g_s under well-watered conditions, may explain the observed significant enhancement in coffee seedling instantaneous WUE under elevated [CO_2]. I acknowledge that there are potential limitations to our study inside the controlled environment system, such as amplified photosynthetic downregulation (Morgan et al., 2001).

For cacao, increases in temperature led to moderate increases in A_{sat}, significantly increased g_s, but a significant reduction in instantaneous WUE. Studies of field grown, as opposed to pot-grown, cacao vary, showing that net photosynthesis declines in plants grown at above-optimal photosynthetic temperatures (31 to 33°C) (Balasimha et al., 1991). However, temperatures in some cacao growing regions reach up to 40°C without having significant negative effects on production (Medina and Laliberte, 2017). When the cacao seedlings in my study grown under elevated temperature were also provided with elevated [CO_2], A_{sat} and instantaneous WUE increased significantly to levels greater than observed under both ambient and elevated temperature alone. Increases in A_{sat} for well-watered seedlings measured at temperatures only slightly above the reported optimum range for cacao (18 to 34°C) (Lahive et al., 2019) may be no great surprise. Here, increased [CO_2] may be facilitating photosynthetic gains in cacao seedlings growing under elevated temperature.

The increased g_s observed under elevated temperature alone was reduced with elevated [CO_2], to levels similar to ambient conditions. Increases in g_s may allow cacao saplings to benefit from increased evaporative cooling (Urban et al., 2017). While such alterations may not be a concern for well-watered experimental seedlings, field-grown plants in water-limited situations may be negatively impacted by enhanced g_s through faster depletion of soil water (Urban et al., 2017). Previous reports of g_s responses to elevated [CO_2] alone in cacao vary and appear somewhat contradictory between studies. At around 700 ppm, Baligar et al. (2008) measured a 65% reduction in g_s and improved iWUE while Lahive et al. (2018) observed no effects of elevated [CO_2] on g_s. Our findings may be more similar to those of Lahive et al. (2018), showing no significant effects of elevating [CO_2] from 400 to 800 ppm on g_s. However, contradictory results may be expected given the reported genotypic variation in growth and photosynthesis of numerous cacao selections in response to predicted future climate change conditions (Lahive et al., 2021).

In this study, elevated [CO_2] ameliorated the significantly negative effect of increased temperature on cacao instantaneous WUE and enhanced sapling WUE to levels significantly greater than found under ambient conditions. Such increases in WUE in response to increased [CO_2] have been reported in various C_3 species, attributed either to increased A, decreases in g_s or a combination of both (Lahive et al., 2018; Wang et al., 2020; Baligar et al., 2021; Lahive et al., 2021).

Leaf phytochemistry

I found that elevated [CO_2] significantly increased the concentration of phenolic compounds in coffee leaves. This finding suggests that part of the extra carbon assimilated as a consequence of increased photosynthesis under elevated [CO_2] may be allocated towards the synthesis of phenolic compounds (Batista et al., 2021) in coffee. While [CO_2] enhancement can change the secondary metabolite concentrations of plant tissues, it can also alter the allocation of C and N resources among plant organs (Salazar-Parra et al., 2015). Thus, for cacao seedlings, in contrast to coffee seedlings, at this developmental age, allocation of nutrients towards supporting structural growth may be prioritised over non-structural compound production.

Previous studies of coffee have found that elevated temperature-mediated impacts on total phenolic content were significantly modified by elevated [CO_2] in both coffee leaves (Martins et al., 2014a) and fruits (Ramalho et al., 2018). Such changes may lead to reductions in leaf damage by herbivorous pests (Batista et al., 2021) and impact both plant and animal nutrition (Dubberstein et al., 2017) among others. In coffee leaves, chlorogenic acid was identified as the major phenolic component, and increased significantly under elevated [CO_2]. Chlorogenic acid, a conjugate of caffeic acid and quinic acid, functions as a defence metabolite in plants, providing protection against different pathogens (Sung and Lee, 2010; Wojciechowska et al., 2014; Martínez et al., 2017) and broad-spectrum anti-herbivore activity (Del Moral, 1972; Leiss et al., 2009). Given this, increases in leaf chlorogenic acid concentration under elevated [CO_2] might suggest an increased resilience of coffee plant leaves, and potentially their fruits, against pests and diseases in future climates. This is an important finding considering the forecasted worsening impact of coffee berry borer (*Hypothenemus hampei*), the most important pest of coffee worldwide, in many coffee producing areas (Jaramillo et al., 2011). Furthermore, though not evaluated here, chlorogenic acids play a vital role in determining coffee beverage taste, aroma and antioxidant properties (Mullen et al., 2011; Awwad et al., 2021). Taken together, the observed alterations in chlorogenic acid concentrations under conditions of elevated [CO_2] might suggest important implications of future climate change on *C. arabica* production, farmer livelihoods, and coffee beverage quality (Jaramillo et al., 2011; Awwad et al., 2021).

Overall leaf N content in coffee was found to be high and with it, a C:N ratio that was unaltered by the experimental treatments. This high N content was likely the result of monthly fertilizer application and well-watered growing conditions. Pompelli et al. (2010) reported N content in coffee seedlings ranging between 20 – 30.6 mg g^{-1} under differing light and N fertilization treatments. However, (Martins et al., 2014a) in their experimental study of 1.5-year-old plants found N ranged between 31.1 and 41.7 mg g^{-1} under differing [CO_2] (380 / 700 ppm) and temperature (25/20; 31/25; 37/30; 42/34; (day/night)) treatment combinations. For coffee seedlings, the observed increase in photosynthesis under elevated [CO_2], combined with unchanged N content across all treatments, suggests that there is an abundance of non-photosynthetic N in coffee leaves that may be involved with the synthesis of plant protective secondary metabolites like alkaloids (e.g., caffeine) (Ashihara, 2006).

For cacao, the average leaf N content was 28.6 ± 1.0 mg g^{-1} for seedlings grown under ambient conditions. These results are consistent with previous studies under ambient glasshouse conditions reporting cacao N levels ranging between 23.9 and 31.7 mg g^{-1}, though with significant genotypic variation between clones (Daymond et al., 2011). However, in contrast to coffee, cacao showed a significant reduction in leaf N content under increased temperature, with further reductions when also supplied with elevated [CO_2]. This impact on leaf N and C:N ratio was observed despite an increase in photosynthetic rate, suggesting that the photosynthetic apparatus (especially RuBisCO content) acclimated to be more efficient under elevated temperatures and [CO_2]. Although not examined here, the implications of reductions in N content on overall plant nutritional value to herbivores may have significant impacts upon trophic cascades (DeLucia et al., 2012).

Leaf P significantly declined in both species when grown under elevated [CO_2], a trend which has also been observed in a range of other C_3 species (Poorter et al., 2022). Such P reductions may potentially indicate a dilution type effect as result of increased non-structural carbohydrates in leaves and overall increases in plant biomass under this treatment (Martins et al., 2014a). Alternatively, the observed P reduction may reflect, or be magnified by, changes in the physiological uptake, or availability of nutrients (Jin et al., 2015). For example, increased C allocation to root growth under elevated [CO_2], and associated increases in root exudates (Pantigoso et al., 2020) may impact the ability of plants to acquire P from the soil (Jin et al., 2015). Combined with enhanced growth under elevated [CO_2], higher P demands may lead to faster depletion of available soil P resources, and potentially drive P limitation in the soil (Jin et al., 2015). This may be particularly relevant in potted plants with root growth restriction (Poorter et al., 2012), even with monthly fertilizer application. Although the pots used in this study were relatively large, they likely restricted the rooting volume and potentially suppressed the plant responses to treatments (Poorter et al., 2012).

Interestingly, leaf N:P ratios increased in coffee in plants grown under elevated [CO_2] but did not change in cacao. The N:P increase in coffee may be due to the relative homeostasis in leaf N concentrations across all treatments, but with decreased leaf P concentrations under elevated [CO_2]. In contrast, in cacao, the concentrations of leaf N and P decreased in a proportionally similar way, leading to no observed differences in leaf N:P across the three treatments. Assuming the general tendency toward decreasing leaf N and P concentrations with elevated [CO_2] is a dilution response resulting from increased C supply (Poorter et al., 2022), the homeostasis of leaf N concentration in coffee may indicate active upregulation of N allocation to match the increased leaf C supply under elevated [CO_2] in this species. This aligns with the high N concentrations observed in coffee. Maintenance of N content in future climates might have positive consequences, as coffee plants are highly N-demanding and adequate N availability is pivotal for plant development and yield (Carelli et al., 2006), as well as in triggering acclimation mechanisms to environmental stressors (Ramalho et al., 2000). My observations of coffee differ from the general decreasing leaf N concentration response to increasing atmospheric [CO_2] of most C_3 plants (Poorter et al., 2022). These observations raise two interesting questions: 1) do other species with naturally high alkaloid concentrations in their tissues respond similarly? and 2) is the observed response of coffee, though perhaps unintended, influenced by the domestication and selection process for investment towards a specific secondary metabolite profile?

Here, we see a glimpse of the combined effects of elevated temperature and [CO_2] on the leaf mineral contents of coffee and cacao seedlings, with potential implications to the fertilization management for these important tropical crops (Le Thiec et al., 1995). Mineral fertilization is considered as a strategy to mitigate the effects of climate changes (Bayu, 2020). Future research addressing the maintenance of nutrient balances to the plant, and in the leaves, in the context of predicted climate changes and global warming is crucial to ensure ongoing vegetative and reproductive development with maintained produce quality in higher C environments, without other mineral limitations.

3.6 Conclusion

Overall, my findings support the potential for elevated [CO_2] to ameliorate temperature-mediated effects on growth parameters and leaf-level photosynthetic traits in coffee and cacao. I also highlight potential alterations to plant structure / architecture and leaf chemistry under future climates. To better understand crop performance under future climate change scenarios, it is crucial to evaluate interactive, rather than singular, climate effects. I recommend that future studies addressing the interactive effects of temperature and [CO_2] on tropical crops simultaneously incorporate factors of water limitations (e.g., drought) or altered nutrient dynamics (e.g., nutrient availability, mobilisation and performance) that are expected to occur in concert with those analysed here. Furthermore, studies that address factors such as plant architecture, nutrient composition and plant-animal interactions, in relation to future productivity and product quality under future climate scenarios, are desirable. This study demonstrates how the growth, physiology, and biochemistry of two globally important tropical crops may be altered by future and interactive climate change drivers, with potential direct and indirect implications for plant susceptibility to pest and disease, future productivity, crop quality (and value), and the livelihoods of farming families who depend their production.

3.7 Supplementary Materials

Supplementary Table 3.1. Day and night chamber conditions for coffee and cacao.

	Treatment	Day			Night		
Species		Temp °C	RH %	$[CO_2]$ ppm	Temp °C	RH %	$[CO_2]$ ppm
Coffee	Temp_{amb} $[CO_2]_{amb}$	29.4 ± 1.8	81.3 ± 3.9	435.4 ± 19.5	24.1 ± 1.4	89.1 ± 1.8	513.4 ± 31.4
	Temp_{elev} $[CO_2]_{amb}$	33.0 ± 1.8	82.3 ± 5.2	429.0 ± 13.4	27.5 ± 1.4	89.3 ± 2.7	508.5 ± 25.0
	Temp_{elev} $[CO_2]_{elev}$	33.0 ± 1.9	82.9 ± 5.6	788.8 ± 57.6	27.4 ± 1.4	90.8 ± 3.1	799.4 ± 38.6
Cacao	Temp_{amb} $[CO_2]_{amb}$	29.5 ± 1.9	81.6 ± 3.8	434.9 ± 20.1	24.1 ± 1.4	89.2 ± 1.7	515.2 ± 31.9
	Temp_{elev} $[CO_2]_{amb}$	33.0 ± 1.9	82.6 ± 5.1	428.4 ± 13.6	27.5 ± 1.5	89.5 ± 2.7	510.3 ± 25.3
	Temp_{elev} $[CO_2]_{elev}$	33.0 ± 1.9	83.5 ± 5.3	788.1 ± 60.5	27.5 ± 1.5	91.1 ± 3.1	799.3 ± 40.6

[z] Values are given as means (± SE) of records taken every five minutes across the 137-day (coffee) and 135-day (cacao) experiments.

[y] Values are averaged by Day (07:00 – 17:00) and Night (17:00 – 07:00) time periods.

[x] Temp, temperature; RH, relative humidity and $[CO_2]$, atmospheric carbon dioxide concentration inside the chamber.

[w] All seedlings were watered to field capacity daily.

Supplementary Table 3.2. Seedling measurements for coffee grown at ambient or elevated temperatures, with either ambient or elevated [CO_2], measured upon the final harvest date.

		Ambient temperature	**Elevated temperature**	
		Ambient [CO_2]	Ambient [CO_2]	Elevated [CO_2]
Character	Initial	137 days	137 days	137 days
Whole plant				
Height (cm)	31.0 ± 0.8	67.2 ± 3.6	76.9 ± 3.0	76.1 ± 3.2
RGR (g g^{-1} d^{-1})		0.017	0.018	0.019
Total dry biomass (g)	5.1 ± 0.8	47.2 ± 2.1	61.9 ± 3.4	64.1 ± 4.8
AG mass (g)	4.0 ± 0.6	30. 8 ± 1.6	41.7 ± 2.1	41.8 ± 3.1
BG mass (g)	5.1 ± 0.8	13.7 ± 0.6	16.0 ± 1.4	16.8 ± 1.6
Leaf number	15.7 ± 1.1	49.1 ± 2.2	76.7 ± 6.1	80.2 ± 4.6
Leaf mass (g, includes petiole)	2.6 ± 0.4	21.0 ± 1.1	29.4 ± 0.6	28.3 ± 1.9
Branch number		7.0 ± 0.4	7.7 ± 0.4	10.3 ± 0.6
Branch mass (g)		2.7 ± 0.2	4.1 ± 0.4	5.5 ± 0.5
Stem mass (g)	1.6 ± 0.2	9.8 ± 0.7	12.3 ± 0.9	13.5 ± 1.4
RC stem diameter (mm)	4.8 ± 0.3	9.5 ± 0.3	10.6 ± 0.4	11.1 ± 0.3
MS stem diameter (mm)		6.6 ± 0.2	7.7 ± 0.2	8.1 ± 0.4
Root mass (g)	1.2 ± 0.2	13.7 ± 0.6	16.0 ± 1.4	16.8 ± 1.6
Leaf and phytochemistry				
Target leaf mass (g)	0.28 ± 0.02	0.43 ± 0.0	0.40 ± 0.0	0.35 ± 0.00
Leaf thickness (μm)	24.7 ± 1.0	25.0 ± 0.4	25.4 ± 0.3	24.6 ± 0.4
Target leaf area (cm^2)		68.3 ± 3.2	59.8 ± 3.7	54.5 ± 3.0
LMA (g m^{-2})		63.5 ± 1.5	66.3 ± 0.8	65.7 ± 3.2
Leaf Nitrogen (mg g^{-1})		40.0 ± 2.7	39.3 ± 3.1	40.2 ± 2.3
Leaf Phosphorus (mg g^{-1})		0.68 ± 0.03	0.61 ± 0.06	0.49 ± 0.03
Leaf Carbon (mg g^{-1})		418.1 ± 3.8	433.5 ± 7.1	430.3 ± 7.6

C: N mass ratio	10.9	±	0.9	11.5	±	0.7	11.0	±	0.6
N: P mass ratio	59.6	±	4.6	67.0	±	7.8	84.9	±	7.3
Leaf Potassium (mg g^{-1})	14.4	±	1.1	14.4	±	0.5	13.2	±	0.4
Leaf Calcium (mg g^{-1})	6.3	±	0.8	6.1	±	0.3	4.8	±	0.3
Leaf Magnesium (mg g^{-1})	3.7	±	0.3	3.9	±	0.2	3.2	±	0.2
Leaf Iron (mg g^{-1})	0.08	±	0.008	0.07	±	0.006	0.05	±	0.002
Leaf Zinc (mg g^{-1})	0.02	±	0.004	0.01	±	0.002	0.01	±	0.003
Phenolic diversity	11.3	±	0.2	10.8	±	0.02	12.2	±	0.5
Phenolic concentration (AU μl^{-1})	2.4	±	0.1	2.2	±	0.15	3.2	±	0.16
Coffee chlorogenic acid absorbance (AU μl^{-1})	1.5	±	0.1	1.7	±	0.12	2.3	±	0.14

[z] Values are given as means (± SE, *n* = 9).

[y] RGR, relative growth rate; AG, above-ground biomass; BG, below-ground biomass; RC, root collar; MS, mid-stem; LMA, leaf mass per unit area; mg g^{-1}, milligrams per gram; AU μl^{-1}, absorption units per microlitre.

[x] Time periods are 0 (initial) and 137 days in treatment.

[w] Leaf tissue phytochemistry evaluations were obtained using the ‘target’ leaves on each seedling at the final harvest.

[v] Leaf and petiole mass was combined for coffee due to reduced petioles.

Supplementary Table 3.3. Seedlings measurements for cacao grown at ambient or elevated temperatures, with either ambient or elevated [CO_2], measured upon the final harvest date.

		Ambient temperature	**Elevated temperature**	
		Ambient [CO_2]	Ambient [CO_2]	Elevated [CO_2]
Character	Initial	135 days	135 days	135 days
Whole plant				
Height (cm)	20.0 ± 0.7	80.4 ± 4.1	95.1 ± 4.5	113.4 ± 5.9
RGR ($g\ g^{-1}\ d^{-1}$)		0.021	0.024	0.024
Total dry biomass (g)	1.3 ± 0.1	38.4 ± 2.4	51.4 ± 3.9	56.5 ± 3.8
AG mass (g)	0.7 ± 0.1	29.8 ± 1.9	41.2 ± 3.4	45.6 ± 2.9
BG mass (g)	1.3 ± 0.1	8.6 ± 0.8	10.1 ± 1.1	10.9 ± 1.0
Leaf number	4.7 ± 0.3	18.4 ± 1.4	26.4 ± 2.6	25.3 ± 3.4
Leaf mass (g)	0.4 ± 0.1	16.6 ± 1.2	22.5 ± 2.5	20.3 ± 1.9
Petiole mass (g)	0.02 ± 0.0	2.36 ± 0.1	3.09 ± 0.2	3.17 ± 0.2
Stem mass (g)	0.3 ± 0.0	10.8 ± 2.8	15.7 ± 1.2	22.2 ± 2.3
RC stem diameter (mm)	4.4 ± 0.2	12.5 ± 0.4	13.1 ± 0.4	14.5 ± 0.6
MS stem diameter (mm)		8.2 ± 0.2	8.8 ± 0.2	9.8 ± 0.5
Root mass (g)	0.6 ± 0.1	8.6 ± 0.8	10.1 ± 1.1	10.9 ± 1.0
Cotyledon mass (g)	1.4 ± 0.0	0.8 ± 0.1	0.6 ± 0.1	0.8 ± 0.1
Leaf and phytochemistry				
Target leaf mass (g)		0.9 ± 0.1	0.9 ± 0.1	0.9 ± 0.1
Leaf thickness (μm)		17.4 ± 0.3	16.2 ± 0.4	16.5 ± 0.5
Target leaf area (cm^2)		184.9 ± 18.0	183.6 ± 26.3	172.2 ± 24.3
LMA ($g\ m^{-2}$)		50.0 ± 1.6	49.2 ± 1.1	52.8 ± 2.5
Leaf Nitrogen (%)		28.6 ± 1.0	21.5 ± 1.1	19.4 ± 0.6
Leaf Phosphorus ($mg\ g^{-1}$)		0.8 ± 0.04	0.8 ± 0.07	0.6 ± 0.05
Leaf Potassium ($mg\ g^{-1}$)		12.0 ± 0.5	13.6 ± 0.9	9.6 ± 0.8

Leaf Carbon (mg g^{-1})	430.6	±	11.1	419.2	±	3.7	428.6	±	10.1
C:N mass ratio	15.9	±	1.1	20.1	±	1.3	22.2	±	0.6
N:P mass ratio	33.5	±	2.3	28.3	±	3.0	34.9	±	2.3
Leaf Calcium (mg g^{-1})	6.4	±	0.7	5.3	±	0.6	3.9	±	0.2
Leaf Magnesium (mg g^{-1}g)	3.7	±	0.3	3.3	±	0.1	2.9	±	0.2
Leaf Iron (mg g^{-1})	0.05	±	0.003	0.05	±	0.005	0.04	±	0.003
Leaf Zinc (mg g^{-1})	0.03	±	0.005	0.03	±	0.002	0.02	±	0.002
Phenolic diversity	17.4	±	0.7	18.4	±	0.6	18.9	±	1.0
Phenolic concentration (AU µl^{-1})	0.3	±	0.03	0.3	±	0.04	0.4	±	0.05

[z] Values are given as means (± SE, *n* = 9).

[y] RGR, relative growth rate; AG, above-ground biomass; BG, below-ground biomass; RC, root collar; MS, mid-stem; LMA, leaf mass per unit area; mg g^{-1}, milligrams per gram; AU µl^{-1}, absorption units per microlitre.

[x] Time periods are 0 (initial) and 135 days in treatment.

[w] Leaf tissue phytochemistry evaluations were obtained using the ‘target’ leaves on each seedling at the final harvest.

Supplementary Table 3.4. Gas exchange measurements for coffee and cacao seedlings grown under ambient or elevated temperatures, with either ambient or elevated [CO_2], measured upon the final harvest date.

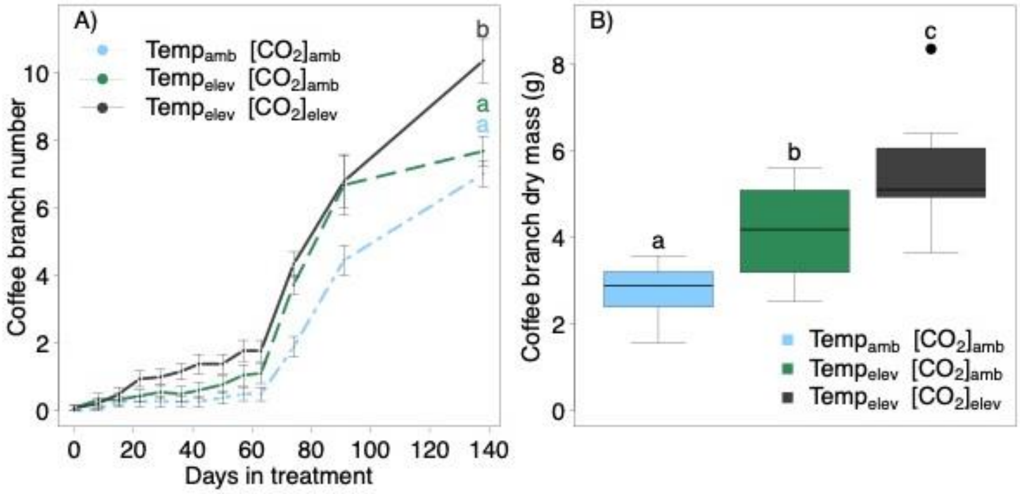
	Ambient temperature		**Elevated temperature**			
	Ambient [CO_2]		Ambient [CO_2]		Elevated [CO_2]	
Measurement	*Coffee*	*Cacao*	*Coffee*	*Cacao*	*Coffee*	*Cacao*
Photosynthesis (A_{sat}, $\mu mol\ m^{-2}\ s^{-1}$)	5.7 ± 2.0	5.1 ± 0.8	4.9 ± 0.8	6.2 ± 1.7	8.9 ± 2.0	10.2 ± 2.3
Stomatal conductance (g_s, $mol\ m^{-2}\ s^{-1}$)	0.05 ± 0.03	0.05 ± 0.02	0.04 ± 0.02	0.09 ± 0.04	0.05 ± 0.03	0.06 ± 0.02
Intracellular CO_2 (Ci, μmol CO_2 $mol\ air^{-1}$)	177.6 ± 50.5	211.3 ± 55.4	171.8 ± 51.8	264.5 ± 24.5	417.5 ± 129.1	439.0 ± 99.0
Leaf transpiration (E, $mmol\ m^{-2}\ s^{-1}$)	0.7 ± 0.4	0.8 ± 0.3	0.8 ± 0.3	1.7 ± 0.6	1.2 ± 0.7	1.1 ± 0.4
Leaf temperature (T_{leaf}, °C)	34.5 ± 0.3	34.2 ± 0.4	36.6 ± 0.9	36.7 ± 0.3	40.2 ± 0.5	38.4 ± 0.7
Air temperature (T_{air}, °C)	32.5 ± 0.2	32.4 ± 0.1	34.8 ± 1.1	35.8 ± 0.1	38. 4 ± 0.6	36.4 ± 0.3
Vapour pressure deficit (*kPa*)	1.45 ± 0.1	1.5 ± 0.2	1.8 ± 0.2	1.9 ± 0.2	2.4 ± 0.2	2.0 ± 0.2
Ci/Ca	0.5 ± 0.1	0.5 ± 0.1	0.4 ± 0.1	0.7 ± 0.1	0.5 ± 0.2	0.5 ± 0.1
Instantaneous WUE (*A/E*)	8.4 ± 1.8	7.2 ± 2.1	7.1 ± 2.5	3.8 ± 0.8	8.6 ± 2.8	9.7 ± 2.4
Leaf Δ temperature ($T_{leaf} - T_{air}$)	2.1 ± 0.4	1.9 ± 0.4	1.8 ± 0.5	0.9 ± 0.2	1.8 ± 0.3	2.0 ± 0.6

[z] Values are given as means (± SE, coffee $n = 9$, cacao n = 9 but $n = 8$ (cacao only) in the $Temp_{amb}$ $[CO_2]_{amb}$ treatment).

[y] Gas exchange measurements were obtained using the third newest fully expanded leaf of each seedling per treatment at the time of harvest. Gas exchange was measured between 08:00 and 12:00 using a Li-Cor 6400 (Li-Cor Inc., Lincoln, NE, USA) with measurements made at a photon flux of 1500 µmol m^{-2} s^{-1} supplied by an artificial LED light source.

[x] The temperature and CO_2 environment within the leaf cuvette were set to reflect the typical midday glasshouse chamber conditions.

[w] Time periods are 137 (coffee) and 135 (cacao) days in treatment and all seedlings were watered to field capacity daily.



Supplementary Figure 3.1. A) Number of initiated branches and B) branch dry mass for coffee seedlings grown in three experimental treatments (Temp$_{amb}$ [CO$_2$]$_{amb}$; Temp$_{elev}$ [CO$_2$]$_{amb}$; Temp$_{elev}$ [CO$_2$]$_{elev}$) for up to 137 days. Statistical analysis was conducted on data obtained from the final harvest only. There were n = 9 seedlings per treatment at the final harvest. Pairwise comparisons were conducted among treatments means. Therefore, means with different letters of the same case (e.g., a, b) represent significant differences between treatments at an alpha level of $P < 0.05$ (Tukey's HSD tests). All seedlings were watered to field capacity daily and provided mineral nutrition once per month.

