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Climate ready sugarcane: Traits for adaptation to high CO_2 levels

Final Report for SRA Project CPI018

C.J. Stokes and N.G. Inman-Bamber
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SRA Research Project

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Name(s) of the Research Organisation(s):	CSIRO
Principal Investigator's name(s), contact phone number, address and Email address:	Chris Stokes ATSIP Building 145 James Cook University Douglas Q 481 chris.stokes@csiro.au
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Executive summary

Recent work in Brazil and Florida had shown strong responses of sugarcane to elevated CO_2, even under well-watered conditions. These results were not expected from current understanding of leaf physiology, given that sugarcane is a C_4 plant, possessing a photosynthetic pathway that concentrates CO_2 and achieves close to optimal rates of carbon fixation even at current low levels of CO_2. The results suggested that the mechanisms by which elevated CO_2 affects sugarcane may not be fully understood or, at least, that there may be genetic variation in responses. Fully capturing the benefits of rising CO_2 could assist the sugarcane industry in adapting to climate change and offsetting potential negative effects associated with rising temperatures and recurring droughts, if they were to become more frequent.

The main objectives of this project was therefore to contribute to the sugar industry's adaption to climate change by conducting experiments to determine the mechanisms by which rising CO_2 affects sugarcane, assessing the genetic variation in CO_2 responses and the potential these provide for selecting 'climate-ready' genotypes for the future, and incorporating these findings into improved modelling approaches to be able to better represent the effects of future CO_2-enriched climates on sugarcane crops.

An experimental system was designed and built in this project specifically to decouple indirect water-related effects of CO_2 on sugarcane, to test for the presence of any direct CO_2 growth responses. These experiments have provided some novel results with important implications for sugarcane breeding programs now and in the future. Two of the most important traits that affect the relative performance of clones, transpiration efficiency (strong responses and evidence) and canopy development (weaker responses and evidence), show indications of being influenced by rising CO_2 levels, and of genetic variation in these responses. The experiments clearly demonstrated that direct effects of elevated CO_2 on sugarcane growth, if any, are small, and that predominant effects are in reducing water use and increasing transpiration efficiency. There was no evidence of any effects of CO_2 on biomass production of stalks, patterns of sucrose accumulation in stalks, or leaf nitrogen, or of any down-regulation (where CO_2 responses would decline with long-term exposure to elevated CO_2). There was however evidence that leaf width and thickness were affected by CO_2, with some evidence that this may affect canopy development.

The strongest responses to CO_2 that were detected were related to reduced water use and increased transpiration efficiency (TE) under elevated CO_2. TE increased 20-60% under well-watered conditions and 10 – 130% under water-stress for an increase in CO_2 from ambient levels of 390ppm to 720ppm. Genetic variation in CO_2 responses among clones was investigated in two experiments (using 7 and 6 clones respectively). The results showed that variation in transpiration efficiency among clones increased under elevated CO_2 and under water stressed conditions. This suggests that the relative importance of transpiration efficiency as a trait and its benefits to crop performance could increase in future CO_2-enriched conditions, particularly if crops are more frequently exposed to water stress.

There were large differences in relative responses of clones to CO_2 in terms of the percentage increase in TE (from 7% to 126%), but these may follow a predictable pattern that does not alter the relative ranking among clones. In Experiment 3, in which we had most confidence in water-use related measurements, TE for 6 clones measured under current CO_2 levels was remarkably strongly correlated with TE for the same clones measured under future elevated CO_2 levels in both well watered and water stress treatments. TE for clones measured under ambient CO_2 accounted for 83% and 96% of the variation in TE measured under elevated CO_2 for well watered and dry conditions respectively. The number of clones (6) is too small to extrapolate across the full genetic diversity of sugarcane, or even that within the Australian sugarcane breeding program, at this stage. But if the result were to hold up generally it would have the important implication that screening for transpiration efficiency at present, would be sufficient to ensure that this trait was still strongly expressed in the future, at which time it could be of greater relative benefit (from preceding paragraph). It would also suggest that, for transpiration efficiency at least, the Australian sugarcane breeding program has not inadvertently selected for traits that predispose clones to

underperform under future CO_2-enriched climates. However, this result was not strongly supported in Experiment 2, and it would be prudent to investigate a broader range of clones before extrapolating this particular finding too far.