Chapter 4. Supplemental and Synchronized Pollination May Increase Yield in Cacao.

Note: References for this published chapter are self-contained.

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Supplemental and Synchronized Pollination May Increase Yield in Cacao

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Additional index words. cherelle wilt, cocoa, dominance, fruit set, artificial pollination, resource competition, *Theobroma cacao*

Abstract. **Artificial pollination management strategies are a potential solution to improving the livelihoods of smallholder cacao farmers by increasing crop productivity in situations when pollination services are limiting. However, field-based research trials evaluating the yield benefits of artificial pollination management strategies within intensified cacao systems are lacking. Thus, in an intensively managed cacao system, we evaluated the effects of artificial pollination condition (i.e., pollen genotype, pollination intensity, and pollination synchrony) on fruit development and yield in three high-yielding cacao clones. Artificial pollination, regardless of intensity, significantly increased fruit set and yield. Pollination synchrony had a significant effect on cherelle survivorship; older cherelles had greater survival rates across all developmental stages than younger cherelles. Yield differed between genotype crosses and varied according to the pollen donor used, highlighting the importance of understanding self- and cross-compatibility when selecting clones for cultivation. Pollination intensity had no significant effect on harvested yield, indicating that more rigorous research is needed to identify the pollination intensity required for optimized yield under artificial pollination conditions. We conclude that strategies to enhance flowering, pollination rates, and pollination synchrony while ensuring adequate tree nutrition may increase productivity in cacao. Future research evaluating numerous cacao clones across multiple years and locations may help us to understand the region-specific effects of intensive management strategies on the long-term sustainability of enhancing cacao tree productivity.**

Cacao is a neotropical species originating in the lowland rainforests of South America (Motamayor et al., 2002) that develops large pods (fruit), each containing an average of 30 to 40 seeds (Lima et al., 2011). After they have been fermented and dried, these seeds constitute the raw product cocoa upon which the chocolate industry is based (Leal et al., 2008). The need to increase crop productivity and farmer income (Edwin and Masters, 2005; Gockowski and Sonwa, 2011), as well as a sustained increase in global cocoa consumption (Lass, 2004; Rice and Greenberg, 2000), has led to a worldwide push for the intensification of cacao cultivation (Bisseleua et al., 2009; Deheuvels et al., 2014). The intensification of cacao production systems has involved advances in agricultural management practices (Blommer, 2011), such as high-density, unshaded, monoculture cacao plantations managed using high inputs of agrochemicals, irrigation, and mechanization. Cacao intensification has also included genetic advances such as the development of high-yielding and disease-resistant cacao cultivars to increase yield production (Edwin and Masters, 2005; Motamayor et al., 2013; Vaast and Somarriba, 2014). However, in field settings where cacao trees are exposed to variable environmental conditions, selectively bred traits to increase yield (e.g., increases in the number and size of pods per tree and seeds per pod) (Cilas et al., 2010) may not be fully realized or may be antagonistic.

Field-based research on increasing cacao yields in intensified cultivation systems is rare (but see Bos et al., 2007; Carr and Lockwood, 2011; De Almeida and Valle, 2007; Goenaga et al., 2009, 2015; Johns, 1999; Zuidema et al., 2005). Furthermore, field trials evaluating the yield of advanced cacao clones under optimal management practices (e.g., fertigation) across multiple locations are not uniformly established. As a result, current yield potential estimations of advanced cacao clones (e.g., Aneani and Ofori-Frimpong, 2013; Bos et al., 2007; Falque et al., 1995, 1996; Goenaga et al., 2009, 2015; Groeneveld et al., 2010) are highly variable and there is a pressing need to evaluate further the yield-limiting factors for numerous clones under intensified management conditions.

Cacao produces a surplus of flowers, with possibly up to 125,000 flowers per tree per year (Lachenaud and Mossu, 1985). However, low fruit: flower ratios are still observed in most producing areas (Aneani and Ofori-Frimpong, 2013). No more than 10% of the flowers produced are naturally pollinated (Groeneveld et al., 2010) and even fewer (0.5% to 5%) develop into mature pods under open pollination conditions, primarily because of fruit abortion (Bos et al., 2007; De Almeida and Valle, 2007; Falque et al., 1995; Young and Severson, 1994). Consequently, pollination and resource availability are widely accepted as two major extrinsic factors limiting cacao production (e.g., Ayre and Whelan, 1989; Bos et al., 2007; Brown and McNeil, 2006; Falque et al., 1995; Groeneveld et al., 2010; Valle et al., 1990; Young, 1982; Young and Severson, 1994; Zuidema et al., 2005).

Although it is well known that most cacao varieties are self-incompatible (Falque et al., 1996), very little is known about pollination services or pollinator ecology in cacao (Toledo-Hernandez et al., 2017). The general consensus suggests that cacao trees strongly depend on the cross-pollination services provided by ceratopogonid midges (Ceratopogonidae) (Kaufmann, 1975; Posnette, 1944; Winder, 1978; Young, 1982). However, low natural midge abundance in cacao systems is widely reported (Winder, 1978), which may drive down pollination rates and limit cacao yield increases (Forbes and Northfield, 2016; Frimpong et al., 2009; Young, 1982; Young and Severson, 1994). In support of pollination limitation, Groeneveld et al. (2010) in

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a low-input agricultural cacao cultivation system found a significant increase in cacao yield when artificially increasing pollination rates from 10% to 40% of flowers pollinated, despite increases in relative fruit abortion. However, Valle et al. (1990) concluded that fruit abortion (cherelle wilt), rather than manipulated pollination rates, was the major factor limiting yield, resulting from variations in fruit-bearing capacity and resource availability.

These results suggest that the upper limit to cacao yield is ultimately set by the number of flowers produced (flowering intensity) (Falque et al., 1995), the number of flowers successfully pollinated (pollination intensity) (Groeneveld et al., 2010), and the resources available to support the developing fruit (Valle et al., 1990). At increased pollination levels, the energetic cost of producing a large number of pods is high (Groeneveld et al., 2010), which may lead to yield reductions via increases in relative cherelle wilt, particularly if resources are limiting (Bos et al., 2007; Valle et al., 1990).

Previous research suggests that the incidence of cherelle wilt may be influenced by the timing of pollinations (pollination synchrony), under the assumption that pods of the same developmental age should not compete strongly with each other until they are no longer susceptible to wilt (i.e., until after 100 d) (Hutcheon, 1977). Therefore, synchronizing pollination efforts into a concentrated time frame may increase the pod production of cacao trees by reducing competitive hierarchy (i.e., reduced competition between pods of different ages) during the wilting stages. However, increases in the per-tree pod number after 100 d may compromise pod size and quality at harvest, resulting from the substantially increased resource requirements of pods during this stage of development (Hutcheon, 1977). Synchronized pollination can increase kernel set in maize (*Zea mays* L.) (Carcova and Otegui, 2001; Carcova et al., 2000; Sarquis et al., 1998), suggesting a negative impact of increasing the interval time between pollinations. However, to date, no research has addressed the potential consequences of altering the natural synchrony of pollination through artificial pollination on cacao yields when nutrients are nonlimiting.

Together, the previous findings suggest that the pollination and resource conditions required for maximizing the pod carrying capacity of an individual cacao tree are unlikely to be fixed. Rather, a spatiotemporal interplay between flower production, pollination intensity, pollination synchrony, genotype compatibility, resource availability, and the genetically predisposed physiological ability of the maternal tree to meet resource demands may be underlying cacao yield potential. Understanding the effects of potential pollination condition (i.e., pollen genotype, pollination intensity, and pollination synchrony) interactions on fruit set, cherelle wilt, and final yield may have large impacts on cacao management and production profitability. Thus, we aimed to identify the pollination conditions required for increased yield in three high-yielding cacao clones within an intensively managed cacao cultivation system in which the nutritional requirements for pod production are presumably met. We pollinated artificially a predetermined percentage of open flowers present on a tree on a given day and examined the effects of 1) pollen donor genotype, 2) pollination intensity, and 3) pollination synchrony on the survivorship of pollinated flowers to harvested pods.

Materials and Methods

We present a series of experiments trial 1 and trial 2 that were carried out to address the research aims detailed earlier. Trial 1 evaluated the effects of artificial pollination and maternal and paternal genotype cross on harvested yield in cacao. This trial was conducted using two successive subtrials, hereafter referred to as trial 1.1 and trial 1.2, which differed by the paternal pollen donor used for artificial pollination as well as the trial timing (season). Trial 2 evaluated the effects of pollination intensity and pollination synchrony on fruit set, development, and harvested yield in cacao.

Trial 1: Assessing the effects of artificial pollination and maternal and paternal genotype cross on harvested yield in three high-yielding cacao clones

Study site, clones, and environment. The study was conducted using a single clonal cacao field (pollination block I) at the Mars Cocoa Research Center (MCRC), in Tarengge Village, South Sulawesi, Indonesia, from Dec. 2013 to July 2014. The experimental field includes 515 trees of 13 *T. cacao* genotypes (clones), organized in linear rows using a 3.5 × 3.5-m planting distance and planted using a randomized block design of eight trees per unique clone block. At the time of the experiment, the trees were 3 years old and producing fruit. The field is under partial shade, with *Gliricidia sepium* [(Jacq.) Kunth ex Walp.] planted at a 7 × 7-m planting distance. All cacao trees within the field are propagated clonally using softwood scion cuttings from plagiotropic material, terminally grafted on rootstocks from open-pollinated M01 seeds. For the three maternal cacao clones described in this article, peak flowering generally occurs between the months of November and March (Supplemental Fig. 1A) and the major harvest period is usually conducted between June and August (Supplemental Fig. 1B), although these events are variable according to yearly rainfall and flowering. After the peak harvest period, a lower number of pods are produced throughout the remainder of the year until a second minor harvest in October through to December (Supplemental Fig. 1B).

The MCRC has an average annual rainfall of 2754 mm, well distributed over 12 months, with higher than average monthly rainfall between April and June (average, 319 mm) and lower than average rainfall between August and November (average, 146.5 mm; the "dry" season) (Spaans, 2016, unpublished data). The MCRC has an annual average temperature range between a minimum of 22.7 °C and a maximum of 31.7 °C, an annual relative humidity between 80% and 85%, and an average monthly solar radiation of 217 W/m^2. The soils at the MCRC are classified as Typic Dystrudepts (Soil Survey Staff, 1999), very homogeneous, and with little spatial variation (Spaans, 2016, unpublished data) (see Supplemental Table 2 for soil composition details).

Tree nutrition and management. A one-sided drip irrigation system (Netafim, Magal, Israel) was fitted to all cacao trees within the experimental field. Each tree had two drippers, each positioned ≈0.5 m on either side of the tree trunk. Additional drip points were positioned every 1 m along the length of the irrigation line. Dripper capacity was 4 L/h and, before Sept. 2014, the system delivered between 2 and 6 mm water/ha every day, depending on the season and incidence of rainfall. In Sept. 2014 (during trial 1.2), the supply of dissolved mineral fertilizers to experimental trees was implemented via fertigation through the Netafim drip irrigation system. Fertigation was applied to trees every second day in 4 to 6 mm water/ha, per day during the dry season and in a more concentrated solution of 2 mm water in months of increased rainfall. Fertigation provided each tree with 15 applications of fertilizer per month of variable nutrient composition and concentration (Supplemental Table 3), developed to complement the cropping cycle of cacao trees in South Sulawesi.

The cacao trees typically had four to five main branches that formed a "cup-shaped" tree architecture. Light sanitary pruning was conducted as required. Heavy structural pruning was conducted once per year after the main harvest period to maintain manageable and uniform tree architecture.

Experimental trial period and tree selection. Before the current study, no experimentation had been conducted on the experimental trees. Trial 1.1 was conducted over a 7-month period from 19 Dec. 2013 to 21 July 2014. Trial 1.2 was conducted during the following 7-month period from 25 Aug. 2014 to 17 Mar. 2015. The results obtained from trial 1.2 may have been influenced by the application of experimental treatments in trial 1.1, as well as fertigation implementation and seasonality, and should be interpreted with this in mind.

Trial 1 evaluated three maternal genotypes (M01, PBC123, and MCC02) and two paternal genotypes: PBC123 (trial 1.1) and BB01 (trial 1.2) (Table 1; see Supplemental Table 1 for clonal evaluations). We selected 40 trees of each maternal genotype (Table 1) that were 1) the same age, 2) mature and bearing flowers, 3) healthy with no symptoms of disease infection, and 4) of uniform tree architecture. Before the trials, any existing immature cacao fruit (cherelles) and larger developing pods were removed from all trees,

Table 1. Flower counts, flowers pollinated, harvested pods and proportional harvested yield for each of the unique maternal and paternal genotype crosses and their associated pollination intensity treatments.

	Genotype		Other variables					
Trial no.	Maternal	Paternal	PI	No. of trees	Open flowers	Pollinated flowers[z]	Harvested pods	Prop. yield[y]
Trial 1.1								
	M01	PBC123	100	16	6,054	6,054	433	0.07
		Open	—	24	27,427	—	230	0.01
	PBC123	PBC123	100	16	6,465	6,465	88	0.01
		Open	—	24	21,605	—	421	0.02
	MCC02	PBC123	100	16	8,653	8,653	463	0.05
		Open	—	24	15,672	—	246	0.02
Trial 1.2								
	M01	BB01	100	16	4,244	4,244	603	0.14
		Open	—	24	4,563	—	85	0.02
	PBC123	BB01	100	16	2,769	2,769	670	0.24
		Open	—	24	2,859	—	109	0.04
	MCC02	BB01	100	16	6,169	6,169	707	0.11
		Open	—	24	2,651	—	69	0.03
Trial 2								
	M01	BB01	100	30	7,845	7,845	1,201	0.15
			80	10	2,716[x]	2,173	486	0.22
			60	10	3,527[x]	2,116	389	0.18
		Open	—	24	16,494	—	194	0.01

[z] Pollinated flowers represents the total number of open flowers counted within the pollination period (Open flowers) that were pollinated artificially.

[y] Prop. yield represents the number of pods harvested within the harvest period, expressed as 1) a proportion of pollinated flowers (for artificial pollination) and 2) a proportion of open flowers (for open pollinations) counted within the pollination period.

[x] Flower counts are approximations only, calculated using the percentage of open flowers pollinated.

PI = pollination intensity treatment.

minimizing possible resource competition and compensation effects from nonexperimental fruit.

Flowering intensity. Before flowering intensity (the number of flowers per tree per observation date) evaluations, the total branch area per tree used for experimental trials was standardized for each experimental tree. For this, the lowest 20 m of branch area was determined and the upper limits of each branch clearly marked with nontoxic red paint (tree branches were not pruned to this length). Branch material existing outside the 20-m experimental branch area or on the trunk was excluded from experimental trial. To evaluate the effects of the experimental treatments on flowering intensity within each trial period, we counted the number of newly opened flowers per tree on 10 observation dates coinciding with the artificial pollination of flowers: between 19 Dec. 2013 and 21 Jan. 2014 for trial 1.1 and between 26 Aug. 2014 and 27 Sept. 2014 for trial 1.2 (Table 1). Flower counts were conducted between 0600 and 1200 HR. Furthermore, natural flowering intensity of open-pollinated trees (see the next section for a description) was monitored continuously between July 2014 and Dec. 2016 (Supplemental Fig. 1).

Artificial pollination. To evaluate the effect of artificial pollination compared to natural pollination on harvested yield, we randomly selected 16 experimental trees per maternal genotype for an "artificial" pollination treatment, whereby 100% of all open flowers within the experimental branch area were pollinated artificially. Furthermore, 24 trees for each genotype were assigned to an "open" pollination treatment, whereby no flowers received artificial pollination. The open pollination treatment was considered to be the control.

The artificial pollination of flowers was conducted on 33 successive pollination dates between 19 Dec. 2013 and 19 Jan. 2014 for trial 1.1 and between 25 Aug. and 27 Sept. 2014 for trial 1.2. Paternal genotype pollen for the artificial pollinations was obtained by collecting freshly opened flowers of known PBC123 and BB01 trees for trial 1.1 and trial 1.2, respectively, between 0600 and 0700 HR. All collected flowers were stored in a sealed plastic container in a cool location and out of direct sunlight. Artificial pollination was conducted by removing three pollen-filled anthers (attached to stamen) from the collected paternal flowers and brushing them along the stigma of each maternal flower (Falque et al., 1995). Artificial pollination was conducted between 0600 and 1200 HR, and each artificially pollinated flower was given a pollination date tag attached to the main branch directly adjacent to the flower pedicel. On average, ≈13 flowers were pollinated per tree per day for trial 1.1 and ≈8 flowers were pollinated per tree per day for trial 1.2 (Table 1) regardless of clone. To reduce the possible effects of pollinators, pollination date and/or time on pollination success, and persons conducting the pollination were circulated randomly throughout the experimental field, and all trees were pollinated at random on each pollination date. For practicality, pollinated flowers were not isolated and artificial pollination was considered supplemental pollination.

A weather station installed at the MCRC on 22 Apr. 2014 showed no significant variation in temperature (average daily mean and average daily maximum) during the pollination period or the entire trial period of trial 1.2.

Maternal and paternal genotype cross. The potential effects of maternal and paternal genotype cross on yield was nested within the broader artificial pollination treatment. For trial 1.1, the artificial pollination of flowers (see previous section) was conducted between the paternal genotype PBC123 and the three maternal genotypes (M01, PBC123, and MCC02) (Table 1). PBC123 was selected as the original pollen donor for its reported self-compatibility (Lee et al., 1993) as well as for its dominant presence in large monoclonal, high-yielding cacao plantations within Malaysia and high popularity among smallholder Indonesian farmers. However, as a result of unexpected results in trial 1.1 indicating self-incompatibility of PBC123, clone BB01 was used as the paternal pollen donor in trial 1.2 (Table 1). Therefore, the genotype labeled PBC123 at the MCRS and used in this study may have been an off type. BB01 was selected as the pollen donor for the second trial as preliminary trials evaluating incompatibility in numerous clones from the MCRS suggested BB01 as the most cross-compatible clone available.

Harvested yield. All ripe cacao pods were harvested on six dates between 22 May and 21 July 2014 for trial 1.1 and between 30 Jan. and 17 Mar. 2015 for trial 1.2. Upon harvest, count data of all harvested pods were recorded per tree and combined as a total pod count per tree for statistical analysis. Harvested pod counts included only healthy, ripe pods and excluded large and/or mature (unripe) pods that were also removed from the experimental trees as a result of cocoa pod borer or *Phytophthora palmivora* infection.

Statistical analysis. To evaluate the effects of flowering intensity, artificial pollination, and unique genotype cross on harvested yield, harvested yield data collected from trial 1.1 and trial 1.2 were combined for analysis. For all analyses, we conducted generalized linear mixed models using the and lmer (flowering intensity) and glm (harvested yield) functions in the nlme package (Pinheiro et al., 2018) in R (version 3.2.4; R Core Team, 2013). To account for possible variation explained by paternal pollen donor and/or trial period differences, the models each included a fixed effect of trial period. To establish the significance of each fixed effect, we performed likelihood ratio tests for each fixed effect and their interactions using the lrtest function in the lmtest package (Zeileis and Hothorn, 2002) in R (R Core Team, 2013). We were unable to determine whether potential differences in the response variables were the result of the effects of trial period or pollen donor because a different pollen donor was used in each of the two successive trial periods [i.e., PBC123 (pat.) in trial 1.1 and BB01 (pat.) in trial 1.2]. Thus, these results should be interpreted accordingly.

Flowering intensity. We evaluated potential differences in flowering intensity between the maternal clone groups subjected to two differing pollination treatments (open, artificial) across two successive trial periods using a general linear mixed model. We included flowering intensity as the response variable; pollination treatment, maternal genotype, and trial period were used as the fixed effects. The models also included all possible interaction terms between the fixed effects as well as a random effect of tree to account for the repeated sampling of trees across time.

Effects of artificial pollination and genotype cross on harvested yield. As result of possible differences in pollination numbers arising from flowering variability, for the analyses, harvested pod counts relative to the number of flowers pollinated artificially (for artificial pollination) or per available flower (for open pollination), hereafter referred to as "proportional harvested yield," were used (Table 1). To evaluate the effects of artificial pollination, unique maternal × paternal genotype cross, and trial period on harvested yield, the generalized linear mixed models included proportional harvested yield as the response variable and pollination treatment, maternal genotype, and trial period as fixed effects. The models also included all possible interaction terms between the fixed effects and assumed a binomial distribution with a logit-link function. Estimates and confidence intervals were relative to the harvested yield obtained by the M01 clone under artificial pollination in trial 1.1.

Trial 2: Assessing the effects of pollination intensity and pollination synchrony on fruit set, cherelle wilt, and harvested yield in M01—a high-yielding cacao clone

Study site, clones, and environment. The study was conducted using a single clonal cacao field (pollination block II) at the MCRC from Feb. 2015 to Aug. 2015. Pollination block II is directly adjacent to pollination block I, separated only by a small road (width, ≈4 m). Pollination block II was planted using a randomized planting arrangement of 10 cacao clones, predominantly clone M01. The experimental trial was conducted using clone M01 as the maternal genotype. This clone was selected because of the adequate number of replicate trees existing at the MCRC, as well as for its wide cultivation throughout Indonesia and known production of a high number of large pods with large beans under open pollination conditions (Supplemental Table 1). The propagation techniques used, environmental characteristics of the region, tree phenology, tree nutrition, and management of pollination block II are as described for pollination block I.

Experimental trial period and tree selection. Trial 2 was conducted over a 7-month period from 3 Feb. to 22 Aug. 2015. Before the research described, no manipulations or experimentation had been conducted on the experimental trees. We selected 50 experimental trees of the M01 clone (per tree selection described earlier) that were used for artificial pollination. In addition, the 24 M01 trees that were assigned previously to the open pollination treatment in trial 1 (described earlier) were used as the controls.

Flowering intensity and artificial manipulation of pollination intensity. Two weeks before beginning trial 2, the experimental trees were, twice weekly, stripped of their existing open flowers, immature cacao fruit (cherelles), and larger developing pods to minimize possible resource competition and compensation effects from nonexperimental fruit [as in Groeneveld et al. (2010)]. Flowering intensity was determined in the same manner as described earlier, regardless of pollination treatment, and was conducted between 3 Feb. and 6 Mar. 2015 (Table 1). To examine the effects of pollination intensity on harvested yield, we conducted artificial pollination on 50 experimental cacao trees within a 31-day pollination period between 3 Feb. 2015 and 5 Mar. 2015. Each experimental tree was selected randomly for one of three pollination intensity treatments including 1) 60% of the open flowers pollinated artificially, 2) 80% of the open flowers pollinated artificially, and 3) 100% of the open flowers pollinated artificially (Table 1). Thus, pollination intensity represents the percentage of all open flowers that were pollinated artificially per tree on each pollination date. Clone BB01 was used as the paternal pollen donor for artificial pollination (Table 1). For the open pollination control trees (0%), no flowers were pollinated artificially.

After flower counting on each pollination date, the number of flowers to be pollinated artificially was determined at the branch level as a percentage of all open flowers on a given branch, according to the pollination intensity treatment assigned. This method was applied to all main branches within each tree. Flowers on each branch were then selected at random and pollinated artificially as described earlier. Because of rainfall events on 7 days within the pollination period, pollination was conducted for 24 d. No significant variation in temperature (average daily mean and average daily maximum) was observed during the pollination period or trial period of trial 2.

Pollination synchrony. We describe pollination synchrony as the temporal distribution of artificial pollination within a 5-week pollination period. All flowers that were pollinated artificially were given unique tags, each coded by pollination date and pollination week (i.e., weeks 1 5). These unique date tags were used to evaluate the success of pollinated flowers and developing cherelles (see next section). The importance of pollination synchrony was determined using the fruit-setting success, as well as the relative survival of cherelles and harvested pods for each of the five pollination weeks.

Cherelle development. The development of cherelles set by artificial pollination was monitored weekly until either cherelle wilt (abortion) or the harvest of a matured pod, with the first monitoring event on 7 Feb. 2015, 4 days after the first pollination date. Cherelle development was monitored by counting the number of surviving cherelles per tree present on each monitoring occasion, grouped by pollination week for practicality. Because cherelle set by natural pollination may incur additional resource competition with and/or between developing cherelles set by artificial pollination, we also tagged and monitored any cherelles set by natural pollination.

Harvested yield. The harvesting of cacao pods in trial 2 was conducted fortnightly during an 8-week harvest period between 27 June 2015 to 22 Aug. 2015 upon observation of ripe pods. Pod harvesting was conducted as described earlier.

Statistical analysis. To evaluate the effects of pollination intensity (60%, 80%, or 100%) and pollination synchrony (pollination weeks 1 5) on fruit development, cherelle survival was analyzed using three distinct time periods including 1) fruit set [0 25 d after pollination (DAP)], 2) cherelle wilt (26 160 DAP), and 3) harvest (161 180 DAP). Cherelle survival was standardized to begin from the date that each flower was pollinated artificially (0 DAP) and was expressed as a proportion relative to the total number of flowers pollinated artificially per tree per pollination date and within each of the five pollination weeks.

Fruit set and harvested yield. To evaluate the effects of pollination intensity and synchrony on proportional fruit set and proportional harvested yield, generalized linear mixed models using the glmer function in the lme4 package (Bates et al., 2015) in R (R Core Team, 2013) were conducted using either 1) the proportion of surviving cherelles within the fruit set period (0 25 DAP) or 2) the proportional harvested yield as the response variables. The fixed effects in the

Table 2. General (flowering intensity) and generalized (harvested yield) linear mixed-model results for flowering intensity and harvested yield in trial 1.

	Flowering intensity			Proportional harvested yield		
Fixed effect[z]	χ^2	df	*P* value[y]	χ^2	df	*P* value[y]
Pollination treatment	77.90	9	<0.0001	102.41	1	<0.0001
Maternal genotype	19.10	8	<0.0001	41.58	2	<0.0001
Trial	812.95	9	<0.0001	18.72	1	<0.0001
Pollination treatment × Maternal genotype	23.98	12	<0.0001	54.07	2	<0.0001
Pollination treatment × Trial	1493.40	12	<0.0001	0.012	1	0.9115
Maternal genotype × Trial	49.86	12	<0.0001	67.28	2	<0.0001
Pollination treatment × Maternal genotype × Trial	116.42	14	<0.0001	22.34	2	<0.0001

[z] Fixed effects included pollination treatment (open, artificial), maternal genotype (M01, PBC123, MCC02), and trial period (trial 1.1 and trial 1.2). The models included both two-way and three-way interaction terms between fixed effects.

[y] All *P* values were calculated using test statistics derived from the mean and variance.

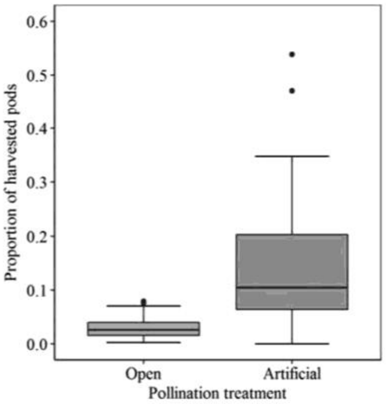

Fig. 1. Proportional harvested yield for each of the two pollination treatments (open, artificial). For the open pollination treatment, harvested yield represents the proportion of available flowers counted that were harvested as pods. For the artificial pollination treatment, harvested yield represents the proportion of pollinated artificially flowers that were harvested as pods. Here, harvested yield data for each unique genotype cross was grouped by pollination treatment.

models included 1) pollination intensity treatment, 2) pollination week, and 3) a pollination intensity-by-pollination week interaction term (used in the analysis of fruit set only as a result of nonconvergence for the interaction term at harvest). Because individual trees were measured repeatedly across time, a random effect of tree was included in the models. The models each assumed a binomial distribution with a logit-link function. To establish the significance of each fixed effect, we performed sequential likelihood ratio tests for each fixed effect and their interactions using the lrtest function in the lmtest package (Zeileis and Hothorn, 2002) in R (R Core Team, 2013).

Cherelle wilt. To evaluate the effects of pollination intensity and synchrony on cherelle survival during the cherelle wilt period (26 160 DAP), a quadratic linear regression was fit to the data using the lm function in R (R Core Team, 2013). The proportion of surviving cherelles was used as the response variable and the main effects included a categorical covariate effect for pollination treatment, a categorical effect for pollination week, as well as effects describing the linear number of days after pollination and the square of days after pollination. The goodness of fit was described using the model's R^2 value.

Results

Trial 1 results

Flowering intensity. Flowering intensity differed between trees subject to each of the two pollination treatments (χ^2 = 77.90, df = 9, $P < 0.0001$; Table 2) and pollination effects depended on the maternal genotype and trial period, as shown by the significant interaction terms between pollination treatment and maternal genotype (χ^2 = 23.98, df = 12, $P < 0.0001$; Table 2) and trial period (χ^2 = 812.95, df = 9, $P < 0.0001$; Table 2). Across both trial periods, mean flowering intensity per tree per day was greatest under open pollination conditions (52, n = 24; Table 1) compared to artificial pollination (22, n = 16; Table 1), suggesting a negative effect of artificial pollination on flowering intensity. We also observed a significant three-way interaction between pollination treatment, maternal genotype, and trial period (χ^2 = 116.42, df = 14, $P < 0.0001$). Across both trials, trees under artificial pollination conditions and maternal genotype MCC02 produced, on average, more flowers per tree per day (29, n = 16; Table 1). However, under open pollination conditions and across both trials, it was maternal genotype M01 that produced the greatest mean flowering intensity (67, n = 40; Table 1). Flowering intensity, regardless of pollination treatment and maternal genotype was greater in trial 1.2 compared to trial 1.1 (Table 1). This may have been a result of seasonality or possible indirect effects on flowering caused by the pollen donor.

Effects of artificial pollination and genotype cross on harvested yield. Across all clones, a total of 34,354 flowers were pollinated artificially during the two successive trials periods, which led to 2964 artificially pollinated ripe pods being harvested (Table 1). A highly significant effect of the pollination treatment was found on harvested yield (χ^2 = 102.41, df = 1, $P < 0.0001$; Fig. 1, Table 2), in which artificial pollination showed a significant positive effect on yield (Fig. 1, Table 2). For trees assigned to the open pollination treatment, 1160 ripe pods were harvested during the two successive trials periods (Table 1).

We found highly significant effects of trial period (χ^2 = 18.72, df = 1, $P < 0.0001$; Fig. 2, Table 2) and maternal genotype (χ^2 = 41.58, df = 2, $P < 0.0001$; Fig. 2, Table 2) on harvested yield. This may be largely driven by differences in compatibility between unique maternal and paternal genotype crosses (Fig. 2), as shown by the significant maternal genotype × trial period interaction term (χ^2 = 67.28, df = 2, $P < 0.0001$; Fig. 2, Table 2), as a different pollen donor was used in each trial period. However, the significant pollination treatment × maternal genotype × trial period three-way interaction term (χ^2 = 22.34, df = 2, $P < 0.0001$; Fig. 2, Table 2) may indicate that the effects of artificial pollination, regardless of pollen genotype, varied across the two trial periods for each of the maternal clone groups and, thus, additional factors unrelated to genotype compatibility, such as environmental factors, may be influencing harvested yield.

The greatest harvested yield (24% of pollinated flowers survived to harvest) was obtained from the PBC123 (mat.) × BB01 (pat.) genotype cross (Fig. 2, Table 1). Furthermore, harvested yield in each maternal genotype was always greater when BB01 was used as the pollen donor (Fig. 2, Table 1).

Trial 2 results

Pollination intensity and synchrony. A total of 12,134 flowers were pollinated artificially across the 50 experimental trees assigned to the artificial pollination treatment during trial 2, regardless of pollination intensity (Table 1). This led to the harvest of 2076 ripe pods during trial 2 (Table 1). For artificially pollinated trees, cherelles arising from natural pollination were observed only in the 60% (n = 798 cherelles) and 80% (n = 486 cherelles) artificial pollination treatments. However, no naturally pollinated cherelles observed on artificially pollinated trees survived to harvest (cherelles wilted). Therefore, cherelle survival and harvested yield data were expressed relative to artificial pollination only. For trees assigned to the open pollination treatment, 194 ripe pods were harvested during the harvest period of trial 2 (Table 1).

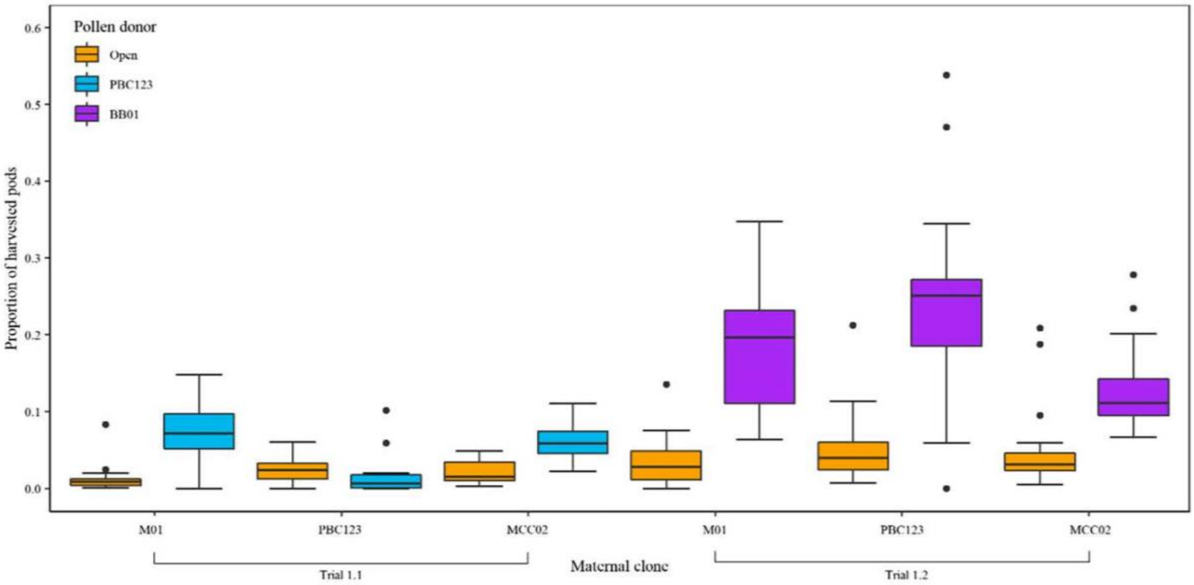

Fig. 2. Proportional harvested yield for each of the unique maternal and paternal genotype crosses. Harvested yield represents the number of harvested pods per maternal clone (M01, PBC123, MCC02) as a proportion of 1) the number of open flowers produced per tree during the pollination period for trees assigned to the open pollination treatment (open, orange) or 2) the number of flowers pollinated artificially [trees pollinated artificially by paternal pollen donors PBC123 (blue) or BB01 (purple)] from each of two successive trial periods (trial 1.1 and trial 1.2).

Fruit set (0 25 DAP). A significant effect of pollination intensity treatment ($\chi^2 = 13.00$, df = 2, $P = 0.001$; Fig. 3A, D, G) and pollination week ($\chi^2 = 267.93$, df = 4, $P < 0.0001$; Fig. 3A, D, G) was found on cherelle survival during the fruit set period, as well as a highly significant interaction effect between pollination intensity treatment and pollination week ($\chi^2 = 718.30$, df = 8, $P < 0.0001$; Fig. 3A, B, D, E, G, H).

Seven of the eight possible pairwise comparisons between the 100% pollination intensity in pollination week 1 and the 60% and 80% pollination intensity treatments and the remaining four pollination weeks showed a statistically significant effect on the proportion of surviving cherelles (Fig. 3G, Table 3).

Cherelle wilt (26 160 DAP). We observed significant linear and squared components of the quadratic relationship between the proportion of surviving cherelles within the cherelle wilt period and the number of days after pollination (F = 3229.80, df = 1, $P < 0.001$; Fig. 3B, E, H) and the square of the number of days after pollination (F = 52.04, df = 1, $P < 0.001$) for each of the three pollination intensity treatments. We also found a significant effect of pollination intensity treatment (F = 223.02, df = 2, $P < 0.0001$; Fig. 3B, E, H) and pollination week (F = 344.50, df = 4, $P < 0.0001$; Fig. 3B, E, H) on cherelle survival within the cherelle wilt period. Across all pollination intensity treatments, cherelle survivorship was greatest for cherelles originating in pollination week 1, with decreasing survivorship in each successive week (Fig. 3B, E, H). Only cherelle survival for cherelles originating in pollination week 2 was found not to be significantly different from pollination week 1, whereas cherelle survival for cherelles originating in weeks 3, 4, and 5 was significantly less than that of pollination week 1. In the model, increases in both the pollination week and the number of days after pollination caused a decreasing proportion of surviving fruit. The model displayed a high adjusted R^2 value of 0.80.

Harvested yield. A significant effect of pollination intensity treatment ($\chi^2 = 22.51$, df = 2, $P < 0.0001$; Fig. 3C, F, I) and pollination week ($\chi^2 = 1091.50$, df = 4, $P < 0.0001$; Fig. 3C, F, I) was found on harvested pod counts, with the model fitting both effects displaying a lower Akaike information criterion value. For this model, we observed that although the pollination intensity treatment effect displayed equal variance, there were unequal variances across pollination weeks, with the residuals following the trend of the harvest data (Fig. 3C, F, I). Contrasts between each of the fixed effects were estimated relative to the 100% pollination treatment and pollination week 1 (Table 4). The 80% pollination intensity treatment displayed the greatest proportion of harvested pods relative to the number of artificial pollination treatments (Table 1). Overall, the greatest harvested yield was obtained from pods originating in pollination week 1 (Fig. 3C, F, I), regardless of pollination intensity treatment. Moreover, we found a clear decreasing trend in the proportion of surviving cherelles and the number of harvested pods, with increasing pollination week (Fig. 3).

Discussion

Artificial pollination and genotype cross effects. In both trials, artificial pollination significantly increased the harvested yield. Open pollination resulted in low flower-to-fruit conversion rates, supporting previous findings documenting pollination as a major extrinsic factor limiting cacao production (Falque et al., 1995, 1996; Groeneveld et al., 2010; Young, 1982, Young and Severson, 1994). Interestingly, the observed yield benefits of artificial pollination for the maternal genotypes varied by paternal genotype in trial 1. Such variation in fertilization success may be the result of physiological differences between the pollen donors (Campbell and Halama, 1993), perhaps regulated by other genes not directly involved with cross-compatibility. We also observed large yield variation between different maternal genotypes when pollinated using the same pollen donor. For example, the benefit of artificial pollination for the cross PBC123 (mat.) × BB01 (pat.) was double the benefit observed for MCC02 (mat.) × BB01 (pat.). These observed maternal effects may be the result of genotype-specific variation in physiological parameters that contribute to pod development, such as photosynthetic rate, which may lead to different pod carrying capacities and harvested yields for the maternal genotypes tested (Daymond et al., 2011). The variation between maternal genotypes in their

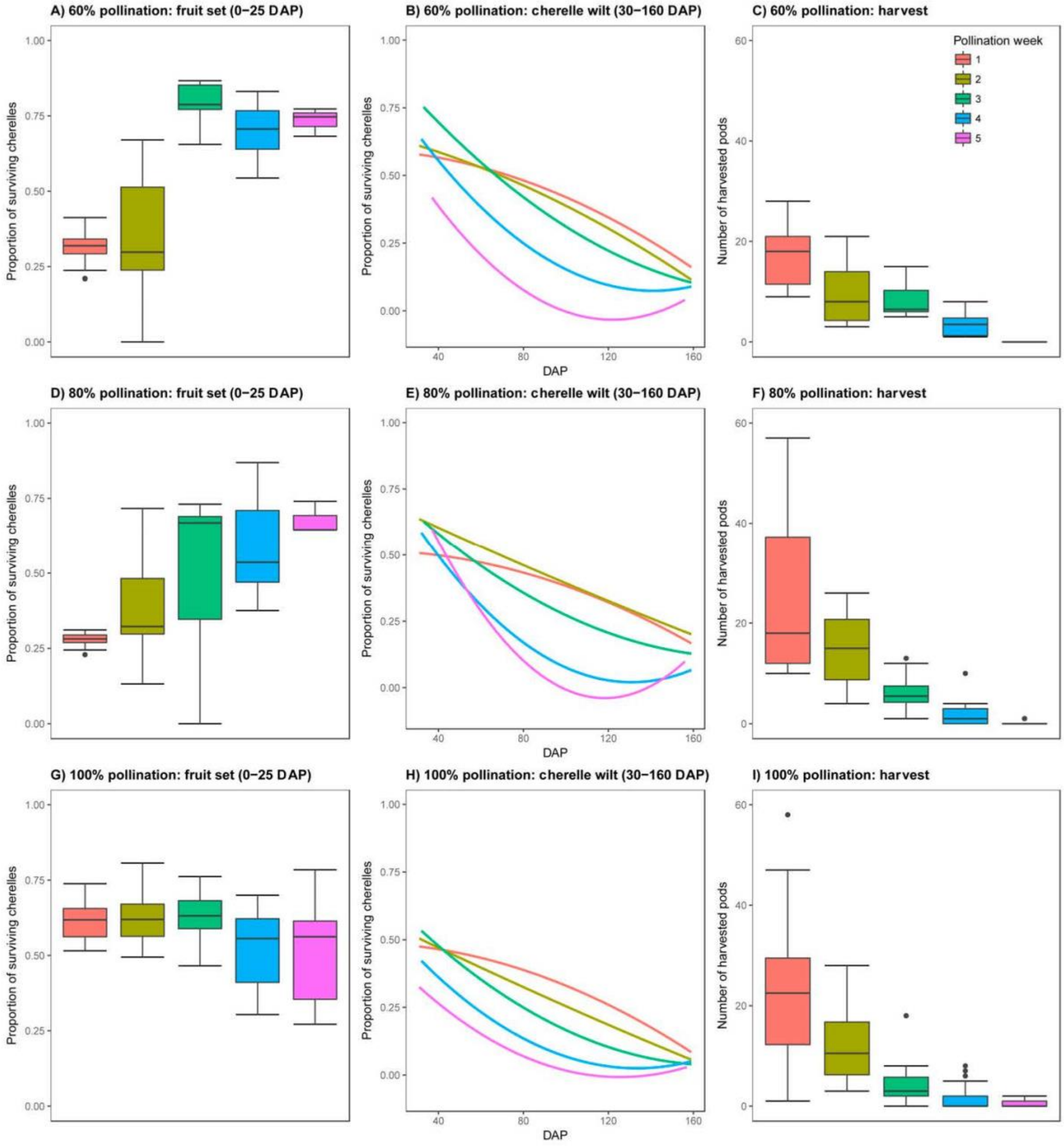

Fig. 3. The mean proportion of surviving fruits for each of the three pollination intensity treatments [(**A–C**) 60% pollination, (**D–F**) 80% pollination, (**G–I**) 100% pollination], originating in each of the five pollination weeks (pollination weeks 1–5 represented by red, gold, green, blue, and purple, respectively) during three distinct fruit developmental stages including fruit set [0–25 days after pollination (DAP) (**A, D, G**)], cherelle wilt [26–160 DAP (**B, E, H**)], and harvest (**C, F, I**). Boxplots are used for the representation of fruit set [0–25 DAP (**A, D, G**); Table 2] and harvest (**C, F, I**; Table 3) data, whereas a quadratic linear regression was fit to represent cherelle wilt [26–160 DAP (**B, E, H**)] data (see trial 2 results; cherelle wilt, 26–160 DAP).

responses to artificial pollination highlights that our results cannot be generalized for all cacao clones, and the benefits of artificial pollination remain genotype-cross specific.

Pollination synchrony. The most novel finding of our study is that we found a significant effect of pollination synchrony on cherelle survivorship during periods of fruit set, cherelle wilt, and harvest. Cherelles originating within earlier pollination weeks (older cherelles) had a progressively greater survivorship than those originating in later pollination weeks (younger cherelles). The greatest proportional harvested yield obtained arose from clustered artificial pollination treatments early during the pollination period, suggesting that synchronized pollination events may maximize cacao yield. Our results support the earlier findings of Hutcheon (1977), who found increased cherelle wilt toward the end of the cropping season and suggested that between-pod competition for a declining reserve of assimilate resources was the causal mechanism. Similar findings

Table 3. Generalized linear mixed-model results for proportional fruit set in trial 2.

Effect[z]	Parameter estimate	SE	z Value	*P* value[y]
Intercept	0.51478	0.06412	8.028	<0.0001
Pollination intensity, 60%	−0.56381	0.12827	−4.396	<0.0001
Pollination intensity, 80%	−0.9502	0.12780	−7.435	<0.0001
Pollination week 2	0.05117	0.03492	1.465	0.1428
Pollination week 3	0.08166	0.04313	1.893	0.0583
Pollination week 4	−0.18092	0.04267	−4.240	<0.0001
Pollination week 5	−0.35617	0.05122	−6.953	<0.0001
Pollination intensity (60%) × Pollination week 2	0.25144	0.07253	3.467	<0.0001
Pollination intensity (60%) × Pollination week 3	0.69936	0.09624	7.267	<0.0001
Pollination intensity (60%) × Pollination week 4	0.77152	0.08249	9.353	<0.0001
Pollination intensity (60%) × Pollination week 5	1.10363	0.17099	6.454	<0.0001
Pollination intensity (80%) × Pollination week 2	0.10092	0.07063	1.429	0.1531
Pollination intensity (80%) × Pollination week 3	1.63755	0.09487	17.262	<0.0001
Pollination intensity (80%) × Pollination week 4	1.58153	0.09415	16.798	<0.0001
Pollination intensity (80%) × Pollination week 5	1.82103	0.14097	12.918	<0.0001

[z] Fixed effects included pollination intensity treatments and pollination week. The intercept was the 100% pollination intensity treatment during pollination week 1.

[y] All *P* values were calculated using test statistics derived from the mean and variance.

Table 4. Generalized linear mixed-model results for proportional harvested yield in trial 2.

Effect[z]	Parameter estimate	SE	z Value	*P* value[y]
Intercept	−1.27616	0.08896	−14.346	<0.0001
Pollination intensity, 60%	0.80037	0.16996	4.709	<0.0001
Pollination intensity, 80%	0.52703	0.16875	3.123	0.00179
Pollination week 2	−0.24867	0.05183	−4.798	<0.0001
Pollination week 3	−1.00385	0.06950	−14.444	<0.0001
Pollination week 4	−2.15847	0.10347	−20.861	<0.0001
Pollination week 5	−3.27340	0.28097	−11.650	<0.0001

[z] Fixed effects included pollination intensity treatments and pollination week. The intercept was the 100% pollination intensity treatment during pollination week 1.

[y] All *P* values were calculated using test statistics derived from the mean and variance.

have been reported in maize (*Zea mays* L.), where synchronized artificial pollination increased kernel set and yield (Carcova et al., 2000; Carcova and Otegui, 2001; Freier et al., 1984; Harris et al., 1976; Sarquis et al., 1998; Struik and Makonnen, 1992). However, access to and competition for a limited supply of local assimilates is also a major driver of postfertilization kernel abortion (Shen et al., 2018).

In cacao, competition for assimilate resources can exist both between structural types (such as between flowers and fruit) or within structural types (such as between developing pods). Flowering intensity (Valle et al., 1990), cherelle survival (Wood and Lass, 1985), and the number and size of seeds per pod (Falque et al., 1995) are all reported to decrease with an increasing number of developing pods, resulting from the competition for resources. Thus, the observed increased survivorship of cherelles originating from clustered pollination events within earlier pollination weeks may be the result of high resource availability at the time of pollination paired with low competitive hierarchy among cherelles. This increased survivorship of older cherelles generally suggests that older fruit (larger resource sinks) may exert dominance in the sequestration of limited assimilates. This is also supported by the observed abortion of all naturally pollinated cherelles in our trial 2. However, it is also possible that the increased survivorship of older cherelles is the result of an alteration in the maternal plants' pattern of assimilate partitioning, mediated by synchronized pollination events in which the maternal tree invests resources preferentially to structures that are more likely to mature, thus selectively aborting weaker structures (De Jong and Klinkhamer 2005; Uma Shaanker et al., 1988; Stephenson 1981). Alterations in the pattern of assimilate partitioning arising from synchronous pollination have been reported previously in maize (Lafitte and Edmeades, 1995), and further investigation of this potential physiological phenomenon in cacao is recommended.

Pollination intensity. Pollination intensity effects were dependent on the pollination week. The interaction between pollination intensity and pollination week observed for trees assigned to the 60% and 80% pollination intensity treatments may be a result of fewer pollinated flowers per tree during the earlier pollination weeks (compared to the 100% treatment). Here, weak sink effects may be underlying the lower fruit set rates during times of presumably unlimited resources and may be further exacerbated by low flowering intensity during the earlier pollination weeks. When flowering intensity is low, increased pollination intensity appears advantageous to fruit set, possibly by establishing a stronger resource sink (e.g., an increased number of developing cherelles) driving the internal plant physiological processes (i.e., assimilate production and/or remobilization, hormone production) that support the fertilization success of subsequently pollinated flowers (Arathi, 2011).

Despite the 100% pollination intensity treatment involving the greatest proportional fruit set (0 25 DAP), the 80% pollination intensity treatment produced the greatest proportional harvested yield. These results indicate that, although the benefits of increased pollination intensity on yield may erode as cherelle wilt increases, this offset may be only partial. The total number of pods harvested per tree in trial 2 remained greatest in the 100% pollination intensity treatment despite increasing rates of cherelle wilt, still reflecting the benefits of enhanced artificial pollination. Future research efforts should evaluate the effects of potential interactions between the reproductive sink size, resource availability, the proximity of developing cherelles to assimilate sources, and resource competition within/between plant parts on fruit development in cacao.

In situations in which pollination rates are of concern and when labor costs allow, artificial pollination management strategies have been implemented for cacao (Francis K. Padi, C.R.I.G., 2018, personal communication) as well as other perennial crops (Pinillos and Cuevas, 2008). A hand-pollination program launched in 2017 by the Cocoa Health and Extension Division of the Ghana Cocoa Board observed greater yields than the previous year (Francis K. Padi, C.R.I.G., 2018, personal communication). However, yields of nonpollinated plots during this time were not monitored simultaneously, making actual yield increases difficult to estimate (Francis K. Padi, C.R.I.G., 2018, personal communication). The group's future efforts to compare artificially and naturally pollinated cacao plots have the potential to quantify the yield benefits of adopting artificial pollination strategies in low-input, smallholder cacao systems using seed-grown hybrid planting materials (Francis K. Padi, C.R.I.G., 2018, personal communication). These yield benefits may be further enhanced if paired with practices that aim to increase pollination synchrony and also consider cacao tree phenology, physiology, and compatibility. For example, by intensively clustering artificial pollination events within a short time period (≈7 14 d) when flowering intensity is high and competition between developing fruit is low, maximized crop productivity for minimal manual labor inputs may be achieved. However, understanding clone compatibility is vital for the evaluation of such efforts. The temporal clustering of artificial pollinations may lead to synchronous pod ripening, condensed harvest events, subsequent increases in harvesting efficiency, and potential quality improvements resulting from the more homogeneous physiological status of the beans to be fermented. Researching these effects will build upon the research presented here. In situations in which conducting artificial pollination is impractical or unachievable, adopting farm management strategies to enhance and support natural pollinator populations (e.g., the provision or organic substrates for pollinator oviposition and larval development) matched with strategies to increase flowering intensity may also lead to increases in crop productivity and harvesting efficiency.

In conclusion, genetically and agronomically driven strategies in cacao cultivation systems that aim to increase flowering, pollination rates, and pollination synchrony may increase productivity in cacao. Whether the effects of such strategies are reflected in final crop yield likely depends on a wide range of site-specific environmental factors (e.g., nutrient availability, pest pressure, local management, climate) as well as the context-dependent fruit-bearing capacity of the cultivars in use (Bos et al., 2007). Thus, artificial pollination management strategies to increase cacao yields should be evaluated further using genetically diverse genotypes that are self- or cross-compatible, and conducted simultaneously with approaches aimed toward improving postpollination pod retention, overall plant health, and resilience to environmental perturbations. This is important for ensuring the sustainability of the cacao industry and farmer livelihoods, particularly given the increased frequency and severity of climate change-related environmental perturbations (Grassini et al., 2013; Ray et al., 2015). In intensified cacao cultivation systems involving continuous artificial pollination to produce unnaturally high fruit loads, cacao trees may be susceptible to resource reserve depletion, particularly when resources are limiting (Hutcheon, 1977). Assimilate resources may become an increasingly limiting factor of yield, apparent only after numerous years (Muller et al., 1988). For this reason, future research efforts to address the effects of intensive yield-increasing strategies should also be evaluated across multiple years to determine the long-term sustainability of enhancing tree productivity.

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Supplemental Table 1. Clonal evaluations of yield parameters.[z]

	Pod and bean parameters					
Clone	Avg pods/tree/yr	Avg dry bean wt/pod (g)	Avg dry bean wt (g)	Avg no. of beans/100 g	Pod index[y]	Yield[x] (t/ha/yr)
M01	57.23	58.4	1.48	67.37	17.06	3.69
PBC123	54.94	35	0.86	115.85	28.49	2.12
MCC02	39.59	57.2	1.61	62.11	17.49	2.49
BB01	42.59	46.1	1.27	78.55	21.62	2.17

[z] Values are calculated from several clone evaluation trials between 2009 to 2016. The evaluations involve 100 mature trees per clone, save the MCC02 evaluation which involved 3000 trees. All trees involved in clonal evaluations were propagated by terminal grafting on M01 rootstocks and were cultivated in South Sulawesi, Indonesia.