The results from this project provide the first measurements of CO_2 effects on water use of whole sugarcane plants. Some data was previously available from small leaf chambers and none was available for parameterizing and calibrating sugarcane crop models. We developed a model (from existing ones) that could make use of the results obtained in our glasshouse experiments to predict the effects of rising CO_2 on sugarcane, based on the simulated linked, flow through effects of elevated CO_2 on conductance, water use, soil moisture and plant growth. Unlike previous approaches, we did not assume any direct stimulation of radiation use efficiency by elevated CO_2, but represented the CO_2 effect entirely in terms of improved transpiration, using a Penman-Monteith approach to account for subsequent changes in gas and energy fluxes. We then tested this model against field observations from a previous experiment, and simulated what effect elevated CO_2 would have had on that crop. The model showed that even in a crop that was considered to be well irrigated, there could be a benefit from 720 ppm CO_2 of 3 to 8% in growth, through different stages of crop growth. The simulations also showed that benefits of CO_2 in reducing water use in the field are likely to be strongest in early growth stages, when canopies are open and plant-atmosphere coupling is strongest, and that these effects would decline as the crop canopy develops and closes.

In the process of designing and building the equipment to conduct the experiments in this project, we have developed a technical resource that is far more capable than originally intended and will be able to assist in further exploring the mechanisms that account for differences in performance between clones, particularly for water-related traits. For example, it has often proved difficult to translate differences in leaf-level responses among clones to differences in their final harvest performance. Being able to dynamically monitor whole-plant patterns of water use and relate these functionally to experimentally-controlled changes in factors controlling the supply and demand for water (e.g., water stress, CO_2, vapour pressure deficit), may provide an intervening step that helps better link leaf-level mechanisms, through whole-plant processes, to final plant performance.

The main target audience for this research is pre-breeders. The main avenue to adoption of this project's results and ultimate industry benefit would be through incorporating our findings into ongoing work related to trait-based selection in sugarcane, particularly work related to water use/stress (e.g. More-Crop-per-Drop) by incorporating considerations of future climate and dynamic whole-plant responses to that work.

SRA is investing in the search for drought resistance and improved water use efficiency. Our work has quantified the amount by which TE will increase under higher CO_2 conditions and shown that the value of TE as a trait, and the difference in TE among clones, could well increase in the future. By incorporating the findings from this project and this line of research into similar TE-related work, such as More-Crop-per-Drop, we should be able to ensure that current breeding initiatives and directions continue to deliver benefits under future climate conditions. In particular, it should be able to assist in identifying the traits that will be of most benefit over the coming decades, and in incorporating these into practical screening approaches. The current average cost of water stress to the industry is $230 million per annum and this is likely to increase. If TE can be improved by 10 % we estimate this would reduce this loss by at least 5% or about $12 million annually.

1 Background

Sugarcane is one of a small number of the world's plant species that possess the CO_2 concentration mechanism (C_4) that involves four carbon compounds. These C_4 species are some of the most important sources of human and animal food and also include maize and sorghum. At current atmospheric levels, photosynthesis is more efficient in crops with a C_4 photosynthetic pathway than in crops like wheat with a C_3 pathway, where CO_2 and energy are lost by photorespiration. As CO_2 levels rise, it would therefore be expected that C_3 plants would directly benefit, as inefficiencies from photorespiration decline, but this direct benefit would not apply to C_4 plants, because of their internal CO_2 concentration mechanism. However, recent work in Florida (Vu and Allen, 2009) and Brazil (de Souza et al., 2008) has indicated that elevated CO_2 may be of substantial benefit to sugarcane, both in terms of improved biomass yield and instantaneous, leaf-level water use efficiency (WUE). In the Florida experiment, photosynthesis of leaf segments was not increased much but WUE increased by 35% and this delayed the onset of water stress (Vu and Allen, 2009). In the longer-term Brazilian experiment photosynthesis was increased by 30%, WUE by 62% and stem biomass by 60% (de Souza et al., 2008). The authors suggested that the unexpected biomass gains were due to reduced water stress even though pots were well watered. These results indicate that the mechanisms of sugarcane response to rising CO_2 may not be properly understood and also suggest that there could well be genetic variation in CO_2 responses. If such variation exists, it could potentially be exploited through selective breeding to develop 'climate-ready' varieties that specifically target future CO_2-enriched climates. The main rationale for this project was therefore to determine the mechanisms by which elevated CO_2 affects sugarcane and to develop an approach to start evaluating the genetic variation in these responses.

The nature and magnitude of the benefit of elevated CO_2 to sugarcane, and the extent to which this can be actively harnessed (e.g., through breeding) to offset negative effects of climate change, is of substantial commercial importance for the whole sugar industry. A comprehensive assessment of the impacts of climate change on the sugar industry value chain was conducted for the Maryborough region by Park et al. (2007). For this region annual mean rainfall is projected to decrease by 1 to 14% by the year 2030, and between 2 and 42% by 2070. Annual mean temperatures are projected to increase by 0.5 to 1.2°C by 2030 and 1.0 to 3.7°C by 2070. An increase in atmospheric CO_2 concentrations up to 450 ppm is expected by 2030 and 700 ppm by 2070. Higher temperatures are expected to increase vapour pressure deficits (the dryness of the air), which would reduce WUE, while higher CO_2 is expected to have some offsetting benefits in this regard. Because no suitable information on sugarcane was available, simulations were conducted assuming that sugarcane would behave like sorghum when responding to these climatic changes. The simulations indicated that sugarcane yields could decline as much as 4% by 2030 and 47% by 2070, with negative effects of reduced rainfall and higher temperatures exceeding the positive effects of increased CO_2. The modelling indicated that increased temperature could hasten crop development but could also increase water stress and so have both positive and negative effects. A negative outlook leading to the closure of just one mill could cost the industry $70 million p.a. ($2bn/28). Opportunities for cogeneration of energy from sugarcane biomass and biofuel production could be underestimated and ultimately foregone leading to losses from potential sugarcane production. If however, some of the more promising experimental response of sugarcane to elevated CO_2 reported above are correct and are harnessed, investment prospects in sugarcane-based industries (sugar, cogeneration, biofuel) may be much more attractive than indicated by current modelling work (Park et al, 2008).

Photosynthesis occurs in two types of tissue in C_4 plants, the bundle sheath (where CO_2 is fixed into a 4-carbon molecule by an enzyme that is not sensitive to O_2 levels) and the mesophyll (where the normal 'light phase' of photosynthesis occurs under protected conditions, where CO_2 is concentrated and O_2 levels are

kept low) , while in the case of C_3 plants, only the mesophyll is involved (with sensitive carbon-fixing enzymes exposed to oxidation in unprotected conditions where $[CO_2]$ is low and $[O_2]$ is high) (Matsuoka et al., 2001). This allows C_4 plants to reach maximum photosynthesis rates at current levels of ambient CO_2 (Ghannoum et al., 2000), and limits the potential for rising CO_2 to have any direct benefit through stimulating photosynthesis directly at the leaf level. However, the most widespread response of plants, both C_3 and C_4, to elevated CO_2 is an increase in WUE as the higher CO_2 level outside the leaf increases the diffusion gradient of CO_2 across stomata (and rate of flow of CO_2 for photosynthesis into the leaf) relative to the diffusion gradient of moisture vapour (and rate of transpiration of water out of the leaf). Under water-limited conditions, this increase in WUE benefits both C_3 and C_4 plants through indirect, moisture-mediated feedbacks, where the lower transpiration rate under elevated CO_2 reduces the rate at which water is extracted from soils, reducing the onset of water stress and prolonging the period of growth before plant-available water is depleted. For this reason reported responses to elevated CO_2 concentrations of increased photosynthesis and consequent biomass accumulation in well-watered C_4 plants, have been difficult to explain. Even in well-watered C_4 plants, elevated CO_2 and consequently reduced stomatal conductance can lead to enhanced leaf growth and photosynthesis through mitigating the effects of transient water stress (Seneweera et al., 1998). Alleviation of transient water stress was thought to be at least partly responsible for an observed 30% increase in sugarcane photosynthesis and a 40 % increase in biomass yield, with twice normal CO_2 (de Souza et al., 2008). Stomatal conductance was reduced by 37% and transpiration by 32% in these plants grown in open top chambers. Vu et al. (2009) reported a similar reduction in conductance (34%) and a smaller reduction in transpiration (25%) when well watered sugarcane plants were provided with 'twice normal' CO_2 (720 ppm) in glasshouse experiments. Ghannoum et al. (2000) listed reports which indicate that assimilation (*A*) and biomass accumulation in well watered C_4 plants both increase under elevated CO_2 and other reports where *A* responded but not growth and yet others where growth responded but not *A*. These conflicting results probably reflect the difficulty of inferring growth responses from short-term measurements of *A* (Ghannoum et al., 2000) often using small segments of young leaves as in the studies by de Souza et al. (2008) and Vu et al. (2009). Ghannoum et al. (2000) argued that indirect effects are dominant in the response of C_4 plants to elevated CO_2. Ghannoum et al. (2003) provided further evidence that even under water stress, elevated CO_2 does not directly enhance *A*, and any enhancement of *A* is most likely due to non-stomatal means.