[y] Pod index is calculated as the number of pods required to produce 1000 g dry cacao beans.

[x] Annual yield per hectare is calculated using a 3 m × 3 m planting density.

Supplemental Table 2. Analysis of soils at the Mars Cocoa Research Center, Tarengge, South Sulawesi.[z]

Soil measurements											
pH	OM (%)	C (%)	N (%)	Texture	P (ppm)	K ($cmol{\cdot}kg^{-1}$)	Ca ($cmol{\cdot}kg^{-1}$)	Mg ($cmol{\cdot}kg^{-1}$)	Na ($cmol{\cdot}kg^{-1}$)	CEC ($cmol{\cdot}kg^{-1}$)	BS (%)
4.6	2.4	1.4	0.2	Clay/loam	5.8	0.1	1.0	1.8	0.05	12.3	24.7

[z] Analyses performed in 2016 at laboratories of CIRAD, Montpellier, France [Spaans (2016, unpublished)], and Hasanuddin University (UNHAS), Indonesia (Mars Inc., internal report, unpublished data).

OM = organic matter; CEC = cation exchange capacity; BS = base saturation.

Supplemental Table 3. Nutrient composition of fertilizer applied each month to experimental trees in trial 2.[z]

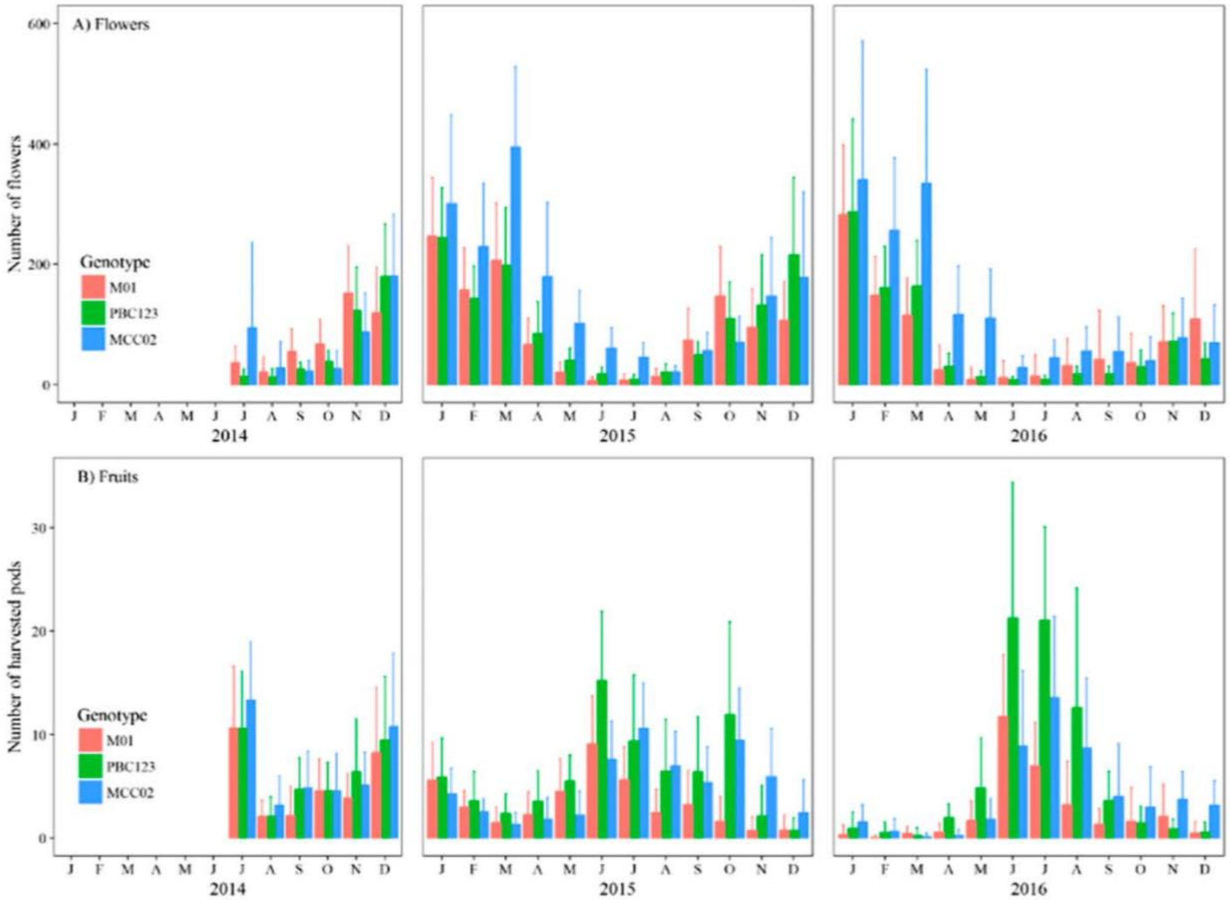
	Month											
Nutrient ($kg{\cdot}ha^{-1}$)	Jan.	Feb.	Mar.	Apr.	May	June	July	Aug.	Sept.	Oct.	Nov.	Dec.
N[y]	70.90	74.3	64.10	33.8	33.8	60.8	70.90	74.3	64.1	33.8	33.8	60.6
P	29.5	18.6	18.3	9.7	11.3	29.0	27.3	18.6	18.3	9.7	11.3	28.9
K	64.5	37.2	71.2	84.5	75.1	71.5	64.5	37.1	71.2	84.5	75.1	71.3
Ca	11.7	6.2	12.9	15.4	13.7	13.0	11.7	6.2	12.9	10.6	13.7	13.0
Mg	3.3	2.5	3.7	3.8	3.4	3.3	3.3	2.5	3.7	4.2	3.4	3.7
S[x]	7.1	7.4	6.4	3.4	3.4	6.1	7.1	6.2	6.4	2.8	3.4	6.1
Zn	0.3	0.2	0.2	0.1	0.1	0.3	0.3	0.2	0.2	0.1	0.1	0.3
B[w]	0.4	0.4	0.3	0.2	0.4	0.7	0.4	0.4	0.3	0.2	0.4	0.7
Cu	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Mn	0.4	0.2	0.4	0.5	0.4	0.4	0.4	0.2	0.4	0.5	0.4	0.4
Fe	0.5	0.3	0.6	0.7	0.7	0.6	0.5	0.3	0.6	0.7	0.7	0.6

[z] Data obtained from Spaans (2016).

[y] Source of nutrients includes Mono-Ammonium Phosphate, Potassium nitrate, and Calcium nitrate.

[x] Source of nutrients includes Magnesium sulfate and Zinc sulfate.

[w] Source of nutrients is Boric acid.



Supplemental Fig. 1. Mean flowers (**A**) and mean fruit (**B**) counted per tree for three cacao genotypes between July 2014 and Dec. 2016. The data shown represent mean values of 24 trees/clone (M01, PBC123, MCC02) existing within a clonal cacao field (pollination block I) at the Mars Cocoa Research Center, Tarengge Village, South Sulawesi, Indonesia. (**A**) Flower data were obtained from the total number of open flowers observed per tree within the dates presented, counted twice per week between 0600 and 1200 HR. (**B**) Fruit data were obtained from the total number of ripe pods per tree, harvested fortnightly within the dates presented.

Chapter 5. Artificial Pollination Impacts Crop Size, Fruit Mass, Seed Quality and Production Sustainability in Cacao (*Theobroma cacao*).

5.1 Abstract

Cacao is widely grown in tropical agroecosystems and cacao fruit production is frequently pollination limited. Artificial pollination is a potential way to increase the short-term yield of cacao but optimized artificial-pollination strategies for in-field application remain poorly developed and have unknown potential consequences on seed quality and longer-term tree productivity. For example, it is unknown whether artificial pollination activities should be condensed or spread more evenly across the flowering period. Furthermore, there may be a threshold at which pollinating an excessively high number of flowers at the same time could lead to short- and long-term declines in yield. In this study, I artificially manipulated pollination intensity at three levels (30, 60, 90 total artificial pollinations per tree, per year), and varied the time window for artificial pollinations within a 3-week pollination period using five different pollination timing levels (Middle, Early, Late, All, Split). I evaluated pollination treatment effects on cherelle survival, harvested pods, individual pod fresh mass, bean dry mass, and bean fat content. This study identified multiple physiological trade-offs at the individual level in the impacts of artificial pollination management in cacao. Firstly, increasing pollination intensity increases the number of fruit produced but reduces fruit size and quality. Secondly, narrowing the pollination time window reduces cherelle (immature fruit) wilting and increases fruit survival and efficiencies, but can lead to reduced production in later years due to associated overcropping. My findings highlight that short-term artificial pollination strategies for improving cacao production that increase pollination intensity or reduce the pollination time window need to be considered in light of potential negative long-term effects.

5.2 Introduction

Natural animal-mediated pollination is an essential ecosystem service for both wild plant communities (Ashman et al., 2004) and agricultural production (Klein et al., 2007). Approximately 87.5% of wild plants benefit from insect pollination (Ollerton et al., 2011) and 75% of cultivated crops globally are estimated to benefit from pollination services provided by animals (Klein et al., 2007). However, ‘pollination limitation’, also referred to as pollination failure or pollination deficit, occurs in pollinator-dependent crops when an insufficient quality or quantity of pollen is deposited on the flowers’ stigma (Wilcock and Neiland, 2002). Pollination limitation is often associated with pollinators that are too few, or in highly specialised species-specific pollination-dependent plants (Wilcock and Neiland, 2002). Pollination limitation can have large implications for agricultural production (Wilcock and Neiland, 2002; Lippert et al., 2021; Wurz et al., 2021). Pollination limitation within a species is generally characterized after experimental results show decreased fruit and/or seed production under natural conditions compared to adequate / optimal pollination conditions (Ashman et al., 2004; Knight et al., 2005; Holzschuh et al., 2012; Miñarro and Twizell, 2015). A variety of factors are reported to contribute to the presence of pollination limitation, such as pollinator declines (Buchmann and Nabhan, 1996; Kremen and Ricketts, 2000; Kremen et al., 2002; Ricketts et al., 2004; Appiah and Agyei-Dwarko, 2013), inefficient pollinators (Ashman and Morgan, 2004), variability in floral phenotypes (Young and Severson, 1994; Knight et al., 2005), seasonality (Frimpong-Anin et al., 2014) pollen genetic origin (Falque et al., 1995; Akoa et al., 2021), pollen viability (García Talledo et al., 2019), and the quantity of deposited pollen (Falque et al., 1995). Changing climatic conditions can further amplify the presence and intensity of pollination limitations, for example, global warming can cause mismatches between plants and their pollinators, with consequences on plant-pollinator interactions (Hegland et al., 2009).

To overcome pollination limitation, interventions such as pollinator habitat management (Adjaloo et al., 2013; Bridgemohan et al., 2017; Forbes and Northfield, 2017; Vanhove et al., 2020), artificial pollination (Wurz et al., 2021) and automated pollination (Li et al., 2022) can be used to enhance or replace natural pollination services. Artificial pollination is also referred to as hand, controlled, assisted, manual or supplementary pollination (Hegland et al., 2009; Wurz et al., 2021). Artificial pollination holds the potential to increase fruit set, harvested yield and improve crop quality by controlling for pollen quantity, quality, genetic origin, as well as the frequency and timing of pollination (Pinto et al., 2005; González et al., 2006; Pritchard and Edwards, 2006).

Cacao (*Theobroma cacao* L.; Malvaceae) is a cauliflorous, tropical perennial tree species that exhibits 'surplus flowering', with some varieties known to produce up to 125,000 flowers per tree per year (Lachenaud and Mossu, 1985). However, it is generally accepted that less than 10% of these flowers are naturally pollinated, and only 0.5 - 5% of pollinated flowers survive to reach fruit (pod) maturity (Falque et al., 1995; Aneja et al., 1999; Bos et al., 2007; De Almeida and Valle, 2007). Indeed, very low fruit-to-flower ratios are reported for cacao (Aneani and Ofori-Frimpong, 2013), and low production is experienced by cacao farmers in many countries (Deheuvels et al., 2012; Gyau et al., 2015; Schroth et al., 2016; Mena-Montoya et al., 2020). Pollination limitation is often considered to be the most important yield-driving factor in cacao, independent of nutrient availability or other environmental stressors (Falque et al., 1995; Groeneveld et al., 2010; Toledo-Hernández et al., 2017; Wanger et al., 2021).

The artificial (hand) pollination of cacao flowers is increasingly being explored in many cacao producing regions as a yield-enhancing agronomical intervention to overcome such pollination limitation (Vera-Chang et al., 2016; Forbes and Northfield, 2017; Forbes et al., 2019; Toledo-Hernández et al., 2020). This practice may be particularly useful for large-scale monoclonal plantings of self-incompatible cacao varieties. Artificial pollination to enhance pollination intensity has been shown on many occasions to increase cacao fruit set and yields (Muntzing, 1947; Valle et al., 1990; Falque et al., 1995; Falque et al., 1996; Bos et al., 2007; Forbes and Northfield, 2017; Toledo-Hernández et al., 2017; Forbes et al., 2019; Toledo-Hernández et al., 2020; Akoa et al., 2021; Toledo-

Hernández et al., 2023; Vansynghel et al., 2023), regardless of fertilizer application (Groeneveld et al., 2010), sometimes tripling yields and increasing cacao farmers' incomes by up to 69% (Toledo-Hernández et al., 2023). Adding to this, previous work by Forbes et al. (2019) suggested that when enhancing pollination intensity for yield benefits, fruit survivorship may be influenced by the specific timing of the artificial pollination event within a defined pollination period. An interesting observation that requires further investigation. However, the abovementioned studies evaluate the short-term and may not capture the long-term interactive effects between enhanced pollination and resource availability. The physical characteristics of cacao beans (e.g., fat content) also remain unevaluated in most studies (Falque et al., 1995; but see Falque et al., 1996), despite being crucial aspects of cocoa industry quality requirements (CAOBISCO, 2015). Deeper insight on the applied components of artificial pollination, such the intensity and timing of artificial pollinations and their effects on cacao production, is needed to ensure any recommended interventions to ameliorate pollination limitation in cacao are achievable, profitable, and sustainable. Thus, while great potential benefits of artificial pollination approaches to cacao production exist, the central question of *how* to optimize these approaches for combined benefits to fruit number, seed quality, production efficiency and long-term sustainability remains, as physiological trade-offs at the individual level are expected to exist between fruit and/or seed number and quality, between annual and longer-term yield production, and between pollination application costs (e.g., labour) and overall production efficiency benefits.

To further elucidate the potential effects of variable levels of combined pollination intensity and pollination timing treatments on cacao yield and quality parameters, I conducted a 3-year artificial pollination study using single high-yielding cacao clone within an intensively managed cacao cultivation system in Sulawesi, Indonesia. I sought to answer the following questions: Does artificially enhanced pollination intensity and narrowed pollination timing windows increase cacao yield and quality? How do these two factors interactively impact cacao yield and quality? What is the best combination of these two treatment factors for cacao yield and quality enhancement? Are there unforeseen longer-term impacts of repeated moderately-intensive artificial pollination strategies on cacao productivity?

5.3 Materials and Methods

5.3.1 Local Environment, Study Site and Planting Material

The study was conducted at the Mars Cocoa Research Station (MCRS, 2°33′09″ S, 120°48′21″ E) in Tarengge Village, Wotu District, East Luwu Regency, South Sulawesi, Indonesia. The region is characterised as a high rainfall area, receiving ~2995 mm per year, well distributed across the year, typically with lower-than-average rainfall between August and November ('dry season' average 95 mm per month) and higher than average monthly rainfall between April and June ('wet season', average 421 mm per month). Annual average temperatures typically range from 19.9 (min) and 36.4 (Gérard et al.) °C, an annual relative humidity between 43.3 - 84 %, and solar radiation of 217 W/m^2.

During the three study years (2018 - 2020), the average monthly rainfall was 288.2 mm (wet season) and 137.3 mm (dry season), and 27.3 °C (wet season) and 26.9 °C (dry season), respectively. Soils at the MCRS are generally classified as Typic Dystrudepts according to the USDA system of soil taxonomy (Soil survey Staff, 1999), homogeneous and with little spatial variability (Spaans, 2016, *unpublished data*).

The study field (hereafter referred to as the ‘pollination plot’) was situated within a 2 Ha monoclonal cacao trial block called the ‘Soil Fertility Trial Block’ (SFTB), which experimentally active between 2014 and 2018, evaluating the effects of various soil amendment combinations on young cacao tree growth, flowering, and soil properties (Lambert et al., 2020). The pollination plot in the current study utilized a corner sub-plot of 132 experimental trees surrounded by border-row trees, after the completion of the previous trial. The experimental treatments in the current study were randomized across the prior soil amendment treatments, to avoid any confounding effects. All cacao trees in the pollination block were planted in 2014 and are of a single, self-incompatible, locally-popular and widely planted clone named ‘MCC02’. Upon initial planting, the cacao trees were organised in rows with 2 x 3 m planting distance. However, in January 2018, every other tree row in the pollination plot was removed but leaving the root systems intact, thereby reducing the tree density to 4 x 3 m spacing. Remaining were 96 experimental cacao trees organised as 8 rows of 12 trees per row. All cacao trees in the pollination plot are clonally grafted, propagated using softwood scion cuttings from known plagiotropic MCC02 material, collected from mature trees growing at the MCRS. The cuttings were terminally grafted onto seed-grown rootstock with seeds obtained from open-pollinated MCC02 pods. The pollination plot is bordered on its S and W sides by other experimental MCC02 cacao trees, and by local access roads on its N and E sides. Clone MCC02 was utilized as the maternal (pod bearing) genotype in this study, due to its acceptable yield and reasonable tolerance to pests and disease. Clone ‘BB01’ was utilized as the paternal (pollen donor) genotype, due to its high cross-compatibility with clone MCC02. Additional information on clones is provided as supplementary information (Supplementary Table 5.1, 5.2).

5.3.2 Pre-Trial Tree Preparation, Evaluation and Management

All trees in the pollination plot were the same age, mature and bearing fruits. However, three trees were considered unsuitable for experimentation due to poor tree health. A total of 93 trees within the plot were deemed healthy, with no signs of disease, of similar uniform architecture and suitable for experimentation. Tree suitability for experimental trial was evaluated annually. I also defined the upper limits of the experimental branch area (the effective branch area within which the artificial pollinations were conducted) as within 2m from the ground (Figure 1).

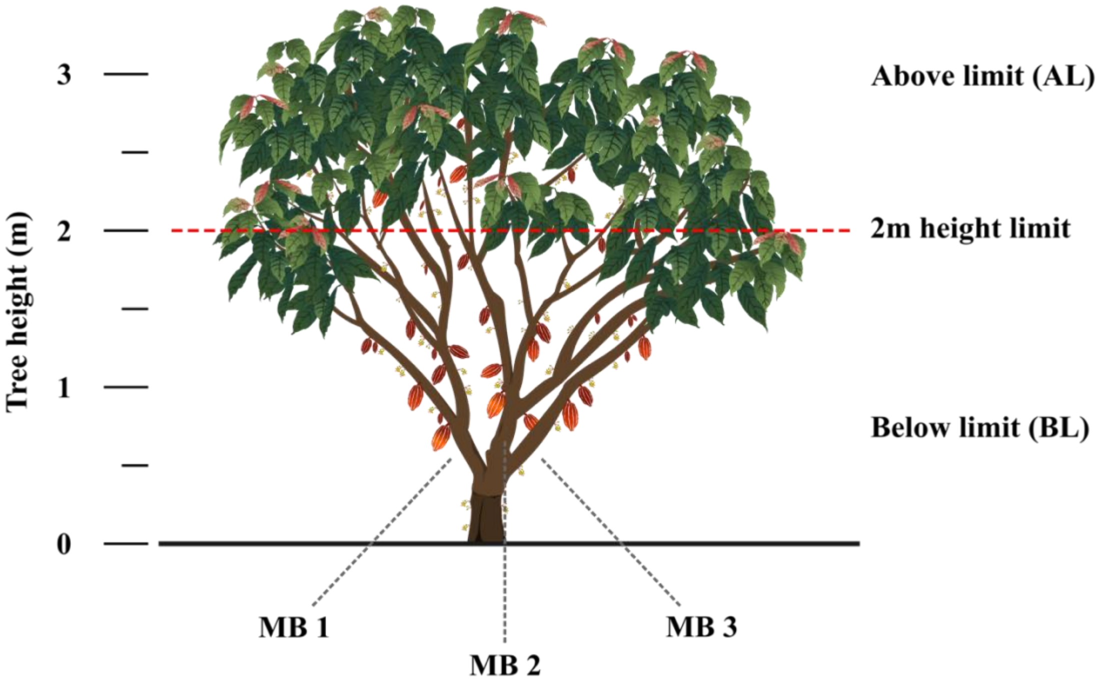


Figure 5.1. An experimental cacao tree, showing the 2m height limit (red line, '2m height limit'), which defines the upper limits of the effective experimental branch area (i.e., the branch area existing below the 2m height limit, 'Below limit (BL)') from the non-experimental branch area existing above the 2 m height limit ('Above limit (AL)'). Also shown is the identification of main branches ('MB1', 'MB2', 'MB3') for which clone MCC02 typically has 1 – 5, but commonly 3, main branches per tree.

During the trial, the agronomic management of experimental trees including fertilization, pruning and weeding management was done according to Mars Inc. standard agronomic practice (Supplementary Text 5.1A, B, C). The control of major pests and diseases was also conducted according to Mars standard agronomic practice (Supplementary Text 5.1D), which included 'pod sleeving' and the application of approved pesticides to control both cocoa pod borer (*Conopomorpha cramerella,* 'CPB') and black pod disease *(Phytophthora palmivora,* '*Phytophthora*'). However, in 2020 plastic sleeves were not used and an increased application frequency of pesticide applications was implemented as the alternative control method as the plastic sleeves were found to enhance *Phytophthora* infection rates in 2019.

5.3.3 Experimental Period and Treatments

The pollination trial was conducted between from February 2018 to July 2020. Within each experimental year, a 3-week pollination period was used to conduct the artificial pollinations. I selected a 3-week pollination period in attempt to narrow the total pollination period, and in response to the findings of Forbes et al. (2019) in their 5-week artificial pollination study who showed progressive fruit survivorship declines with each successive pollination week, regardless of pollination intensity. Each of the 93 experimental cacao trees were randomly assigned to 1 of 15 pollination intensity × pollination timing treatment combinations, with between 4 – 8 replicate trees per treatment (Table 5.1). The pollination intensity × pollination timing treatments were applied to the same experimental trees for each of three years to evaluate any longer-term effects and sustainability of these artificial pollination conditions. In this paper, I hereafter refer to the juvenile cacao fruits as ‘cherelles’, the mature fruits as ‘pods’ and the seeds as ‘beans’.

Pollination intensity (the total number of artificially pollinated flowers per tree) involved three treatment levels (30, 60, 90). Here, the experimental trees received a total of either 30, 60 or 90 artificially pollinated flowers per tree during the 3-week pollination period each year (Table 5.1). Pollination timing (the specific timing of, or the degree of spread between the artificial pollinations) involved five different treatment levels (Table 5.1) including: ‘Middle’ (highly condensed, applied within the middle of the pollination period during week 2 alone), ‘Early’ (moderately condensed, applied earlier in the pollination period within weeks 1 and 2), ‘Late’ (moderately condensed, but applied later in the pollination period during weeks 2 and 3), ‘All’ (poorly condensed, spread evenly across all 3 pollination weeks) and ‘Split’ (poorly condensed, applied in pollination weeks 1 and 3, with a one-week rest in between). For this, the total number of artificial pollinations prescribed to each experimental tree (i.e., the pollination intensity treatment) was nested within the five pollination timing treatments (Table 5.1). This design was used to evaluate if the total number of artificially pollinated flowers per tree, and the timing of those artificial pollinations within the pollination window, influenced cacao production metrics of: cherelle survival (90 / 110 days after pollination, ‘DAP’), the number of harvested pods (counts), individual fresh pod mass (g), dry bean mass (kg) and bean fat content (the % of bean sample nib mass that was fat). Single bean mass (g) was evaluated in 2018, but not in 2019 or 2020 due to internal miscommunications with the bean analytics lab. For this reason, I do not present the results for single bean mass. This design also allowed further investigation of the potential competitive dominance and increased survivorship of older cherelles, as suggested by (Forbes et al., 2019), while simultaneously investigating some of the potential physiological trade-offs between crop size, crop developmental timing and harvested fruit size and quality. Additional information on experimental treatments is provided as supplementary information (Supplementary Text 5.2). Clonal screening results for clone MCC02 yield performance (Supplementary Table 5.1) are also provided as supplementary information for contextualisation of the results.

Table 5.1. The fifteen pollination intensity × pollination timing treatment combinations and associated artificial pollinations applied to the 93 experimental trees within the 3-week pollination period each year.

Treatments			Artificial pollination application		
No. trees	**Pollination intensity**	**Pollination timing**	**Week 1**	**Week 2**	**Week 3**
8	30	Middle	-	30	-
8	60	Middle	-	60	-
7	90	Middle	-	90	-
4	30	Early	15	15	-
4	60	Early	30	30	-
4	90	Early	45	45	-
4	30	Late	-	15	15
4	60	Late	-	30	30
4	90	Late	-	45	45
7	30	All	10	10	10
8	60	All	20	20	20
8	90	All	30	30	30
7	30	Split	15	-	15
8	60	Split	30	-	30
8	90	Split	45	-	45

[z] No. trees, number of replicate trees.

Each experimental tree was given a unique identification tag to identify the trees' unique treatment allocation and the number of pollinations to be conducted per pollination date. I also labelled each main branch per tree (Figure 5.1) to facilitate flower counting and the artificial pollination process. The unique treatments assigned to each experimental tree were maintained on the same trees for the duration of the 3-year study period. This was done to assess the longer-term sustainability of artificial pollination on tree health and productivity. Consequently, cacao tree performance results in later years may have been influenced by the previous year's experimental treatment effects on cacao production and physiological status, and the results should be interpreted with this in mind.

5.3.4 Flowering Intensity

Flowering intensity, representing the number of freshly opened flowers per tree, was typically evaluated every 3 days throughout the entire study period, as old and unpollinated flowers usually abscise and drop within 3 days. The location of counted flowers was recorded as either below ('BL') or above ('AL') the 2 m height limit (Figure 5.1). Flower counts were conducted in this manner to enable evaluations of whole-tree flowering production. All flower counts, regardless of location, were conducted per main branch. During the 3-week pollination period each year, flowering intensity was evaluated daily for all BL flowers (experimental flowers that may receive artificial pollination). Flower counts were conducted in the morning hours between 7:00 am and 10:00 am, immediately before the artificial pollination of experimental flowers. In the current study, I report only on flowering intensity within the 3-week pollination period. Literature on the flowering phenology of cacao trees is relatively scarce (Wibaux et al., 2024). However, is generally accepted that cacao trees in Sulawesi produce flowers year-round with two distinct peak flowering events per year, the "major flowering" and the "minor flowering" events (Mars Incorporated, *personal communication*). These two flowering events are directly associated with the two annual harvest periods, commonly referred to as the "main-crop" and the "mid-crop". The exact timing of these peak flowering events is under both external (e.g., local climatic factors such as rainfall, temperature) and internal (e.g., cultivar) control. However, for clone MCC02 in the study region, peak flowering for the main-crop is generally expected to occur from December – February and from June – July for the mid-crop (Mars Incorporated, *personal communication*). All data are available in Dryad (Forbes et al., 2024).