There is therefore a need to establish the mechanism by which sugarcane responds to elevated CO_2, and the magnitude of responses for biomass, integrated whole-plant transpiration efficiency (TE) and other aspects of plant growth. SRA (and formerly, SRDC) has already invested in the search for drought resistance and improved TE. If TE is going to increase with increasing CO_2 to the extent indicated by recent research then we should start looking for germplasm with traits that will allow the industry to harness this advantage to the full. The current average cost of water stress to the industry is $230 million (Inman-Bamber, 2007) and this is likely to increase. Being able to better understand, model and offset these impacts under future, CO_2-enriched conditions will increase the ability of the industry to adapt to climate change and assist in introducing traits for improved response to CO_2 in breeding programs.

This project makes significant advancements through:

- developing experimental techniques to decouple direct and indirect effects of elevated CO_2 on photosynthesis and transpiration (Chapters 3 and 4);
- applying these techniques to start quantifying genetic variation in responses to CO_2 under both well-watered and water-stressed conditions (Chapter 4); and
- improving approaches to simulate the effects of CO_2 enrichment on sugarcane growth and water use in crop modelling (Chapter 5).

2 Objectives

The overall objectives of this project was to contribute to the sugar industry's adaption to climate change by contributing to strategies that will maximise the benefits of increasing CO_2 levels, ultimately through more effective varieties. There were four specific goals within this, each of which is listed below together with a brief summary of how each of these was achieved:

• To reassess the impact of climate change on the industry by establishing the physiology of sugarcane growing in elevated CO_2.

An experimental system was designed and built in this project specifically to decouple indirect water-related effects of CO_2 on sugarcane so that we could test for the presence of any direct CO_2 growth responses (Chapter 3). These experiments have provided some novel results with important implications for sugarcane breeding programs now and in the future. They have clearly demonstrated that direct effects of elevated CO_2 on sugarcane growth, if any, are small, and that the predominant effects are in reducing water use and increasing transpiration efficiency (Experiments 2 and 3, Chapter 4). Such effects would only be expected to translate into increased crop growth and yields where water supplies are not fully adequate. However, the modelling (Chapter 5) showed that even in a crop that was considered to be well irrigated, there could be a benefit of rising CO_2 (from current levels of 390ppm to 720ppm) of 3 to 8% in growth, at different stages of crop growth. There was no evidence of any effects of CO_2 on whole plant biomass, stalk biomass or patterns of sucrose accumulation in stalks. Neither was there any indication that the strength of CO_2 responses changed with long-term exposure to elevated CO_2, or that leaf nitrogen levels were negatively affected (which is often associated with down regulation of photosynthesis, where this occurs in other plants). There was however some evidence that elevated CO_2 increased the area of sugarcane leaves (probably through indirect effects), although these response were weaker than those for TE. There was also genetic variation in leaf responses. Leaf responses were associated with increases in total plant leaf area of 8 to 10%, although this was not statistically significant. Differences in canopy development are also a trait of major practical importance in determining the relative performances of different clones, so potential effects of CO_2 on canopy development warrant further investigation.

• To assess adaptive strategies for the sugarcane plant in terms of improved transpiration efficiency and photosynthesis.