5.3.5 Artificial Pollinations

Effort was made to conduct the artificial pollinations within the major peak flowering events for clone MCC02 each year. For this, I utilized the continuous flowering intensity evaluations to predict the start of the peak flowering events. However, precise prediction of peak flowering for best-timed artificial pollinations was not always achieved due to inter-annual variability in the timing and intensity of peak flowering events, and potentially altered by the previous year's production. Each year, the artificial pollination of flowers was conducted during a 3-week period and only on 5 days, for a total of 15 hand pollination days, per yearly pollination period. The pollination periods were between 19 February – 09 March in 2018, between 14 February – 01 March in 2019, and between 27 January – 15 February in 2020. On each pollination date and immediately after flower counts, the number of flowers to be pollinated per tree (BL) were artificially pollinated using anthers from the paternal clone BB01. Artificial pollinations were conducted using the 'three-anther method' (Falque et al., 1995). After pollination, each artificially pollinated flower was given a unique identifier-tag specifying the tree ID, treatment ID, main branch number and pollination date. All artificial pollinations were made between the hours of 08:00 am and 12:00 pm. During events of high wind, heavy rainfall or extreme heat, artificial pollinations were postponed to next suitable day. All artificially pollinated and un-pollinated flowers (BL and AL) remained open (not isolated) to the prospect of natural pollination. Additional information on artificial pollination methods is provided as supplementary information (Supplemental Text 3).

5.3.6 Cherelle Development and Pod Harvesting

After artificial pollination, cherelle survival during the cherelle development phase ($10_{\text{(initial)}}$ –

$170_{(\max.)}$ DAP was monitored by counting the number of surviving and healthy, artificially pollinated cherelles present on each experimental tree, on each observation date. Observations of cherelle survival were conducted every 20 days in each, 2018 and 2019, every 10 days in 2020, and continued until the onset of pod harvesting. During cherelle monitoring, any aborted, wilting or highly pest and/or disease infected artificially pollinated cherelles were removed from the tree and experimental field to reduce further pest / pathogen spread. Similarly, any naturally pollinated cherelles observed during the cherelle development phase were continuously counted, recorded by location (BL or AL), and removed to reduce potential resource competition with experimental cherelles. Any artificially pollinated cherelles that were removed due to pest and/or disease infection (as opposed to physiological cherelle wilt) during the cherelle development phase were not included in the final yield estimates, reflected in the data as decreased proportionate cherelle survival during the cherelle development phase. This practice was particularly prominent in 2019 when the natural incidence of *Phytophthora* was high. Thus, cherelle survival rates in 2019 likely underestimate their true potential in the absence pest and/or disease attack.

The pod harvesting phase was between 06 – 23 August in 2018, between 12 June – 14 August in 2019, and between 05 June – 15 July in 2020. Pod harvesting was conducted upon the observation of pod ripening and was done on a weekly basis until all artificially pollinated pods were harvested. However, in 2019 pod harvesting was initiated prematurely as pods reached the ‘mature’ size class (> 15 cm) to avoid excessive pod losses to *Phytophthora* infection when incidence of this pathogen was notably high. All prematurely harvested pods were counted as final yield (i.e., included in the total number of pods harvested), opened, and visually assessed for bean development and acceptability. Harvested pods with fully developed (filled) beans were used for further pod and bean-level evaluations and pods with immature (unfilled) beans were discarded. Thus, the 2019 estimates of individual pod fresh mass and dry bean mass likely underestimate their true potential in the absence of severe *Phytophthora* outbreak. Furthermore, it is possible that the bean quality results may have been impacted by premature harvesting, despite my best efforts to discard immature and unfilled beans. Upon each harvesting event, all harvested pods per tree were placed directly underneath their respective maternal tree. The total number of harvested pods per tree were then separated by pollination week, counted and individual fresh pod mass (g) was determined before pod opening and further processing & evaluation of cacao beans.

5.3.7 Cacao Bean Evaluation

Upon pod opening, each pod and its beans were visually assessed and classified by the infection level of CPB and *Phytophthora*, using a grading scheme common to Mars Inc. field research technicians. The grading scheme includes ‘A’ - healthy pod with all acceptable beans (acceptable beans are suitable for post-harvest use), B - infected pod with all beans able to be extracted by hand and some wastage beans, and ‘C’ - highly infected pod with no acceptable beans (unacceptable beans are unsuitable for post-harvest use). All beans were extracted, and all unacceptable beans were discarded. After bean extraction and sorting, the mass (g) of wet, acceptable cacao beans per pod was determined. The remaining acceptable beans were dried in a solar dryer for 7 days, with bean turning occurring daily. Beans were considered as dry when they had a 7 - 8% moisture content. The beans were combined to form a single bean sample per tree and per treatment. From each cacao trees' total dry cacao beans, a 150 - 300 g sub-sample of dry cacao beans was obtained and shipped directly to the Mars quality laboratory, Makassar, for bean quality analysis. Between 1 - 3 bean samples were obtained per tree, per year. The average bean fat content (% of nib sample mass) was determined using near-infrared spectroscopy (NIR) and the Mars Inc. standard for cacao bean fat content of 51 % or greater, is used as the comparison.

5.3.8 Statistical Analysis

To evaluate the effect and interactive effects of pollination intensity, pollination timing and year on cacao production metrics of cherelle survival, the average number of harvested pods (counts) per tree, the average individual fresh pod mass (g), average dry bean mass (kg) per tree, and average fat content (%) of cacao beans, over time, I fit generalized linear models using the glmmTMB function in the glmmTMB (Bolker et al., 2009; Brooks, 2017) package in R (R Core Team, 2021). The models included fixed effects of each, pollination intensity (30, 60, 90), pollination timing (Middle, Early, Late, All, Split) and year (2018, 2019, 2020), and where possible, included all interaction terms. In each of these models, I used a stepwise AIC-minimizing approach (Burnham and Anderson, 2004), dropping the highest order, non-significant interaction terms if it improved the AIC values.

Cherelle survival was analysed at 110 DAP for the 2018 data, but at 90 DAP for both 2019 and 2020 data, due to the earlier onset of pod harvesting in 2019 and the high rates of pod losses due to *Phytophthora* in 2020 experienced at and beyond 100 DAP. Cherelle survival was analysed using the number of surviving cherelles (counts) and the proportion of surviving cherelles (i.e., cherelle survival as a proportion of the total number of initial artificial pollinations made, expressed as a percentage). For the models describing the number of surviving cherelles (counts) and the number of harvested pods (counts), I used a negative binomial distribution with a log-link function to better describe the positive relationship between mean and variance. For surviving cherelles I ran 'dredge' in the MuMIn package (Barton, 2009) to produce the best fit model as ranked by the AIC as the full model was over-parameterized. For the model describing the proportion of surviving cherelles, I used a hurdle model with a beta-binomial distribution and a logit-link function to model a zero-inflated beta distribution. Here, I was only able to model the main effects as there was not enough statistical power to include interactions between main effects in the model. For the models describing each, individual fresh pod mass (g) and bean fat content (%), I used a gamma distribution with a log-link function to account for the positive relationship between the mean and variance. The model describing individual dry bean mass (g), used a normal distribution with an identity-link function to maintain the additive interaction terms when applicable (i.e., so that the interaction was not additive on a log-scale).

For all statistical models, I performed sequential likelihood ratio tests for each fixed effect and their interactions (where relevant) using the lrtest function in the lmtest package (Zeileis and Hothorn, 2002) in R (R Core Team, 2021). Where a fixed effect or interaction term was found to have a significant effect on a response variable, Tukey's HSD post-hoc tests were used to determine where these differences occurred, using the emmeans package (Lenth, 2016) in R (R Core Team, 2021). An alpha level of $P = 0.05$ was used for the interpretation the results. Unless otherwise stated, all error bars and intervals represent standard errors. The data that support the findings of this study are openly available in Dryad (Forbes et al., 2024).

5.4 Results

5.4.1 Flowering Intensity

Across the study period, flowering intensity (the average number of freshly opened flowers per tree and per pollination date) under the 2m height limit and regardless of treatment generally increased from 2018 (37.4 ± 1.8) to 2019 (117.67 ± 4.6) and reduced again in 2020 to levels below those found in 2018 (27.2 ± 1.8) (Supplementary Table 5.3). Flowering intensity above the 2m height limit also increased from 2018 (7.2 ± 0.5) to 2019 (13.0 ± 0.6) but remained constant in 2020 (13.2 ± 0.6) (Supplementary Table 5.3).

5.4.2 Cherelle Survival

Across all years and regardless of treatment, I observed an expected decline in the number and proportion of surviving cherelles over time during the cherelle development phase, regardless of treatment (Figure 5.2A: F).

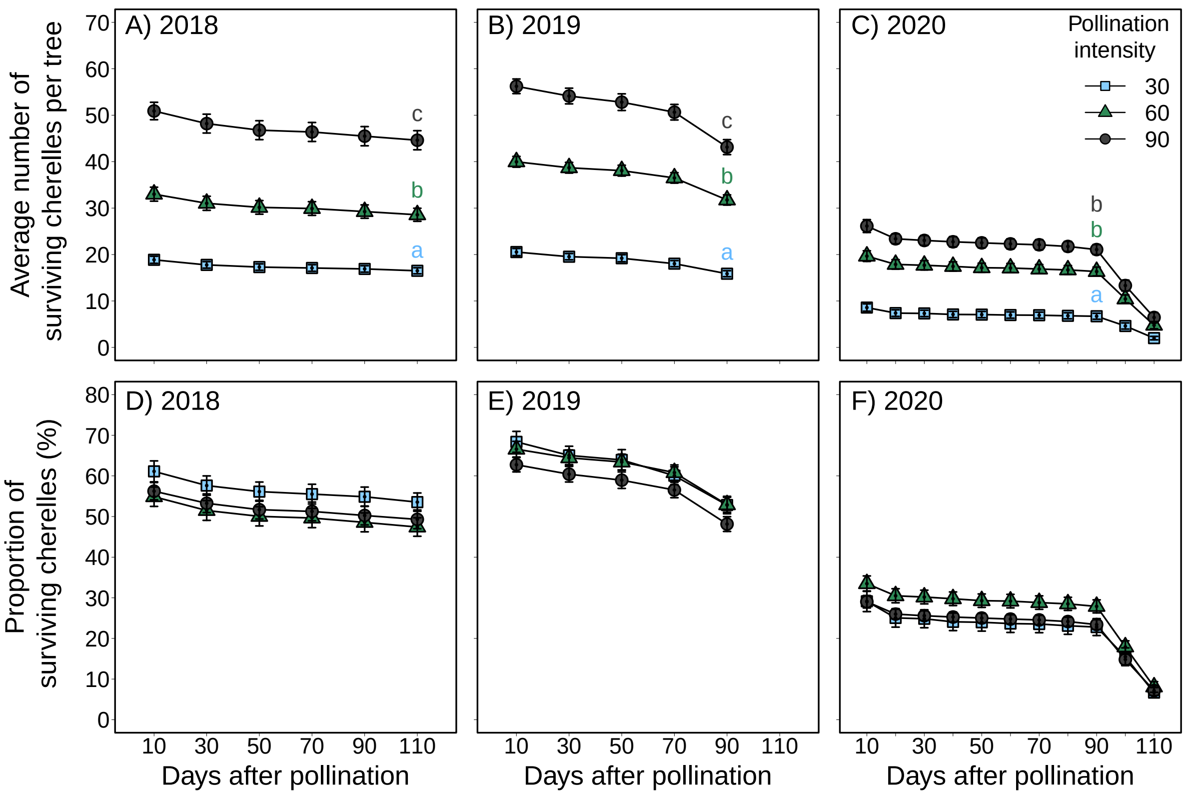


Figure 5.2. Mean (± SE) number of surviving cherelles (A: C) and proportion (%) of surviving cherelles (D: F) present on experimental trees assigned to one of three pollination intensity treatments (30, 60, 90; n = 30, 32, and 31 replicate trees per pollination intensity treatment, respectively) and across three study years (A) 2018, B) 2019, and C) 2020). Means with different letters of the same case (e.g., a, b) represent significant differences between treatments at an alpha level of $P < 0.05$ (Tukey's HSD tests).

5.4.2.1 Number of surviving cherelles (counts)

I found significant main effects of pollination intensity ($\chi^2 = 316.0$, $df = 2$, $P < 0.0001$; Table 5.2) and year ($\chi^2 = 244.3$, $df = 2$, $P < 0.0001$; Table 5.2) on the number of surviving cherelles (Supplementary Table 5.4), and a significant pollination intensity × year interaction ($\chi^2 = 11.7$, $df = 4$, $P = 0.0195$; Table 5.2; Figure 5.2A: C). Although no significant main effect of pollination timing treatment was detected (Table 5.2), I did find a significant pollination timing × year interaction ($\chi^2 = 93.7$, $df = 8$, $P < 0.0001$; Table 5.2; Supplementary Figure 5.2A: C).

Regarding pollination intensity, a general increasing trend in the number of surviving cherelles with increasing pollination intensity is observed across all years with the highest counts of surviving cherelles consistently obtained from the '90' pollination intensity treatment (Figure 5.2A: C; Supplementary Table 5.4). However, Tukey's HSD post-hoc test revealed that in 2020, the average number of surviving cherelles obtained from the '90' pollination intensity treatment was not significantly greater than was obtained from the '60' pollination treatment (Figure 5.2C;

Supplementary Table 5.4) at an alpha level of $P < 0.05$. Interestingly, in the third year of the study, the success of artificial pollinations had markedly declined, with average cherelle survivorship at 10 DAP in 2020 being ~ 50 % lower than in previous years.

Regarding pollination timing, Tukey's HSD post-hoc test determined an effect of pollination timing on the number of surviving cherelles in 2018 only (Supplementary Figure 5.2A: C). Here, the highly condensed 'Middle' pollination timing treatment appeared to benefit the number of surviving cherelles, with a significantly greater number of surviving cherelles on trees assigned to this treatment as compared to those assigned to the poorly condensed 'Split' pollination timing treatment (Supplementary Figure 5.2A; Supplementary Table 5.4). The observed benefits of highly condensed pollination in 2018 were no longer observed in 2019 and 2020 (Supplementary Figure 5.2B, C).

Table 5.2. Results of likelihood ratio tests evaluating main effects and their possible interactions on the response variables describing cacao production.

Response variable	**Effects**	χ^2	***df***	***P***
Number of surviving cherelles (counts)	Pollination intensity × year	11.7	4	**< 0.0195**
	Pollination timing × year	93.7	8	**< 0.0001**
	Pollination intensity	316.0	2	**< 0.0001**
	Pollination timing	2.5	4	0.6401
	Year	244.3	2	**< 0.0001**
Proportion of surviving cherelles (%)	Pollination intensity	3.4	2	0.1785
	Pollination timing	0.8	4	0.9354
	Year	222.6	2	**< 0.0001**
Number of harvested pods (counts)	Pollination intensity × pollination timing × year	15.0	16	0.5214
	Pollination intensity × year	10.1	4	**0.0381**
	Pollination timing × year	51.8	8	**<0.0001**
	Pollination intensity × pollination timing	6.3	8	0.6119
	Pollination intensity	276.0	2	**<0.0001**
	Pollination timing	5.3	4	0.2546
	Year	284.9	2	**<0.0001**
Individual pod fresh mass (g)	Pollination intensity × pollination timing × year	12.6	16	0.6992
	Pollination intensity × year	16.8	4	**0.0021**
	Pollination timing × year	9.9	8	0.2751
	Pollination intensity × pollination timing	12.0	8	0.1523
	Pollination intensity	50.3	2	**<0.0001**

	Pollination timing	6.9	4	0.1409
	Year	37.4	2	**<0.0001**
Dry bean mass (kg)	Pollination intensity × pollination timing × year	2.1	16	0.8471
	Pollination intensity × year	26.8	4	**0.0004**
	Pollination timing × year	37.0	8	**<0.0001**
	Pollination intensity × pollination timing	7.9	8	0.5116
	Pollination intensity	53.3	2	**<0.0001**
	Pollination timing	67.0	4	**<0.0001**
	Year	1.6	2	0.4394
Bean fat content (%)	Pollination intensity × pollination timing × year	13.8	16	0.6166
	Pollination intensity × year	10.9	4	**0.0281**
	Pollination timing × year	9.7	8	0.2847
	Pollination intensity × pollination timing	3.9	8	0.8638
	Pollination intensity	18.4	2	**<0.0001**
	Pollination timing	11.0	4	**0.0268**
	Year	139.1	2	**<0.0001**

[z] Proportion of surviving cherelles represents the number of surviving cherelles as a proportion of the total number of initial artificial pollinations made per tree, expressed as a percentage.

[y] Bean fat content (%) represents the percentage of the bean sample nib mass per tree that was determined as fat by near-infrared spectroscopy analysis.

[x] *P*-values were calculated using test statistics derived from the mean and variance and assumed an alpha level of $P = 0.05$.

5.4.2.2 Proportion of surviving cherelles (%)

Despite a significant main effect of pollination intensity on the number of surviving cherelles (Table 5.2), no such effects were observed on the proportion of surviving cherelles ($\chi^2 = 3.4$, $df = 2$, $P = 0.1785$; Table 5.2). However, a significant main effect of year ($\chi^2 = 222.6$, $df = 4$, $P < 0.0001$; Table 5.2) on the proportion of surviving cherelles was observed. Here, overall and regardless of treatment, the proportion of surviving cherelles at the end of the experimental cherelle development phase slightly increased from 2018 (50 %, 110 DAP) to 2019 (51 %, 90 DAP), but significantly decreased in 2020 (25 %, 90 DAP). There was no observed main effect of pollination timing ($\chi^2 = 0.8$, $df = 4$, $P = 0.9354$; Table 5.2) on the proportion of surviving cherelles.

5.4.3 Harvested Pods

5.4.3.1 Number of harvested pods (counts)

A total of 6723 pods were harvested from the 93 experimental trees during the 3-year study. Across all years, the total number of pods harvested increased slightly from 2018 (2528) to 2019 (2818) but declined significantly in 2020 (1377) (Supplementary Table 5.4) (year effect: $\chi^2 = 284.9$, $df = 2$, $P < 0.0001$; Table 5.2).

A significantly positive main effect of pollination intensity ($\chi^2 = 276.0$, $df = 2$, $P < 0.0001$; Table 5.2) was found on the total number of harvested pods per tree. However, this effect varied according to the year (pollination intensity × year interaction: $\chi^2 = 10.1$, $df = 4$, $P = 0.0381$; Table 5.2; Figure 5.3A: C). Tukey's HSD post-hoc test revealed a significantly positive relationship between increasing pollination intensity and the total number of harvested pods per tree in 2018 and 2019 (Figure 5.3A, B). However, in 2020, the number of harvested pods was not significantly different between the '60' and '90' pollination intensity treatments (Figure 5.3C). The greatest annual cumulative harvested pod yield was obtained from trees receiving 90 artificial pollinations in 2019 (Figure 5.3B), producing on average 43 ± 1.4 pods per tree. In comparison, the '30' pollination intensity treatment yielded the lowest overall harvested pod counts across all years, averaging only 7 ± 0.6 pods per tree in 2020 (Figure 5.3C; Supplementary Table 5.4).

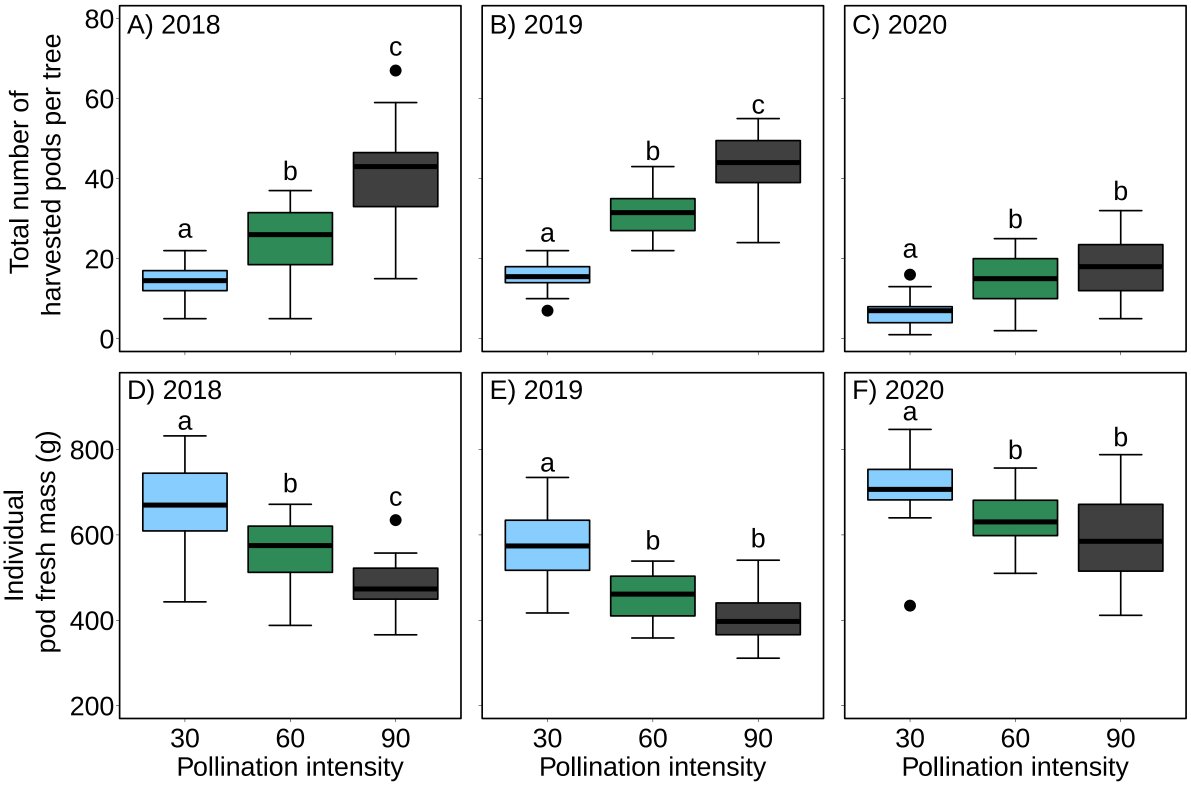


Figure 5.3. Number of harvested pods (A: C) and individual pod fresh mass (D: F) from experimental trees assigned to one of three pollination intensity treatments (30, 60, 90; n = 30, 32, and 31 replicate trees per pollination intensity treatment, respectively) and across three study years (2018 (A, D), 2019

(B, E), and 2020 (C, F). Means with different letters of the same case (e.g., a, b) represent significant differences between treatments at an alpha level of $P < 0.05$ (Tukey's HSD tests).

There was no observed main effect of pollination timing ($\chi^2 = 5.3$, $df = 4$, $P = 0.2546$; Table 5.2) on the total number of harvested pods per tree. However, I did observe a significant pollination timing × year interaction ($\chi^2 = 51.8$, $df = 8$, $P < 0.0001$; Table 5.2; Supplementary Figure 5.3A: C) on the total number of harvested pods per tree. Tukey's HSD post-hoc test revealed similar effects as observed in the number of surviving cherelles, whereby in 2018, trees assigned to the highly condensed 'Middle' pollination timing treatment produced significantly greater numbers of harvested pods, as compared to trees assigned to the poorly condensed 'Split' pollination timing treatment (Supplementary Table 5.4; Supplementary Figure 5.3A, B).

5.4.3.2 Individual fresh pod mass (g)

Contrary to the results of harvested pod counts, a significantly negative main effect of pollination intensity was found on the average mass of individual cacao pods ($\chi^2 = 50.3$, $df = 2$, $P < 0.0001$; Table 5.2; Figure 5.3D: F), but this effect varied over time (pollination intensity × year interaction: $\chi^2 = 16.8$, $df = 4$, $P = 0.0021$; Table 5.2; Figure 5.3D: F). Increasing pollination intensity and associated increases in the number of harvested pods (Figure 5.3A: C) was generally associated with reductions in the mass of individual pods (i.e., smaller pods) (Figure 5.3D: F; Supplementary Table 5.4), though the magnitude of this effect was greatest in 2018. In 2018, significant reductions in individual fresh pod mass were observed with each increase in pollination intensity (Figure 5.3D). While the general trend of reducing individual fresh pod mass with increasing pollination intensity is still evident in both 2019 and 2020, differences were not statistically significant between the '60' and '90' pollination intensity treatments in these years (Figure 5.3E, F).

In 2019, when the number of harvested pods per tree was highest (Supplementary Table 5.4), the average individual pod fresh mass (408 ± 10.4 g) across treatments, reached its lowest of all experimental years (Supplementary Table 5.4; Figure 5.3F). However, a rebound effect was observed in 2020 when the number of harvested pods per tree reached its lowest, but the average individual pod mass (647 ± 14.7 g) across treatments, was higher than in all previous experimental years (Figure 5.3F; Supplementary Table 5.4). No main effect of pollination timing was found on the average mass of individual cacao pods ($\chi^2 = 6.9$, $df = 4$, $P = 0.1409$; Table 5.2).

5.4.4 Bean Yield and Quality

5.4.4.1 Dry bean mass (kg)

Like the results for the number of harvested pods, I found a significantly positive main effect of pollination intensity on the average dry bean mass (kg) per tree ($\chi^2 = 53.3$, $df = 2$, $P < 0.0001$;

Figure 5.4A: C, Table 5.2). However, this effect varied with time (pollination intensity × year interaction: $\chi^2 = 26.8$, $df = 4$, $P = 0.0004$; Table 5.2; Figure 5.4A: C).