The experiments in the project sought to establish whether there were genetic interactions in the biomass and water use responses of sugarcane to elevated CO_2 (Chapter 4). As noted above, the results provided no evidence of any direct benefit of elevated CO_2 to photosynthesis and growth, and little indication of genetic interactions that might present opportunities for adaptation by selecting for direct photosynthesis or growth responses to elevated CO_2.

The strongest responses to CO_2 that were measured were related to reduced water use, expressed as increases in instantaneous leaf-level water use efficiency and corresponding increases in integrated whole-plant transpiration efficiency under elevated CO_2 (20 – 60% improvement in TE under well-watered conditions and 10 – 130% under water-stress) (Chapter 4). There were genetic interactions in these responses indicating some potential for adaptive strategies to select for such response (discussed under

next following objective). The results also showed that variation in TE among clones increases under elevated CO_2 and under water stressed conditions. This suggests that the relative importance of TE as a trait and its benefits to crop performance could increase in future CO_2-enriched conditions, particularly if droughts occur more frequently.

• To assess the opportunity for selecting for greater response to elevated CO_2 in terms of improved transpiration efficiency and sucrose and biomass production.

There was no evidence of any effects of CO_2 on biomass production of stalks, patterns of sucrose accumulation in stalks, or whole plant biomass. Neither was there, therefore, an indication that there was potential for selecting for CO_2 responses in these variables. As noted above, the strongest CO_2 responses were related to altered patterns of transpiration (Chapters 3 and 4).

Genetic variation was investigated in Experiments 2 and 3, using 7 and 6 clones respectively (Chapter 4). The results showed clear evidence of genetic variation in water-related responses to CO_2. Analyses focused on genetic variation in transpiration efficiency, because this was the water use metric that translates most directly into differences among clones in final growth performance under water-limited conditions. There were two key aspects to these interactions. The first was how CO_2 influenced the magnitude of genetic variation and amount of separation between TEs of different clones (discussed above).

The second aspect to genetic variation in TE was how this affected relative rankings among clones. There were large differences in relative responses of clones to CO_2 in terms of the percentage increase in TE (from 7% to 126%), but these may follow a predictable pattern that does not alter the relative performance among clones (Chapter 4). In Experiment 3, in which we had most confidence in water-use related measurements, the amount of correspondence in TEs between current and future CO_2 conditions was remarkably strong: TEs under ambient CO_2 accounted for 83% (wet conditions) and 96% (water-stressed) of variation among clones under elevated CO_2. This is too small a number of clones (6) to extrapolate across the full genetic diversity of sugarcane, or even that within the Australian sugarcane breeding program, at this stage. But if the result were to hold up generally it would have the important implication that screening for TE at present, would be sufficient to be reasonably confident that this trait was still expressed in the future, at which time it could be of greater relative benefit (given the increased variation in TE among clones noted above). The strong correlation may be an inadvertent consequence of screening approaches within the Australian breeding program and the specific subset of genetic variation this has selected. Many traits are highly correlated with each other so it is common that breeding approaches that favour one target trait can simultaneously alter other non-target traits within the selected population. There is therefore no guarantee that varieties that are selected for current climates will continue to be the best varieties in future climates, and inadvertent selection at present for traits that affect future performance could just as easily have negative consequences as positive ones. If the results from Experiment 3 are a consequence of the particular pool of genetic variation that has been selected in Australian breeding, then the results indicate, for TE at least, that such potential maladaptation has not occurred and that selecting high TE clones within this breeding population will continue to have benefits in the future. The results for Experiment 2 showed much weaker correspondence, with TEs at ambient CO_2 accounting for less than 20% of variation in TEs among clones under elevated CO_2. It is not clear whether the extra unexplained variation was because of genetic interactions in CO_2 in responses of the different set of clones used (where differing CO_2 responses were changing the ranking of TE among clones), or because of extra experimental error associated with the lower accuracy of measuring water use in Experiment 2. Whatever the case, it would be prudent to investigate a broader range of clones before extrapolating this particular finding too far.

• To assess the properties of elevated CO_2 for mitigation of increased water stress expected with climate change.