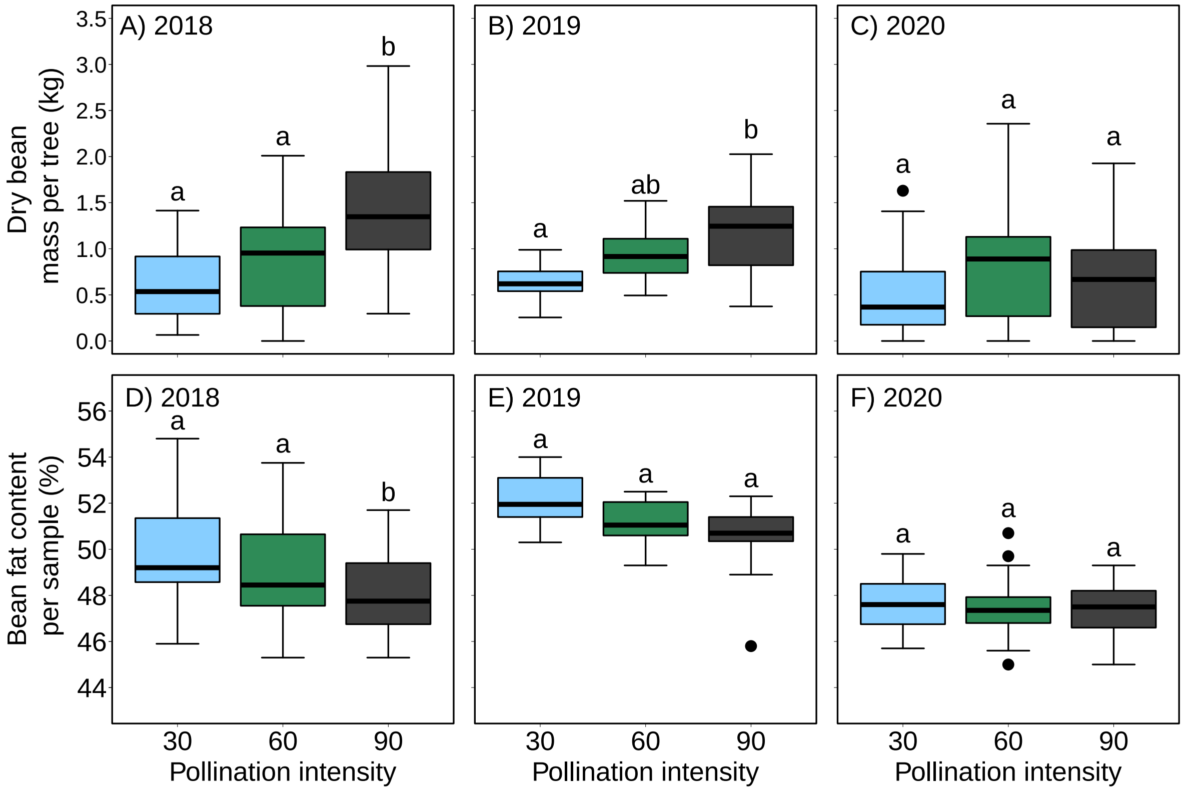


Figure 5.4. Mass (kg) of dry cacao beans (seeds) per tree (A: C) and average fat content (%) of dried cacao beans per sample (D: F) from experimental trees assigned to one of three pollination intensity treatments (30, 60, 90; n = 30, 32, and 31 replicate trees per pollination intensity treatment, respectively) and across three study years (2018 (A, D), 2019 (B, E), and 2020 (C, F). Means with different letters of the same case (e.g., a, b) represent significant differences between treatments at an alpha level of $P < 0.05$ (Tukey's HSD tests).

Overall, the average dry mass of beans per tree for each pollination intensity treatment was similar between 2018 and 2019 (Figure 5.4A, B; Supplementary Table 5.5), despite larger differences in the numbers of harvested pods (Supplementary Table 5.4). Tukey's HSD post-hoc test revealed that in 2018, trees assigned to the highest '90' pollination intensity treatment produced, on average, significantly greater dry bean mass (1.5 ± 0.1 kg) per tree, than trees assigned to the lower '60' (0.9 ± 0.3 kg) and '30' (0.6 ± 0.1 kg) pollination intensity treatments (Figure 5.4A; Supplementary Table 5.5). Similar but reduced effects were observed in 2019 and all effects of pollination intensity on dry bean mass had diminished by 2020 (Figure 5.4C; Supplementary Table 5.5). Premature pod harvesting in 2019 and potential reductions in dry bean mass from immature beans and the exclusion of unfilled beans may have limited the 2019 bean dry mass, regardless of treatment, and these results should be interpreted with this consideration.

Regarding pollination timing, I found a significant main effect of pollination timing on dry bean mass ($\chi^2 = 67.0$, $df = 4$, $P < 0.0001$; Table 5.2) that depended on year (pollinating timing × year interaction, $\chi^2 = 37.0$, $df = 8$, $P < 0.0001$; Table 5.2). Here, Tukey's HSD post-hoc test revealed that in 2020, trees assigned to the poorly condensed 'All' pollination timing treatment produced significantly higher average dry bean mass (1.0 ± 0.1 kg), as compared to trees assigned to the highly condensed 'Middle' pollination timing treatment (0.4 ± 0.1 kg) (Supplemental Figure 5.4C; Supplementary Table 5.5).

5.4.4.2 Bean Fat Content (%)

The fat content (the % of bean sample nib mass that was determined as fat) in cacao beans varied significantly by year ($\chi^2 = 139.1$, $df = 2$, $P < 0.0001$; Table 5.2), with the highest values observed in 2019 (average 51 %) and very low values observed in 2020 (average 47 %) across all treatments (Figure 5.4D: F; Supplementary Table 5.5). Overall, the highest average bean fat content values (52 ± 0.6 %) were obtained from trees assigned to the lowest '30' pollination intensity treatment in 2019 (Figure 5.4B, Supplementary Table 5.5). In contrast to the results of dry bean mass (kg), I found a significant negative main effect of pollination intensity on the average fat content (%) of cacao beans ($\chi^2 = 18.4$, $df = 2$, $P < 0.0001$; Table 5.2; Figure 5.4D: F), but this effect varied over time (pollination intensity × year interaction, $\chi^2 = 10.9$, $df = 4$, $P = 0.0281$; Table 5.2; Figure 5.4D: F). Tukey's HSD post hoc test revealed that the significant effects of pollination intensity on the average bean fat content were present in 2018 only (Figure 5.4A; Supplementary Table 5.5). In 2018, trees under the highest '90' pollination intensity produced significantly lower average bean fat content values (48 ± 0.6 %) than trees assigned to any other pollination intensity treatment (Figure 5.4A; Supplementary Table 5.5). Although a decreasing trend in bean fat content values with increasing pollination intensity was also observed in 2019 (Figure 5.4E), these effects were not significantly different. The effects of pollination intensity on bean fat content completely diminished in 2020 (Figure 5.4F).

There was also a moderately significant effect of pollination timing on cacao bean fat content ($\chi^2 = 11.0$, $df = 4$, $P = 0.0268$; Table 5.2; Supplementary Figure 5.4D: F) that was consistent across years (pollination timing × year interaction, $\chi^2 = 9.7$, $df = 8$, $P = 0.2847$). Tukey's HSD post-hoc test revealed that overall, cacao bean fat content values in the poorly condensed 'All' and 'Split' pollination timing treatments were significantly higher as compared to those obtained from the highly condensed 'Middle' pollination timing treatment (Supplementary Figure 5.4D: F).

5.5 Discussion

In this 3-year study, I found that increasing pollination intensity and increasing the concentration of artificial pollinations led to increases in cherelle survival, harvested yield and the mass of dry cacao beans per tree. These factors but reduced pod size (individual pod fresh mass) and bean dry mass & quality (reduced fat content). However, these patterns varied substantially by year, suggesting that climate and other environmental factors also play an important role in how artificial pollination impacts cacao production. This study highlights the crucial importance of multi-year studies to determine the long-term impacts and sustainability of intensive artificial pollination management strategies.

Pollination intensity

In two of the three study years, I observed an increase in the per-tree number of surviving cherelles and harvested pods with increasing pollination intensity. However, these effects were less pronounced (number of cherelles and harvested pods) in 2020. For trees assigned to the highest ‘90’ pollination intensity treatment, in the first two study years I achieved near the annual years expected production (~ 45 pods per tree) in a single cropping season. For dry bean mass, the initially observed benefit of high (‘90’) pollination intensity was reduced in 2019, and absent by 2020. These results support previous findings documenting the short-term positive effect of increasing pollination intensity, via artificial pollination strategies, on cacao harvested yield (Groeneveld et al., 2010; Forbes et al., 2019; Toledo-Hernández et al., 2020). However, previous studies only cover one growing season, and raised the central questions of *i.* how long can increased cacao yield be sustained under intensive artificial pollination? and *ii.* what approaches might improve both the viability and/or efficiency of artificial pollination strategies?

My results showed that the documented short-term benefits of increasing pollination intensity on cacao pod yield declined over time, when repeatedly applied over three consecutive years. This result suggesting the potential for an alternate bearing pattern to emerge in cacao trees artificially managed to produce unnaturally high yields over consecutive reproduction events. The variability in treatment effects was likely magnified by year-to-year variability in both pathogen prevalence and environmental conditions. Furthermore, although increases in pollination intensity increased the number of pods harvested per tree, the mass of individual pods decreased with increases in pollination intensity and the total number of pods per tree. This relationship between pod weight and pollination intensity supports previous findings (Groeneveld et al., 2010), and is likely driven by within-tree resource limitation under high pollination levels. Under increased pod load, the total number of pods requiring assimilate supply for development may exceed within-tree resource (carbohydrate) availability (overcropping) (McKelvie, 1960). Under natural conditions, cherelle wilt may act here to preferentially abort smaller resource sinks (younger cherelles / pods) and thereby regulate total resource demands (pod loads) to match internal resource availability (Valle et al., 1990). However, in contrast to the findings of previous studies (Groeneveld et al., 2010; Forbes et al., 2019; Toledo-Hernández et al., 2020), I did not observe any effect of increasing pollination intensity on rates of cherelle wilt. The artificial pollination intensity (the percentage of the total number open flowers present under 2m height that are artificially pollinated per tree) levels utilized in these previous studies were up to 100% of open flowers pollinated daily for up to 60 days. In contrast, pollination intensity in the current study was altered to represent three very 'modest' pollination intensity levels of 30, 60 and 90 artificial pollinations (total) per tree, all made within a short 21-day period. Thus, the pollination intensities used here may have been high enough to reduce individual fruit size, but their application not spread out enough to facilitate any measurable increases in cherelle wilt.

Despite the overall yield (i.e., harvested pods and dry bean mass) increases that can be achieved through increasing pollination intensity, reductions in individual pod weight with increasing crop size may not be a profitable or efficient outcome. Per pod bean content and characteristics depend on many factors (e.g., pod location, pollen quantity and quality, management, environmental and genetic factors), but also can depend on the size of the pod (Toxopeus and Wessel, 1970). For example, beans from smaller pods have lower macronutrient concentrations than beans from larger pods, throughout all development stages (Goudsmit et al., 2023). Thus, artificial pollination strategies to increase the total number of harvested pods, may come at a consequence to pod size, single bean weight and bean composition, with subsequent potential implications for post-harvest pod and bean processing efficiencies. It remains unknown if the timely provision of optimal mineral nutrition to support pod and bean development can ameliorate the enhanced crop load mediated reductions in pod size and bean composition.

My study revealed significant reductions in bean fat content in beans obtained from trees subject to the highest '90' pollination intensity treatment in 2018, although this effect was absent in 2019 and 2020. This study is the first to my knowledge, investigating the potential negative effects of intensifying artificial pollination on cacao bean fat content. This finding is important considering fat (lipid) content is one of the most important quality factors of the cacao bean (Torres-Moreno et al., 2015). A major determinant of its market price (Afoakwa, 2016), the unique fatty acid profile of cacao beans contributes to the desirable textural characteristics of chocolate and other confections (Mustiga et al., 2019). Fat typically represents half of the cacao bean's weight and is used to produce cocoa butter, one of the most valuable products of the cacao bean (Afoakwa, 2016) and one of the most expensive edible fats in the world (McHenry and Fritz, 1987). Despite its importance, little is reported on the pre-harvest factors that control fat content and fatty acid composition in cacao beans. However, genotype (Liendo et al., 1997; Pires et al., 1998; Vázquez-Ovando et al., 2015), origin (Lipp and Anklam, 1998; Chaiseri and Dimick, 1989), and environmental conditions (Lehrian et al., 1980; McHenry and Fritz, 1987) are known to influence fatty acid composition and total fat levels in cacao beans. The 2018 results may reflect a dilution effect, whereby a limited amount of within-tree assimilates for fat allocation may be spread more thinly between a greater number of pods per tree, as found in the ‘90’ pollination intensity treatment. The year-to-year variability in bean fat content, potentially influenced by other interacting factors such as disease load, tree age, tree nutrition, bearing cycles, environmental factors and more, suggests that the results must be interpreted with caution.

Pollination timing

The results showed that condensing the temporal window over which pollination occurs increased cherelle survival and the number of harvested pods in the first year of the study. However, as compared to the most poorly condensed artificial pollination treatments, highly condensing pollinations also reduced the mass of dry cacao beans per tree in 2020, and bean fat content values in all years. My findings firstly support the previous research by Forbes et al. (2019) who in their five-week intensive artificial pollination study, observed greater survival of older cherelles than younger cherelles, and who suggested that narrowing the pollination time window may maximize cacao yield. However, the conclusions from this study were over a single growing season and did not evaluate bean-level quality, or track production over multiple years.

My results from 2018 reveal that condensing the pollination time window holds great potential to enhance cacao production. However, the multi-year results incorporating important bean quality measurements reveal two opposing suggestions. Firstly, condensing pollination time windows for artificial pollinations reduced bean fat content. In 2018, the ‘Middle’ pollination timing treatment, where the total number of artificial pollinations were condensed within a 5-day period, led to increases in the number of harvested pods, but decreases in bean fat content values. In contrast, in this same year, bean fat content values were highest under the two poorly condensed pollination timing treatments where artificial pollinations took place across three weeks, ‘All’ and ‘Split’. These results suggest that condensing the time windows for artificial pollination applications can have a negative effect on cacao quality. Such approaches may not lead to profitable outcomes for cacao farmers who access markets with set quality standards. Bean fat content values followed this trend across all experimental years and could potentially decrease the market value of cacao beans (Afoakwa, 2016) for both farmers and cocoa processors. Secondly, based on the results of Forbes et al. (2019), I intentionally reduced the pollination period from 5 weeks to a very condensed 3 weeks, to increase overall production efficiencies. By doing this, I *i*. reduced the total time (labour hours) spent pollinating, *ii*. anticipated reduced cherelle wilting, and *iii*. found that condensing pollination also decreased the total harvesting time due to increased synchrony in pod ripening. However, condensing the artificial pollinations may have reduced the amount of cherelle wilt and resulted in potential overcropping (excessive pod loads that exceed the trees resource reserves or carrying capacity for reproduction). Similar sink-strengths and auxin levels of similarly aged artificially pollinated cherelles (McKelvie, 1960) may partly explain the avoidance of cherelle wilt. The avoidance or absence of cherelle wilt under condensed timing of artificial pollinations is a significant finding with great potential to increase the number of pods retained on trees. However, trees artificially manipulated into overcropping, and repeated overcropping over successive years, may experience increased resource depletion.

From a physiological perspective, high-yielding cultivars such as clone MCC02 are suggested to take advantage of the available natural resources more efficiently, with better uptake of resources or allocation of assimilates (partitioning) towards reproductive organs (flowers, cherelles, and pods). However, when manipulated into repeated overcropping with artificial pollination regimes, these high-yielding cacao cultivars may exploit available (limiting) inputs at a faster rate to sustain enhanced crop loads causing *i.* increasing between-pod competition for limiting resources, *ii.* an increased rate of within-tree resource depletion and leading to *iii.* subsequent abnormal flowering patterns, fruit abortion, and suboptimal yields in following years (Valle et al., 1990). Especially in the absence of precision agronomic management. Given the above, declining within-tree resource reserves from one, or multiple years of artificially intensified crop loads seems plausible and may explain the observed reduction in the effect of increasing pollination intensity on both cherelle survival and harvested pod yield by the end of the 3-year study. The above may be suggestive of a potential natural biennial bearing cycle in cacao (Cheesman, 1948), driven by assimilate depletion and perhaps magnified by overcropping under artificially enhanced pod loads. Discovering the conditions needed to support repeated artificially enhanced (overcropped) pod loads (e.g., via precision nutrition or timely management of “off” years according to within-tree nutritional status) may be crucial to fully realise the benefits offered by increased and condensed artificial pollination. Multi-year studies of field- or pot-grown cacao trees under heavy cropping, in combination with controlled nutrition treatments, non-structural carbohydrate and hormone monitoring and strategies to control bearing cycles, may facilitate such learnings.

5.6 Conclusion

In the current study, I identified multiple physiological trade-offs at the individual level in the impacts of cacao pollination management. Increasing pollination intensity increases the number of pods produced to the cost of pod size and quality, and narrowing the pollination time to improve cherelle survival and production efficiency can lead to overcropping, resource depletion and reduce bean quality. Thus, short-term strategies to improve cacao yield through increasing pollination intensity or condensing pollination time windows may have unintended consequences on bean quality and long-term production sustainability. Despite this, the ‘avoidance of cherelle wilting’ with narrowed pollination windows is an exciting prospect for future artificial pollination management strategies in cacao, if its potential can be harnessed. Further exploration of cherelle wilt avoidance to enhance yield in cacao is desirable but should be evaluated in combination with management strategies designed to support sufficient pod and bean growth under enhanced and/or overcropped pod loads. While sustainable cacao production indeed depends on the annual quantity of cacao bean production, identifying factors that impact bean quality (e.g., bean size, fat content) is equally important, particularly as changes can alter cacao prices for cacao farming families (McHenry and Fritz, 1987). Thus, I also recommend future research includes relevant bean physical and quality characteristics.

This multi-year study also revealed the crucial role that inter-annual variability plays in understanding the viability of artificial pollination strategies to enhance yield in cacao. I therefore recommend that longer-term evaluations of artificial pollination strategies continue to be investigated. Although not identified as a key factor here, long-term studies evaluating fruit production in other systems have identified that increasing short-term yield can lead to biennial bearing cycles (Wolstenholme, 2010; Thorp, 2011; El-Sayed et al., 2017; Martínez-Lüscher and Kurtural, 2021). My results suggest the potential for biennial bearing patterns in cacao, mediated by enhanced resource depletion under overcropping from moderately intensive artificial pollination. Adding to my previous suggestions, methods are needed to understand the within-tree non-structural carbohydrate reserve thresholds that are required for cacao fruiting (and artificially enhanced fruiting), and the conditions (e.g., nutritional inputs, time) needed to reaccumulate depleted reserves back to the required thresholds. Integrated approaches (e.g., crop modelling or path analysis) incorporating important crop and environmental parameters (e.g., cultivar, age, pollen donor and compatibility, environment, agronomic management, previous year’s production, internal tree carbohydrate reserves etc), may help farm managers to identify if, when and how (e.g., pollination intensity and timing) to implement artificial pollination strategies. Such approaches will also help to identify the capacity of, and conditions needed by, cacao trees to support maximised pod loads, without compromising pod size, single bean size or bean quality. Only then may the efforts of this labour- and resource-intensive yield enhancing strategy be truly fruitful.

5.7 Supplementary Materials

Supplementary Text 5.1. Mars standard agronomic practice for experimental cacao tree management (2018 – 2020).

A. Pre-trial tree preparation

Tree suitability for experimental trial was evaluated annually, with all 93 trees deemed suitable for experimentation upon each annual review. In each year the study was conducted, and one month prior to the first pollination date, all experimental trees were lightly pruned, and had their flowers and developing fruits (regardless of size) removed with secateurs. This practice is generally assumed to *i*. promote flowering without causing competition effects between leaf flushing, flowering, and fruit setting, *ii*. increase physiological homogeneity between experimental trees and *iii*. minimize possible resource competition and compensation effects from existing and non-experimental fruits.

B. Tree nutrition

A one-sided drip irrigation system (Netafim, Fertikit) was fitted to all remaining cacao trees within the pollination plot (post tree thinning of every other row), giving each experimental cacao tree two drippers, each positioned 0.5 m on either side of the tree trunk. Additional drippers were positioned every 1 m along the irrigation line. This system provided dissolved mineral fertilizers to experimental trees every second day in 4 to 6 mm water/ha, per day during the dry season and in a more concentrated solution of 2 mm water in months of increased rainfall. The composition and variable dosage of applied fertilizers via fertigation was to complement the cropping cycle of cacao trees in South Sulawesi according to annual conditions and tree age.

Fertilizer type and dosage applied each year and month to experimental cacao trees across the 3-year study.

Year	Fertilizer	Month and fertilizer dosage (kg-ha^{-1})											
		Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec
2018													
	MerokeMAP -Monoammonium phosphate (N 12%, Phosphate P_2O_5 61%)	48.22	30.58	30.14	15.88	18.52	47.78	44.98	30.58	30.14	15.88	18.23	47.63
	MerokeKALINITRA-Potassium nitrate (K_2O 45%, N 13%)	163.17	95.11	182.13	216.09	192.13	183.02	164.79	94.96	182.13	216.09	192.13	182.28
	MerokeMAG-SM - Magnesium sulfate (MgO 16%, S 13%)	21.90	16.32	24.40	25.28	22.49	21.46	22.05	16.32	24.40	27.93	22.49	24.40
	Neo Kristalon Boron - (B_2O_3 55%)	2.21	2.35	1.86	1.03	2.35	4.12	2.21	2.35	1.91	1.03	2.35	4.12
	Zinc sulfate ($ZnSO_4$, Zn 21%)	1.62	1.03	1.00	0.44	0.59	1.62	1.47	1.03	1.03	0.44	0.59	1.62
	Karate Plus Boroni - Calcium nitrate (CaO 26%, N 15.5%) plus Boron (0.3%)	236.82	348.83	196.98	0.00	5.15	157.58	242.84	357.36	196.98	0.00	5.15	157.00
2019													
	MerokeMAP -Monoammonium phosphate (N 12%, Phosphate P_2O_5 61%)	48.22	30.58	30.14	15.88	18.52	47.78	44.98	30.58	30.14	15.88	18.23	47.63
	MerokeKALINITRA-Potassium nitrate (K_2O 45%, N 13%)	163.17	95.11	182.13	216.09	192.13	183.02	164.79	94.96	182.13	216.09	192.13	182.28
	MerokeMAG-SM - Magnesium sulfate (MgO 16%, S 13%)	21.90	16.32	24.40	25.28	22.49	21.46	22.05	16.32	24.40	27.93	22.49	24.40
	Neo Kristalon Boron - (B_2O_3 55%)	2.21	2.35	1.86	1.03	2.35	4.12	2.21	2.35	1.91	1.03	2.35	4.12
	Zinc sulfate ($ZnSO_4$, Zn 21%)	1.62	1.03	1.00	0.44	0.59	1.62	1.47	1.03	1.03	0.44	0.59	1.62
	Karate Plus Boroni - Calcium nitrate (CaO 26%, N 15.5%) plus Boron (0.3%)	236.82	348.83	196.98	0.00	5.15	157.58	242.84	357.36	196.98	0.00	5.15	157.00

2020													
	MerokeMAP -Monoammonium phosphate (N 12%, Phosphate P_2O_5 61%)	19.68	12.36	6.11	3.20	3.71	9.57	9.09	6.21	6.11	3.20	3.78	11.55
	MerokeKALINITRA-Potassium nitrate (K_2O 45%, N 13%)	66.60	38.40	36.80	43.68	38.43	36.61	33.31	19.20	0.64	43.68	38.85	44.13
	MerokeMAG-SM - Magnesium sulfate (MgO 16%, S 13%)	8.94	6.60	4.93	5.12	4.51	4.29	4.48	3.30	4.93	4.93	4.54	5.92
	Neo Kristalon Boron - (B_2O_3 55%)	0.90	0.96	0.42	0.22	0.48	0.83	0.45	0.45	0.38	0.19	0.48	1.02
	Zinc sulfate ($ZnSO_4$, Zn 21%)	0.66	0.42	0.22	0.10	0.13	0.32	0.29	0.19	0.19	0.10	0.10	0.38
	Karate Plus Boroni - Calcium nitrate (CaO 26%, N 15.5%) plus Boron (0.3%)	96.66	141.00	39.81	0.00	1.02	31.52	49.09	70.43	75.97	0.00	1.06	38.02

[z] Information contained in this table was obtained from Mars Incorporated, 2023.

C. Pruning and weed management

The cacao trees typically had three to five main branches that together formed a low ‘cup-shape’ tree architecture. Pruning management was conducted to support this desirable tree architecture as well as physical homogeneity between experimental trees. Heavy structural pruning of hardened chupons and branches was conducted once per year after the main harvest was complete, while a smaller light sanitary pruning event to manage new chupons, undesirable leaf biomass and understory vegetation (e.g., weedy species) occurred when developing pods were approximately 3 months old, to increase light and airflow, and thereby reducing the incidence of *Phytophthora*.

D. Pest and disease control

During the trial, chemical control of the cocoa pod borer (*Conopomorpha cramerella,* ‘CPB’) was conducted every two weeks by targeted application of Prevathon (DuPont™, active constituent: Rynaxypyr® Chlorantraniliprol) insecticide. A knapsack sprayer was used to apply insecticides as a light mist on the ventral surfaces of cacao tree branches where adult insects commonly harbour during the daylight hours. In 2018 and 2019, pod ‘sleeving’, whereby a plastic sleeve is applied to encapsulate mature sized pods, was conducted to prevent cocoa pod borer (‘CPB’, *Conopomorpha cramerella*) oviposition. However, this practice was discontinued in 2020 after observations in 2019 of enhanced black pod disease (*Phytophthora palmivora,* ‘*Phytophthora*’) infection rates, and subsequent increased pod losses of sleeved pods. The weekly application of insecticide at the same dosage was implemented as the alternative to sleeving. Chemical control of *Phytophthora* on cacao pods was controlled by application of Score (Syngenta$_{®}$, active constituents: Difenoconazole) and Ridomil gold (Syngenta$_{®}$, active constituents: Mancozeb + Metalaxyl-M) fungicides. Fungicides are applied on fortnightly basis using a knapsack sprayer, directly onto the pod, from the initial observation of a developing pod, through until harvest. Control of *Phytophthora* on the tree trunk (stem canker) is by targeted application of a concentrated Nordox (NORDOX, active constituent: Copper (Cu) present as Cuprous Oxide (Cu_2O) solution (20 g Nordox per L of water), after infected tissues have been removed with a sharp, clean blade. Due to increased *Phytophthora* rates in 2019 and 2020, weekly application frequency was implemented at the same dosage.

Supplementary Text 5.2. Additional information on the experimental treatments.

The three artificial pollination intensity levels (30, 60, 90 artificial pollinations per tree) were developed *i.* with the expectation of ~ 50% pod losses from cherelle wilt post-pollination, *ii.* in knowing the young age of the experimental trees, *iii*. considering previous artificial pollination studies by Forbes et al. (2019) showing final harvested pod yields of ~ 42 pods per tree when receiving more than 200 artificial pollinations per tree, *iv.* assuming yields calculated at 1000 trees per hectare, *v*. in knowing that excessive pod loads combined with extreme environmental perturbations (e.g., flooding or pest and disease outbreaks) can cause tree mortality (Mars Inc., *unpublished data*) and, *vi*. with the intention of conducting two pollination periods per year, reflecting the major and minor yield peaks of cacao in Sulawesi. As the lower limit, I selected the '30' pollination intensity treatment as an annual yield of approximately 30 harvested pods per tree (assuming 50% pod losses but with two distinct pollination periods per year) is expected from this clone and likely sustainable over time. For the mid-range, I selected the '60' pollination intensity treatment under the assumption that an annual yield of 60 harvested pods would be doubling natural yield expectations whilst remaining as a non-excessive pod load that is potentially sustainable over time for young trees. Finally, I selected the '90' pollination intensity treatment as the upper limit, under the assumption that an annual yield of 90 harvested pods would be tripling current yields but potentially excessive and unsustainable over three consecutive years. Despite my best efforts, delayed flowering prevented a second pollination event during any experimental year, and I thus only achieved one pollination period per year.

Supplementary Text 5.3. Additional information on artificial pollination methods.

A team of 10 people ('pollinators') conducted the daily artificial pollinations for this trial over the 3-year period. Each pollinator can achieve 250 (maximum) artificial pollinations (uncontrolled) per day. To make daily artificial pollinations on all 93 experimental trees achievable by the team in the morning hours, the total number of flowers to be artificially pollinated per tree (Table 5.1) was distributed evenly across the total number of pollination days for each treatment. The total number of pollinations to be made per tree and per pollination period (i.e., 30, 60 or 90) was equally distributed between all main branches. Furthermore, within each main branch, effort was made to distribute the artificial pollination of flowers across the whole branch area, avoiding flowers emerging from the same cushion. This was done in attempt to evenly distribute the resource demands of developing cherelles / pods across the experimental branch area. Collection of the BB01 genotype (paternal pollen-donor) flowers was at 08:00 am on each pollination date, and flowers were stored in a sealed plastic container in a cool location out of direct sunlight until used. Additional BB01 flowers were harvested as required. The identifier tags were attached with cotton string to the main branch directly adjacent the pollinated flower's pedicel. This done to avoid creating open wound sites for pathogen infection that can be caused by other tagging methods such as metal pins. The total number of flowers pollinated per tree, per day, was recorded to ensure that the total number of artificial pollinations required per tree was achieved. To reduce potential 'time-of-pollination' effects on artificial pollination success, artificial pollinations were always started in on a different cacao tree on each pollination day. Furthermore, the 10 pollinators were trained in the same manner and randomly assigned to trees within the experimental field on each pollination day to potential pollinator effects. Artificial pollinations were considered as supplemental to natural pollinations, as flowers are not protected from pollination by insects or wind.

Supplementary Table 5.1. Clone MCC02 information.

General clone information

Clone MCC02 (also known as ‘MP45’ or ‘Clone 45’) originates in Tingkara Village, Malangke Barat District, North Luwu Regency, South Sulawesi, Indonesia. The material was first identified in 2006 by a local cacao farmer for its high pod production under open pollination conditions, prompting screening tests and field testing with farmers in 2009-2012 as requested by the Indonesian Cocoa Research Institute. The plant material was subsequently released by the national government in 2014 as ‘clone MCC02’ and now has a wide distribution across Sulawesi (more than 50 million seedlings distributed across Sulawesi). Clonal screening suggests that clone MCC02 is known to produce approximately 30 pods per tree under the (suboptimal) farm design and management strategies that are common in Sulawesi. However, clone MCC02 is suggested to yield much higher when grown under optimal planting design and recommended agricultural practice. The pods are long, oval-shaped, red in colour and generally have a fresh pod mass of 380 – 830 grams (average 605 g). Pods take approximately 6 months from pollinated flower to ripe harvestable fruit. Each pod is expected to yield approximately 42 flattened oval-shaped beans per pod, with per bean dry masses typically ranging between 1.4 – 1.6 g per bean (equivalent to ~ 71 beans per 100g). The obtained fat content values of bean nib samples for this clone are between 49.9 – 51 %. Clone MCC02 is highly self-incompatible with 0% fertilization success after controlled self-pollination (Mars Inc., *unpublished data*). Thus, this clone requires pollen from a different cacao genotype for successful fertilization and adequate bean development, and it is strongly recommended that farmers who utilize this clone, do so using multi-clonal plantings to facilitate cross-pollination and avoid potential yield losses to insufficient pollination from self-incompatibility. Clone MCC02 is a popular choice by farmers, as when planted in multi-clonal designs, the clone offers *i*. high yield, *ii*. ease of management (pruning; a small / medium sized tree and not overly vigorous), *iii*. reasonable tolerance to CPB, *Phytophthora* and VSD, *iv*. good bean size and *v*. good availability of budwood for propagation. However, the high yield and other desirable agronomic characters has led this clone to become almost ‘too popular’ with many cacao farmers choosing to plant mono clonal MCC02 farms, despite the strong advice from qualified farm extensionists and knowledgeable others that multi-clonal plantings are crucial for yield optimization. As result, issues of low yield production have arisen with this clone in Sulawesi, presumably due to low clonal diversity and the subsequent effects on pollination success. More recently, this clone has become a popular choice as a locally adapted and disease-resistant rootstock for other desirable cacao clones, as it is vigorous in the nursery and has a high resistance to trunk canker. Clone MCC02 has been taken for trial in other countries, such as the Philippines and Vietnam, but the performance of this clone in other regions remains unknown.

Variable	**Value**	**Description**

Tree characteristics		
Growth habit	Erect	The architecture of plant growth.
Plant vigour	Small / medium tree with low vigour	The reported level of vigour in plant growth.
Leaf shape	Moderate – long elliptic	The shape of individual cacao leaves.
Leaf flush colour	Red	The colour of the new (young leaves) during leaf flushing.
Flowering	High flowering, early	The reported level of flowering intensity measured using Mars Inc. flowering assessment classifications.
Compatibility	Highly self-incompatible	The degree of self-compatibility, and cross-compatibility (where relevant).
Fruiting season	Continuous throughout the year but with two distinct peaks	The reported periods of annual major and minor peak fruit production events.
Main harvest(s)	End April – end July (major) October – December (minor)	The reported periods of annual major and minor peak pod harvesting events.
Fruits (pods)		
Harvested pods per tree	30 pods	Annual average number of ripe harvested pods per year obtained from a single MCC02 tree under open pollination conditions.
Fruit shape	Oval and long	The reported average shape of MCC02 pods.
Surface colour	Red (cherelle and mature) with flush to orange with yellow sectors indicating ripeness	The colour of MCC02 pods during the cherelle, mature and ripe stages of fruit development.
Surface rugosity	Intermediate	The reported level of rugosity (topographic complexity, roughness) of the pods exterior surface.
Basal constriction	Intermediate	The reported level of the presentation of a basal constriction in MCC02 cacao pods.
Apex form	Obtuse	The reported appearance of the apex form in MCC02 cacao pods.
Primary furrow	Intermediate	The reported level of the presentation of primary furrows in the MCC02 pod’s exterior surface.
Fruit (pod) fresh mass	380 - 830 g (av. 605 g)	The average fresh mass (g) of a single MCC02 pod after harvest.
Pod index	18.5 - 22	The number (count) of pods required to produce 1kg of dry cacao beans.
Fruit length	19 cm	The average length (cm, base to apex length) of a mature MCC02 cacao pod.
Fruit diameter	10 cm	The diameter (cm) of the mature fruit, measured at its widest girth.
Husk thickness	1.7 cm	The thickness (cm) of the mature fruits husk wall.

Seeds (beans)		
Number of beans per pod	42 beans	The average number of cacao beans per pod.
Dry bean mass	1.4 - 1.6 grams	The average mass of an individual dry bean.
Bean shape	Flattened oval	The reported shape of a typical MCC02 cacao bean.
Cotyledon colour	Light purple 90% with moderate purple surrounding	The colour of the fresh, un-dried cotyledon.
Butter / fat content	49.9 - 51 %	The expected percentage fat content to be obtained from a MCC02 dry bean sample, as measured by NIR.
Shell content	11.53 %	The expected percentage shell content to be obtained from a MCC02 dry bean sample.
Bean count (100 grams)	71 beans	The average number (count) of beans in a 100 g dry bean sample.
Pest and Disease		
Vascular Streak Dieback (VSD) resistance	Medium	Reported resistance level of clone MCC02 to VSD.
Phytophthora palmivora (Phytophthora) resistance	Low - Medium	Reported resistance level of clone MCC02 to *Phytophthora*.
Cocoa Pod Borer (CPB; *Conopomorpha cramerella*) resistance	Medium	Reported resistance level of clone MCC02 to CPB.

[z] Information contained in this table was obtained from Mars Incorporated, 2023.

Supplementary Table 5.2. Clone BB01 information.

General clone information

Clone BB01 originates in Buntu Batu Village, Luwu Regency, South Sulawesi, Indonesia. The material was first identified in year 2005 by a local cacao farmer for its high pod production and lower level of pest and disease incidence. Clone BB01 is still in the process of clone screening for release from the national government of Indonesia. Despite this, clone BB01 is present in the local community with approximately 180,000 cacao plants distributed across 2,300 farms, achieved by both side grafting on existing trees and replanting. Clonal screening suggests that clone BB01 has a slightly perpendicular-horizontal branching with rapid branch growth. Mature trees produce 55 - 62 oval to round and green coloured pods per year that average 610 grams (fresh pod mass). Each pod is expected to yield approximately 44 beans per pod that are shaped like unequal triangles, with per bean dry masses typically averaging 1.40 g per bean (equivalent to ~ 73.3 beans per 100g). The obtained fat content values of bean nib samples for this clone are approximately 50.0 %. Clone BB01 is self-incompatible with 0% fertilization success after controlled self-pollination (Mars Inc., *unpublished data*). While clone BB01 yields well, it is a popular choice by farmers as a pollen donor (source) tree, as this clone results in as high as 74 % pollination success when used as the pollen donor in controlled pollinations with other (non-BB01 maternal) clones (Mars Inc., *unpublished data*). Clone BB01 is the most highly cross compatible clone known in the region at present (Mars Inc., *unpublished data*). Thus, having this clone present in a well thought out planting design with other desirable clones can help reduce pollination limitation caused by self-and cross-incompatibility.

Variable	**Value**	**Description**
Tree characteristics		
Growth habit	Semi erect	The architecture of plant growth.
Plant vigour	Vigorous	The reported level of vigour in plant growth.
Leaf shape	Obovate	The shape of individual cacao leaves.
Leaf flush colour	Light brown	The colour of the new (young leaves) during leaf flushing.
Flowering	High with flowering Index of 3.49 through the year	The reported level of flowering intensity according to Mars Inc. classifications.
Compatibility	Highly self-incompatible, Highly cross-compatible	The degree of self-compatibility, and cross-compatibility (where relevant).
Fruiting season	Most fruits developing between March – May and August – October	The reported periods of annual major and minor peak fruit production events.

Main harvest(s)	May - August (major) and November – January (minor)	The reported periods of annual major and minor peak pod harvesting events.
Fruits (pods)		
Harvested pods per tree	55 - 62 pods	Annual average number of ripe harvested pods per year obtained from a single BB01 tree under open pollination conditions.
Fruit shape	Round	The reported average shape of BB01 pods.
Surface colour	Medium green with reddish sectors (cherelle and mature pod)	The colour of BB01 pods during the cherelle, mature and ripe stages of fruit development.
Surface rugosity	High, rough	The reported level of rugosity (topographic complexity, roughness) of the pods exterior surface.
Basal constriction	Intermediate	The reported level of the presentation of a basal constriction in BB01cacao pods.
Apex form	Obtuse	The reported appearance of the apex form in BB01 cacao pods.
Primary furrow	Intermediate	The reported level of the presentation of primary furrows in the BB01 pod's exterior surface.
Fruit (pod) fresh mass	370 - 780 grams (av. 610 g)	The average fresh mass (g) of a single BB01 pod after harvest.
Pod index	15.7 - 20	The number (count) of pods needed to produce 1kg of dry cacao beans.
Fruit length	15 - 18 cm	The average length (cm, base to apex length) of a mature BB01 cacao pod.
Fruit diameter	8 - 10 cm	The diameter (cm) of the mature BB01 fruit, measured at its widest girth.
Husk thickness	1.42 - 1.74 cm	The thickness (cm) of the mature fruits husk wall.
Seeds (beans)		
Number of beans per pod	38 - 51 beans (av. 44)	The average number of cacao beans per pod.
Dry bean mass	1.4 grams	The average mass of an individual dry bean.
Bean shape	Unequal triangles	The reported shape of a typical BB01 cacao bean.
Cotyledon colour	Dark purple	The colour of the fresh, un-dried cotyledon.
Butter / fat content	50 %	The expected percentage fat content to be obtained from a BB01 dry bean sample, as measured by NIR.
Shell content	12.10 %	The expected percentage shell content to be obtained from a BB01 dry bean sample.
Bean count (100 grams)	73.3 beans	The average number (count) of beans in a 100 g dry bean sample.
Pest and Disease		
Vascular Streak Dieback (VSD) resistance	Medium	Reported resistance level of clone BB01 to VSD.

Phytophthora palmivora (*Phytophthora*) resistance	Low	Reported resistance level of clone BB01 to *Phytophthora*.
Cocoa Pod Borer (CPB; *Conopomorpha cramerella*) resistance	Low – Medium	Reported resistance level of clone BB01 to CPB.

[z] Information contained in this table was obtained from Mars Incorporated, 2023.

Supplementary Table 5.3. Mean (± SE) flowering intensity of experimental cacao trees during the 3-week pollination period and across three years.

Year	**Treatment details**		**Average number of open flowers counted per tree and per pollination date**			
	Pollination intensity	**Pollination timing**	**Week 1 BL flowers**	**Week 2 BL flowers**	**Week 3 BL flowers**	**Total AL flowers**
2018						
	30	Middle	25.73 ± 4.9	40.34 ± 8.0	47.53 ± 8.5	4.85 ± 1.0
	60	Middle	23.68 ± 4.6	34.20 ± 8.3	46.93 ± 10.3	6.97 ± 1.2
	90	Middle	26.63 ± 4.8	34.29 ± 6.7	47.74 ± 9.1	7.54 ± 0.7
	30	Early	23.25 ± 5.6	52.29 ± 16.8	48.50 ± 11.8	4.22 ± 0.6
	60	Early	31.32 ± 8.9	57.46 ± 12.1	65.50 ± 11.5	11.59 ± 2.9
	90	Early	15.25 ± 5.4	37.57 ± 11.7	50.75 ± 9.9	4.59 ± 1.1
	30	Late	23.50 ± 6.8	40.79 ± 13.7	44.25 ± 11.2	5.59 ± 1.6
	60	Late	26.93 ± 7.9	53.43 ± 20.8	45.35 ± 11.5	9.24 ± 3.8
	90	Late	17.89 ± 4.1	29.93 ± 7.3	39.35 ± 5.1	7.37 ± 1.5
	30	All	22.61 ± 5.2	49.00 ± 12.5	63.66 ± 13.7	8.18 ± 2.0
	60	All	28.34 ± 6.0	50.63 ± 11.5	50.83 ± 7.5	8.65 ± 1.4
	90	All	13.61 ± 3.9	24.29 ± 6.6	28.98 ± 5.4	4.76 ± 1.4
	30	Split	26.88 ± 7.0	47.98 ± 10.3	48.43 ± 10.6	7.62 ± 2.1
	60	Split	24.52 ± 6.3	50.05 ± 13.7	58.33 ± 13.7	8.90 ± 2.5
	90	Split	21.04 ± 5.3	50.09 ± 12.0	51.43 ± 9.8	7.57 ± 2.5
2019						
	30	Middle	111.70 ± 19.4	76.07 ± 14.2	61.66 ± 10.6	8.54 ± 1.3
	60	Middle	141.95 ± 24.8	100.52 ± 16.7	82.16 ± 11.9	9.57 ± 0.7
	90	Middle	185.47 ± 32.2	117.92 ± 16.1	113.24 ± 19.8	17.97 ± 1.8
	30	Early	224.71 ± 42.6	172.21 ± 36.0	141.14 ± 24.0	15.85 ± 2.6
	60	Early	118.54 ± 30.9	103.54 ± 15.1	87.00 ± 13.0	12.08 ± 1.6
	90	Early	132.07 ± 37.3	108.04 ± 21.6	104.04 ± 26.0	10.44 ± 1.8
	30	Late	166.18 ± 45.1	123.86 ± 38.3	106.07 ± 18.1	14.76 ± 3.5
	60	Late	156.68 ± 28.1	115.54 ± 21.8	117.25 ± 24.4	16.04 ± 2.1
	90	Late	154.46 ± 25.8	111.96 ± 17.1	98.93 ± 13.2	14.83 ± 1.8
	30	All	116.78 ± 16.4	99.78 ± 15.9	84.82 ± 12.4	11.26 ± 1.6
	60	All	158.59 ± 25.2	113.00 ± 13.7	110.41 ± 15.6	16.00 ± 2.6
	90	All	89.25 ± 18.6	71.82 ± 14.5	65.93 ± 13.0	10.02 ± 1.4
	30	Split	160.47 ± 22.8	115.18 ± 14.6	100.71 ± 15.5	11.96 ± 1.6
	60	Split	134.13 ± 22.5	117.71 ± 17.1	93.02 ± 10.8	14.74 ± 2.0

	90	Split	192.46 ± 31.4	139.23 ± 23.1	115.64 ± 17.9	14.74 ± 2.8
2020						
	30	Middle	15.88 ± 4.1	10.79 ± 4.5	42.70 ± 14.0	9.75 ± 1.9
	60	Middle	22.29 ± 7.6	8.63 ± 4.3	43.15 ± 16.6	12.88 ± 1.3
	90	Middle	21.29 ± 4.2	4.79 ± 4.2	42.57 ± 15.0	9.70 ± 1.9
	30	Early	32.08 ± 11.6	13.79 ± 5.8	81.55 ± 34.0	17.82 ± 4.5
	60	Early	16.88 ± 5.2	9.17 ± 5.2	51.10 ± 11.6	12.88 ± 1.5
	90	Early	22.38 ± 9.7	6.21 ± 4.4	50.60 ± 14.9	13.24 ± 2.7
	30	Late	20.75 ± 5.5	12.75 ± 7.6	43.65 ± 18.1	9.99 ± 2.5
	60	Late	34.38 ± 9.3	26.92 ± 13.2	74.70 ± 20.6	15.84 ± 1.2
	90	Late	16.17 ± 3.8	16.75 ± 9.8	51.45 ± 11.7	13.29 ± 1.4
	30	All	26.67 ± 6.1	24.05 ± 6.9	75.03 ± 18.5	18.88 ± 1.9
	60	All	18.46 ± 5.9	16.31 ± 6.8	44.85 ± 17.0	14.06 ± 2.6
	90	All	9.63 ± 2.9	10.50 ± 4.9	25.50 ± 8.3	10.12 ± 1.5
	30	Split	17.90 ± 4.7	28.26 ± 8.1	50.11 ± 12.0	14.81 ± 2.8
	60	Split	18.88 ± 5.1	23.08 ± 5.5	52.38 ± 17.5	13.85 ± 1.5
	90	Split	16.67 ± 5.0	24.69 ± 7.5	40.43 ± 12.7	13.71 ± 1.1

[z] 'BL' the experimental branch area existing below the 2m height limit, within which artificial pollinations were conducted; 'AL', the non-experimental branch area existing above the 2m height limit.

[y] Flowering intensity for flowers existing 'BL' are separated between three pollination weeks (Week 1, Week 2, Week 3).

[x] The three pollination intensity levels (30, 60, 90) represent the annual total number of artificial pollinations made per tree.

[w] Pollination timing categories (Middle, Early, Late, All, Split) alter the degree of spread between artificial pollinations within the pollination period and are listed in order of assumed increasing spread.

[v] Number of replicate trees per unique pollination intensity × pollination timing treatment is n = 8,8,7,4,4,4,4,4,4,7,8,8,7,8,8, respectively.

Supplementary Table 5.4. Mean (± SE) number and proportion of surviving cherelles, number of harvested pods, and individual pod fresh mass (g) for experimental cacao trees across three years.

Year	**Treatment details**		**Average values for artificially pollinated cherelles and harvested pods per tree**			
	Pollination intensity	**Pollination timing**	**Number of surviving cherelles (counts)**	**Proportion of cherelles surviving (%)**	**Number of harvested pods (counts)**	**Individual pod fresh mass (g)**
2018						
	30	Middle	18.38 ± 0.9	61.25 ± 3.2	17.50 ± 1.3	606.59 ± 44.9
	60	Middle	34.63 ± 1.2	57.60 ± 2.1	31.50 ± 1.7	563.02 ± 27.0
	90	Middle	58.14 ± 3.6	64.48 ± 3.9	54.00 ± 3.1	446.11 ± 14.2
	30	Early	14.50 ± 2.9	44.64 ± 9.5	12.25 ± 2.9	702.23 ± 47.3
	60	Early	32.25 ± 2.3	53.50 ± 3.6	29.00 ± 3.2	607.34 ± 23.2
	90	Early	51.25 ± 2.0	56.94 ± 2.2	47.25 ± 1.6	496.85 ± 25.3
	30	Late	19.00 ± 2.0	62.19 ± 6.3	16.25 ± 2.3	650.39 ± 48.5
	60	Late	32.75 ± 1.4	54.58 ± 2.3	30.00 ± 1.4	565.68 ± 26.6
	90	Late	41.25 ± 5.1	45.83 ± 5.7	37.50 ± 4.3	483.96 ± 39.4
	30	All	16.00 ± 0.8	50.02 ± 3.0	14.29 ± 1.0	676.14 ± 21.6
	60	All	28.88 ± 2.0	47.36 ± 3.1	23.25 ± 2.4	543.82 ± 31.5
	90	All	40.13 ± 2.5	44.19 ± 2.9	36.75 ± 2.6	464.63 ± 17.1
	30	Split	14.57 ± 1.1	48.41 ± 3.6	12.57 ± 1.4	723.32 ± 27.1
	60	Split	18.25 ± 2.3	30.66 ± 3.5	16.25 ± 2.2	559.92 ± 31.9
	90	Split	35.63 ± 2.6	39.01 ± 2.9	33.88 ± 3.2	514.75 ± 24.2
2019						
	30	Middle	13.38 ± 1.6	44.58 ± 5.2	13.50 ± 1.3	539.11 ± 30.8
	60	Middle	28.13 ± 1.7	46.88 ± 2.8	28.25 ± 1.4	466.14 ± 18.8
	90	Middle	36.00 ± 3.4	40.00 ± 3.8	38.57 ± 2.3	459.80 ± 21.4
	30	Early	15.25 ± 1.3	50.83 ± 4.4	14.75 ± 1.0	602.87 ± 41.7
	60	Early	32.25 ± 4.5	53.75 ± 7.6	31.50 ± 4.0	451.86 ± 25.3
	90	Early	47.25 ± 1.3	52.50 ± 1.5	47.50 ± 2.4	384.04 ± 28.9
	30	Late	15.00 ± 0.9	50.00 ± 3.0	15.25 ± 1.3	610.18 ± 53.7
	60	Late	32.50 ± 4.0	54.17 ± 6.7	32.50 ± 3.6	468.03 ± 21.7
	90	Late	46.50 ± 3.8	52.62 ± 4.4	45.75 ± 4.3	394.88 ± 14.8
	30	All	17.29 ± 1.1	57.34 ± 3.7	17.29 ± 1.3	551.57 ± 26.2
	60	All	33.63 ± 2.1	56.07 ± 3.5	33.50 ± 2.0	455.87 ± 24.1

	90	All	42.00 ± 2.1	47.04 ± 2.4	42.50 ± 2.3	375.29 ± 17.3
	30	Split	18.14 ± 0.9	60.48 ± 3.1	17.29 ± 0.9	599.93 ± 22.4
	60	Split	32.88 ± 1.9	54.69 ± 3.2	31.75 ± 1.8	442.80 ± 18.6
	90	Split	46.75 ± 4.1	51.94 ± 4.5	45.13 ± 3.8	413.06 ± 19.2
2020						
	30	Middle	7.13 ± 2.0	23.75 ± 6.6	7.00 ± 2.0	665.15 ± 37.7
	60	Middle	12.38 ± 1.4	22.33 ± 2.5	13.63 ± 2.2	637.78 ± 20.2
	90	Middle	17.14 ± 2.6	19.05 ± 2.8	15.71 ± 2.4	551.99 ± 40.6
	30	Early	6.25 ± 1.0	20.83 ± 3.4	5.80 ± 0.7	727.13 ± 31.3
	60	Early	16.75 ± 2.0	27.92 ± 3.3	10.33 ± 2.7	628.71 ± 28.1
	90	Early	20.75 ± 1.9	23.06 ± 2.2	16.00 ± 2.3	631.49 ± 21.1
	30	Late	4.60 ± 1.3	18.00 ± 3.7	6.33 ± 1.7	715.09 ± 38.6
	60	Late	13.00 ± 4.0	21.67 ± 6.7	10.20 ± 3.6	633.89 ± 22.8
	90	Late	20.25 ± 1.7	22.50 ± 1.8	18.20 ± 2.6	609.95 ± 30.6
	30	All	6.57 ± 0.8	21.90 ± 2.8	6.80 ± 0.7	717.73 ± 26.4
	60	All	18.50 ± 1.5	30.83 ± 2.6	16.33 ± 2.3	640.23 ± 22.3
	90	All	20.00 ± 1.8	22.22 ± 2.0	16.90 ± 2.2	584.69 ± 34.1
	30	Split	8.14 ± 1.1	27.14 ± 3.7	6.25 ± 1.0	750.90 ± 14.5
	60	Split	19.63 ± 1.5	33.58 ± 2.8	15.10 ± 2.0	626.57 ± 18.1
	90	Split	26.13 ± 2.0	29.03 ± 2.3	18.20 ± 3.2	593.45 ± 28.5

[z] Cherelle survival (counts and proportion) was analysed at 110 DAP for the 2018 data, but at 90 DAP for the 2019 and 2020 data.

[y] Proportion of surviving cherelles represents cherelle survival as a proportion of the total number of initial artificial pollinations made, expressed as a percentage.

[x] The three pollination intensity levels (30, 60, 90) represent the annual total number of artificial pollinations made per tree.

[w] Pollination timing categories (Middle, Early, Late, All, Split) alter the degree of spread between artificial pollinations within the pollination period and are listed in order of assumed increasing spread.

[v] Number of replicate trees per unique pollination intensity × pollination timing treatment is *n* = 8,8,7,4,4,4,4,4,4,7,8,8,7,8,8, respectively.

Supplementary Table 5.5. Mean (± SE) cacao bean dry mass (kg) and fat content (%), for experimental cacao trees across three years.

Year	**Treatment details**		**Average values for artificially pollinated cacao beans**						
	Pollination intensity	**Pollination timing**	**Bean dry mass (kg)**				**Bean fat content (%)**		
						No. samples			
2018									
	30	Middle	0.62	±	0.2	6	48.93	±	1.3
	60	Middle	1.19	±	0.2	8	48.08	±	0.7
	90	Middle	1.38	±	0.3	8	47.08	±	0.8
	30	Early	0.55	±	0.2	6	50.72	±	0.8
	60	Early	1.15	±	0.2	6	48.91	±	1.3
	90	Early	1.77	±	0.4	8	47.65	±	0.6
	30	Late	0.95	±	0.2	8	48.30	±	0.4
	60	Late	1.16	±	0.2	7	49.11	±	0.8
	90	Late	1.49	±	0.3	8	48.06	±	0.8
	30	All	0.52	±	0.1	7	50.89	±	0.7
	60	All	0.65	±	0.2	10	49.53	±	0.8
	90	All	1.58	±	0.2	12	48.49	±	0.5
	30	Split	0.53	±	0.1	19	49.99	±	0.5
	60	Split	0.54	±	0.2	9	50.63	±	1.2
	90	Split	1.23	±	0.2	8	48.51	±	0.6
2019									
	30	Middle	0.53	±	0.1	4	52.25	±	0.7
	60	Middle	0.96	±	0.1	5	51.78	±	0.3
	90	Middle	1.18	±	0.1	5	49.64	±	1.0
	30	Early	0.63	±	0.0	3	51.77	±	0.8
	60	Early	1.02	±	0.2	1	51.30	±	NA
	90	Early	1.23	±	0.2	2	50.85	±	0.6
	30	Late	0.64	±	0.0	3	52.93	±	0.6
	60	Late	0.85	±	0.1	2	50.45	±	0.3
	90	Late	1.02	±	0.2	1	50.40	±	NA
	30	All	0.68	±	0.1	3	51.80	±	0.7
	60	All	0.96	±	0.1	1	52.30	±	NA
	90	All	1.08	±	0.1	2	51.95	±	0.3
	30	Split	0.74	±	0.1	3	51.97	±	0.4

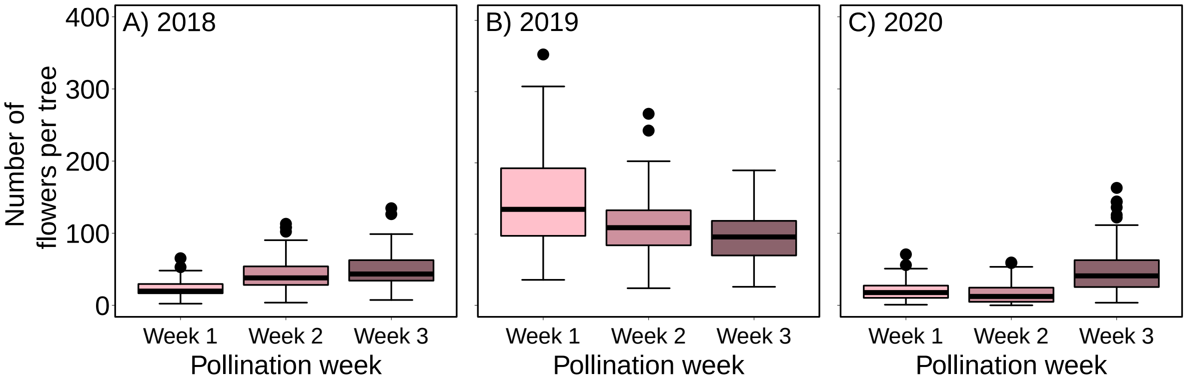
60	Split	0.88	±	0.1	3	50.17	±	0.4
90	Split	0.70	±	0.10	5	50.76	±	0.6
2020								
30	Middle	0.22	±	0.1	6	47.33	±	0.4
60	Middle	0.57	±	0.2	8	47.29	±	0.6
90	Middle	0.41	±	0.2	6	46.62	±	0.3
30	Early	0.47	±	0.2	3	47.23	±	0.4
60	Early	0.42	±	0.2	6	47.25	±	0.2
90	Early	0.46	±	0.2	8	47.36	±	0.6
30	Late	0.54	±	0.2	4	47.43	±	0.9
60	Late	0.47	±	0.1	5	47.88	±	0.6
90	Late	0.28	±	0.2	5	47.90	±	0.5
30	All	0.70	±	0.2	8	47.77	±	0.3
60	All	1.12	±	0.2	14	47.26	±	0.4
90	All	1.12	±	0.2	14	47.67	±	0.4
30	Split	0.37	±	0.1	8	47.92	±	0.4
60	Split	0.71	±	0.2	12	47.71	±	0.4
90	Split	0.59	±	0.1	15	47.59	±	0.5

[z] The three pollination intensity levels (30, 60, 90) represent the annual total number of artificial pollinations made per tree.

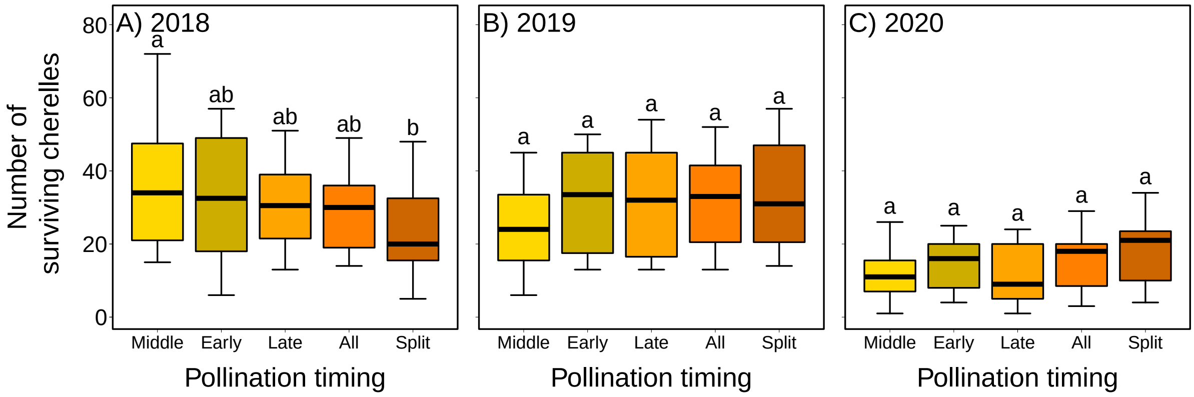
[y] Pollination timing categories (Middle, Early, Late, All, Split) alter the degree of spread between artificial pollinations within the pollination period and are listed in order of assumed increasing spread.

[x] No samples, the number of bean samples per treatment analysed for bean fat content.

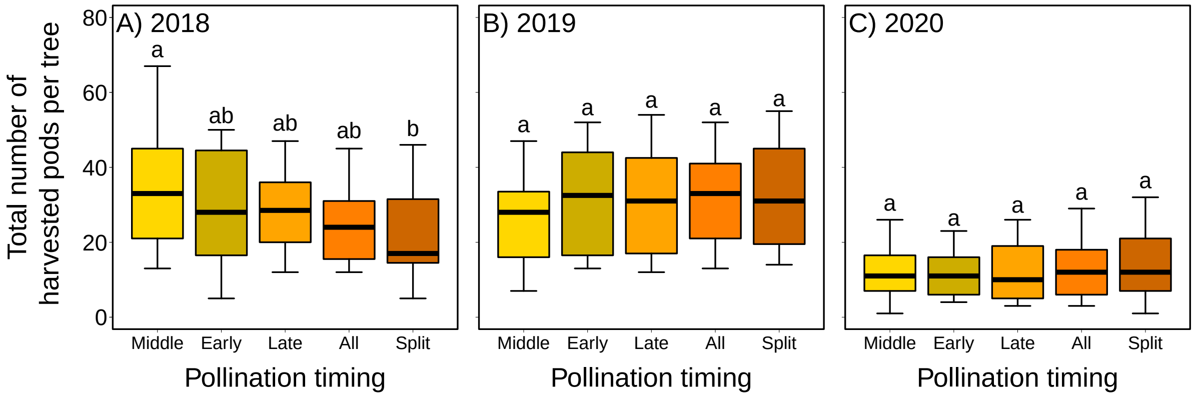
[w] Number of replicate trees per unique pollination intensity × pollination timing treatment for bean dry mass is n = 8,8,7,4,4,4,4,4,4,7,8,8,7,8,8, respectively.



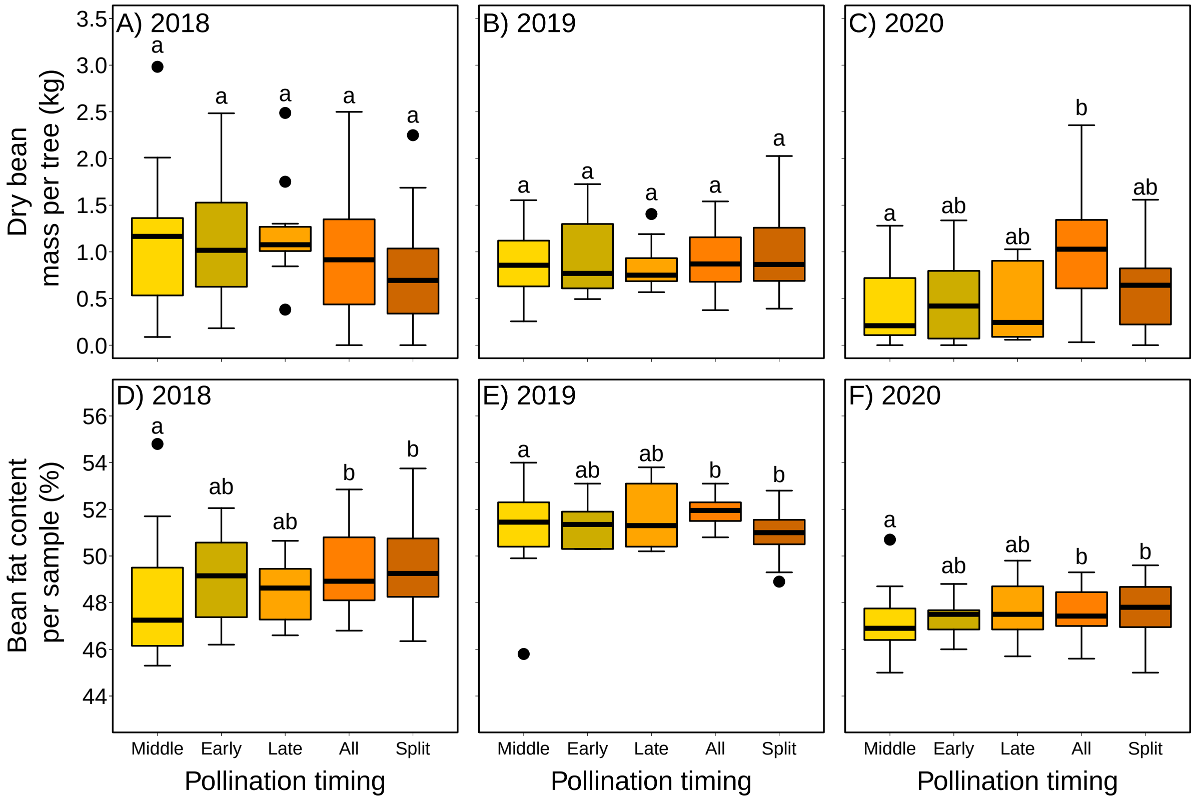
Supplementary Figure 5.1. Flowering intensity, the average number of freshly opened flowers counted per tree and per pollination date (n = 93 replicate trees), within the three pollination weeks (Week 1, Week 2, Week 3) and across three study years (A) 2018, B) 2019, and C) 2020).



Supplementary Figure 5.2. Number of surviving cherelles present per tree within five pollination timing treatments (Middle, Early, Late, All, Split) and across three study years (A) 2018, B) 2019, and C) 2020). Means with different letters of the same case (e.g., a, b) represent significant differences between treatments at an alpha level of $P < 0.05$ (Tukey's HSD tests). n = 23, 12, 12, 23, 23 replicate trees per pollination timing treatment.



Supplementary Figure 5.3. Number of harvested pods (fruits) per tree (A: C) within five pollination timing treatments (Middle, Early, Late, All, Split) and across three study years (A) 2018, B) 2019, and C) 2020). Means with different letters of the same case (e.g., a, b) represent significant differences between treatments at an alpha level of $P < 0.05$ (Tukey's HSD tests). n = 23, 12, 12, 23, 23 replicate trees per pollination timing treatment.



Supplementary Figure 5.4. Mass (kg) of dry cacao beans (seeds) per tree (A: C) and bean fat content (% of bean sample nib mass) (D: F) within five pollination timing treatments (Middle, Early, Late, All, Split) and across three study years (A, D) 2018, B, E) 2019, and and C, F) 2020). For the mass of dry cacao beans (A, B, C), pairwise comparisons were conducted among means within, but not between years. As there was no significant treatment × year interaction for bean fat content (D, E, F), pairwise comparisons were conducted among treatment means averaged across all years. Means with different letters of the same case (e.g., a, b) represent significant differences between treatments at an alpha level of $P < 0.05$ (Tukey's HSD tests). n = 23, 12, 12, 23, 23 replicate trees per pollination timing treatment.

Chapter 6. General Discussion and Conclusions

6.1 Study Summary

My thesis focused on addressing knowledge gaps I identified in the direct, indirect and interactive effects of future-predicted climate change. Specifically, I examined the direct and interactive impacts of elevated temperature and carbon dioxide concentration ([CO_2]) on key tropical crops and how changes in pollination intensity, an indirect impact of climate change, may impact crop yields and quality. In my studies I utilized three focal crop species: coffee, cacao and cassava, due to their tropical distributional ranges where the impacts of climate change are disproportionately larger than temperate zones, and due to their key roles in ensuring the economic livelihoods (coffee and cacao) and food and nutrition security (cassava) in many of the world's poorest and most food insecure regions. The results from these crops as studied in Indonesia (field studies of cacao) and Australia (controlled glasshouse studies of all crop species) can provide insight into their growth and use in other countries across their native ranges.

My first objective **(Objective 1)**, addressed in **Chapter 2 and 3**, was to evaluate the direct and interactive effects of future-predicted elevated air temperature and elevated [CO_2] on the physiology, tissue biochemistry and resource partitioning of the three study species. Overall, the results indicate that the effects of elevated air temperature and [CO_2] on the physiology, biochemistry and growth of these three globally important tropical crops will be generally positive for food production and safety. For the staple crop cassava, as evidenced in **Chapter 2**, the combined effects of elevated temperature and [CO_2] ranged from modified plant architecture to modified yield quantity and quality. Cassava plants grown under elevated temperature, in the presence of elevated [CO_2], showed increased plant biomass, tuber initiation and tuber mass. I also found that the negative effects of elevated temperature on tuber production may be ameliorated and indeed enhanced with simultaneous elevated [CO_2]. **Chapter 2** also revealed that elevated temperature decreased both tuber and leaf tissue cyanide concentrations, thus potentially improving food safety. However, the effect of temperature on cyanide concentration for cassava tubers, but not leaves, was reduced when supplemented with elevated [CO_2]. In this case, reduction in leaf cyanide concentration may improve the quality of cassava leaves for human (and perhaps livestock) consumption and perhaps reduce the need for processing before consumption. However, reduced leaf cyanide concentration may indirectly lead to increased herbivore pressure, with a cascade of subsequent potential reducing effects on yield and food security under future climate change.

Further to the main findings from **Chapter 2**, examining the commodity crops of coffee and cacao in **Chapter 3** also showed significant biomass increases when grown under non-extreme elevated temperature and non-limiting soil moisture. For coffee, increases in total plant biomass were expressed as increased lateral branching, which could have positive effects on final plant yield potential when trees reach fruit bearing age (da Silva Angelo et al., 2019), given coffee berries are produced on the lateral branches. However, in cacao I observed potential temperature-dependent growth abnormalities in cacao seedlings grown under increased temperature, that were potentially magnified by elevated [CO_2] fertilization effects. Cacao seedlings with significant stem elongation under elevated temperatures, were 'bent over' by the weight of new leaf flushes and required structural support. Our results support previous work (Batista and Alvim, 1981; Wood and Lass, 1987) and suggest that temperature-driven stem elongation during the orthotropic growth phase of establishing cacao seedlings could lead to undesirable plant architecture in well-watered and fertilized conditions.

Interestingly, **Chapter 3** also revealed that increased [CO_2] helped cacao seedlings to moderate the temperature-mediated fluctuations in stomatal conductance and instantaneous water-use efficiency (WUE) and led to overall improvements in photosynthesis (A_{sat}) and instantaneous WUE. This highlights the important role of [CO_2] in mitigating the stress responses of cacao to increased temperature (i.e., increased stomatal conductance for evaporative cooling under heat stress, and associated declines in instantaneous WUE), at least in this well-watered study. Finally, **Chapter 3** also revealed increased phenolic compound concentrations, particularly chlorogenic acid, in coffee leaves grown under elevated [CO_2], suggesting that part of the extra carbon assimilated as a consequence of increased photosynthesis under elevated [CO_2] may be allocated towards the synthesis of phenolic compounds (Batista et al., 2021) in coffee.

To summarize, the major findings of **Chapter 2** and **3**, I found that under future-predicted conditions of elevated temperature and [CO_2] each *i.* increased cassava yield and edible tissue cyanide composition, *ii.* increased coffee branching and leaf chemistry, *iii.* may have promoted growth abnormalities in cacao, and *iv*) increased photosynthesis (A_{sat}) and instantaneous WUE for all species. These studies generally support the potential for increased [CO_2] to ameliorate, or potentially magnify, temperature-mediated effects on plant growth parameters and leaf-level photosynthetic traits. These studies also highlight some important potential alterations to plant structure and biochemistry under future climate scenarios, with both direct and indirect implications on plant growth, physiological performance, productivity, crop quality (value) and susceptibility to pests and disease. Such alterations may subsequently drive irreversible consequences on farm profitability and the livelihoods of farming families.

In my second objective **(Objective 2)** discussed in **Chapter 4 and 5**, I aimed to evaluate the impacts of pollination intensity on crop production and if artificial pollination (a generally accepted but scarcely evaluated adaptation strategy to ameliorate pollination limitation) could drive sustainable yield enhancements in cacao, without compromising pod size or bean quality. I also aimed to identify the pollination conditions (i.e., pollen genotype, pollination intensity, and pollination timing) that are needed to achieve this. In addressing this objective, in **Chapter 4**, I firstly found that artificial pollination, regardless of pollination intensity (the percentage of open flowers artificially pollinated), significantly increased fruit set and yield. This finding supporting the potential of this management strategy to reduce pollination limitation in cacao. Secondly, I found that yield (the number of harvested pods) differed between genotype crosses and varied according to the pollen donor used. This finding highlights the importance of understanding self- and cross-compatibility rates when *i*. selecting clones for cultivation, *ii.* considering high-productivity farm designs, and *iii*. selecting pollen sources (donors) for artificial pollination practices. Third, the most novel finding of **Chapter 4**, I found that pollination timing (the temporal distribution of artificial pollinations) potentially plays a role in determining cherelle survival, whereby older cherelles had greater survival rates across all developmental stages than younger cherelles. Finally, I found that pollination intensity had no significant effect on final harvested yield, indicating that more rigorous research is needed to identify the pollination intensity levels that can optimise yield under artificial pollination conditions. While conducting the research of **Chapter 4**, some major observations and limitations were revealed, notably 1) the artificial pollination strategy used (pollination 100% of open flowers for more than 30 consecutive days) was likely unachievable for smallholder cacao farmers; 2) excessive pollination lead to overcropping and branch losses (branches broke under the weight of pods) and was linked to later tree mortality; and 3) the study was only conducted over a single harvesting season, not long enough to capture effects of previous yield on following year production.

Thus, in **Chapter 5**, I sought to explore and resolve some of the shortcomings of, and questions raised in **Chapter 4**. Notably, I adopted a very ‘modest’ artificial pollination strategy, designed for farmer achievability, long-term sustainability, and which was investigated over a three-year period. This chapter revealed multiple physiological trade-offs at the individual level in the impacts of cacao pollination management. Specifically, increasing pollination intensity increases the numbers of fruit produced to the cost of fruit size and quality, and narrowing the pollination time to improve fruit survival and production efficiency can lead to overcropping, resource depletion and reduced bean quality. Thus, short-term strategies to improve cacao yield through increasing pollination intensity or condensing pollination time windows may have unintended consequences on bean quality and long-term production sustainability. Such management-induced reductions to tree productivity, bean value and overall farm profitability can seriously impact the economic stability and sustained livelihoods for cacao farming families. This multi-year study also highlighted the crucial role that inter-annual variability plays in understanding the viability of artificial pollination strategies to enhance yield in cacao.

6.2 Study Limitations and Future Research Recommendations

The findings of this thesis highlights, and helps explain, two key limitations of climate change studies: [CO_2] and experimental duration, both of which were found to reduce treatment effects. Specifically, elevated [CO_2] can help offset some of the negative effects of elevated temperature on plants, and longer-term experimental studies may find that short-term observed impacts of artificial pollination strategies on yield dissipate over time. Thus, I demonstrate the importance of including these considerations in future evaluations of climate scenarios and impacts. Due to logistical limitations, it is not always feasible to evaluate all effects simultaneously. However, when possible, including [CO_2] increases with concomitant increases in temperature, or conducting multiple-year experiments will help inform our understanding of the potential impacts of future climate scenarios and the limitations and opportunities they provide.

Future investigations on the impacts of climate change on tropical crops would also benefit from integrated approaches that include the combined impacts of each, temperature, [CO_2], rainfall, and the potential frequency of extreme weather events (e.g., drought, flood or tropical storms) on both the quantity (e.g., growth, yield) and quality (e.g., chemical composition or nutritive value) of the target species. For example, while my findings showed benefits of elevated [CO_2] for cacao plants grown under well-watered conditions, Lahive et al., 2017, in their study incorporating the influence of water deficit (drought risks) for cacao, showed an even greater advantage of elevated [CO_2] to cacao plants growing under water limited conditions. Furthermore, additional incorporation of a greater comparison of genetic diversity, changes in nutrient availability, plant ontogenetic shifts and architecture, and interactions with other species (both plants and animals) in future research efforts may also help to better-develop effective climate change adaptation and mitigation strategies in agriculture.

In future examinations of existing adaptive management strategies, such as artificial pollination in cacao, integrated approaches that consider crop (e.g., cultivar profile, tree age, self- and cross-compatibility rates, previous year’s yield production, internal tree carbohydrate reserve status), agronomic (e.g., nutrient management, pruning regime, planting design), and environmental (e.g., temperature, rainfall, fertility of agricultural soils) parameters may help farm managers to identify if, when, and how to implement such strategies without diminishing returns. Lastly, though not evaluated in this thesis, greater understanding of the return on investment and overall profitability of this technically-and labour-intensive strategy is also needed.

6.3 Concluding Remarks

Climate change is impacting agricultural systems and communities in every region across the globe, and climate models predict further negative effects on agricultural productivity in the coming decades (IPCC, 2021). These projected environmental changes are likely to threaten the availability of food, water and shelter, especially for developing nations in the tropics and subtropics, where large constraints to food production (e.g., drought, nutrient poor soils, low-to-no farmer access to best farming practices or agricultural inputs) already exist (St.Clair and Lynch, 2010). This represents a significant challenge for agricultural production, as estimates suggest that food production will have to increase by 60% in the coming decades to meet world food demands (Wild, 2003)

My thesis has shown potential increases in plant biomass, yield, chemical composition and physiological functioning (gas exchange) of three important tropical crops under future-predicted conditions of elevated temperature and [CO_2], at least in well-watered and controlled situations. I also showed that cacao yields can be enhanced, in the short-term, by artificial pollination. My findings can contribute to improved practice in smallholder farms, as well as towards the development of management strategies and/or future research efforts for sustainable agriculture production. However, whether driven by elevated temperature and [CO_2] (i.e., **Chapter 2 and 3**), or adaptive management interventions (i.e., **Chapter 4 and 5**), lasting increases in plant growth, physiology, biochemistry or productivity, under future climate scenarios can only be maintained if there are concomitant improvements in plant nutrition. Put simply, this theses highlights that direct or indirect influence of future environmental change, or the benefits of improved adaptive management strategies, on crops will somewhat depend on plant nutrient availability. Indeed, my long-term evaluations of artificial pollination of cacao in **Chapter 5**, saw the benefits of this existing adaptation strategy reduce over time, an observation I suggest is driven by resource depletion. Other studies also report that the negative impacts of climate change on soil fertility and mineral nutrition of crops will far exceed the beneficial effects, which would further intensify food insecurity, particularly in developing countries (St.Clair and Lynch, 2010).

To support increased agricultural production under future climate conditions, the simplest solution for traditionally low-input smallholder farming systems may be to utilize fertilizers to supplement plant nutrition (St.Clair and Lynch, 2010). Indeed, current yields in smallholder agriculture are well below those that are estimated could be obtained if farmers were to adopt better agronomic practices, including fertilizer use and integrated soil fertility management (Vanlauwe et al., 2011). But while improving fertilizer access and use is often a key element in private company programs supporting the supply chains they depend upon for business (Mars Inc., *personal communication*), smallholder farming families typically lack the finances and access for obtaining fertilizers, as well as the information for their proper and most effective use (Lobell et al., 2008; Cohn et al., 2017; Holland et al., 2017). Combined with current increasing trends in fertilizer prices for the poorest nations (Knepper, 2002; Sánchez et al., 2002), it is unlikely that low-income smallholder farmers will be able to fertilize their way to sustainable agricultural productivity. Furthermore, continued agricultural production on already depleted lands will likely require ever more external inputs such as fertilizers, that are not only unattainable for farmers, but which may further exacerbate soil degradation and existing constraints to agricultural production. Therefore, it is vital that smallholder farmers use sustainable fertilization approaches that do not degrade the soil and lead to poverty traps (Tittonell and Giller, 2013). Given these challenges, climate-resilient agricultural approaches, such as agroforestry (coffee, cacao) (Gusli et al., 2020; Niether et al., 2020; De Leijster et al., 2021) or intercropping (cassava) (Gezahegn et al., 2022), may be a more feasible and promising strategy for smallholder farmers in the face of climate change (IPCC, 2021). Such approaches can provide a multitude of biological and socioeconomic co-benefits in addition to climate change adaptation, including synergies with climate change mitigation through carbon sequestration, increased food security and income opportunities, the provisioning of ecosystem services, and contributions to biodiversity conservation (Duguma et al., 2014; Tschora and Cherubini, 2020; van Noordwijk et al., 2021).

Nevertheless, as discovered in this thesis, adaptive approaches intended to generate resilience and sustainability in agricultural production systems require better understanding of the long-term impacts of management actions, especially for long-lived tree crops. While this thesis and similar research works towards identifying the responses of agricultural plants, and appropriate adaptation options for agriculture under future climate change, we must acknowledge that we are still limited to our current understanding of the here and now. Thus, while taking our best actions now is crucial, the adaptation strategies we develop today need to be adaptable, as the conditions we will face in the 21st century may fundamentally alter the effectiveness of our chosen strategies for the future management of agricultural crops.

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