There was only limited evidence that CO_2 had any direct effect in alleviating water stress for plants growing under conditions where soil moisture levels were equivalent (as they were purposely controlled and maintained in experiments, Chapter 4). Under field conditions however, reductions in transpiration under elevated CO_2 would reduce the rate at which soil moisture was depleted and delay the onset of water stress. The magnitude of CO_2 effects on TE (improvements of 20-60% under well-watered conditions and 10 – 130% under water-stress) show this benefit is substantial, and likely to increase as plants become more stressed. As noted above, crop modelling showed that even in a crop that was considered to be well irrigated, there could be a benefit of 3 to 8% in yield (for an increase in CO_2 from current levels to 720ppm), through different stages of crop growth, from alleviating transient water stress (Chapter 5).

For Experiments 2 and 3 (Chapter 4), a similar analysis to that conducted for genetic interactions in TE responses to CO_2 mentioned above, was also applied to water treatments. These analyses tested whether TE under well-watered conditions was a good indicator of TEs under water-stressed conditions. Relationships were reasonably strong, accounting for 33% to 72% of variation in TEs among clones under stressed conditions, suggesting that TEs under well-watered conditions provide at least a good first approximation of clones' TEs under stress. Further, several (but not all) analyses showed that the improvement in TE under water stressed conditions was greater under elevated CO_2, and that genetic variation in TE increased both with water stress and exposure to elevated CO_2. Collectively these results suggests that the benefits of high TE clones, as an option for mitigating water stress, could be greater under higher CO_2 conditions.

Full details of the research approach and findings of the project are provided in the technical section of the report in the following three chapters.

3 Designing and testing a system for measuring sugarcane responses to elevated CO_2

3.1 Introduction

The first part of the project involved developing an innovative new experimental system that would be able to isolate and independently measure the direct and indirect effects of elevated CO_2 on sugarcane under controlled conditions. This involved setting up a new Tall Plant Facility and testing and configuring it for the requirements of the CO_2 work; developing and setting up an automatic demand-driven watering system that could control soil moisture levels in each pot independently of CO_2 and the water demand of the clone used; and running the first experiment, with sequential sampling to determine how different growth stages of sugarcane are affected by elevated CO_2.

3.1.1 NEW TALL PLANT FACILITY

This project built on previous sugarcane work by Inman-Bamber and colleagues that involved pot experiments in the controlled environment chambers of the Tall Plant Facility (TPF) at the CSIRO Davies Laboratory in Townsville. Just after the start of the project the Davies Laboratory and its facilities were decommissioned and CSIRO staff moved to new facilities at the Australian Tropical Science and Innovation Precinct on the James Cook University campus in Townsville. This new precinct included a new Tall Plant Facility, to duplicate and expand on the capabilities of the previous TPF. The new TPF first had be commissioned and brought to the specified standards of operation required for our experiments (Figure 1). It also required some modifications to meet the additional requirements of this project, particularly the additional equipment needed for the delivery and safe control of CO_2 in the chambers. The new TPF has two controlled environment chambers, each measuring approximately 6m x 9m by 7m tall (Figure 2).

The methodologies for the use of the Davies Laboratory TPF were well established through experience in the previous experiments. This included using measurements of inflow and outflow gases to determine whole-of-chamber plant gas exchange (CO_2 and water vapour). By adding CO_2 treatments and measurements of transpiration to this experimental system, a number of new challenges had to be considered and overcome:

- a control system was needed to be able to accurately regulate CO_2 levels within chambers to defined treatment levels (including options for setting CO_2 both above and below ambient levels);
- any experimental additions of CO_2 needed to be accurately measured (to calculate whole-of-chamber plant gas exchange) and the systems for controlling and measuring CO_2 had to be compatible;
- the flow rate of air through the chambers needed to be sufficient to maintain humidity near ambient levels (especially in irrigated treatments) without being so large that CO_2 usage in CO_2-enriched treatments (a major expense) is too high or placing excessive energy demands on the temperature control system;
- safety systems were needed to monitor CO_2 levels and vent enclosed spaces in the event of CO_2 build up;
- facilities and equipment were needed for storing, delivering and replenishing the large amount of CO_2 that was required by the experiment; and
- the design of the new facility also had to meet the requirements of additional, alternative uses (e.g., meeting quarantine standards).

Figure 1. Construction of the new Tall Plant Facility at the Australian Tropical Sciences and Innovation Precinct (ATSIP) on the James Cook University campus, Townsville (7 December 2009).

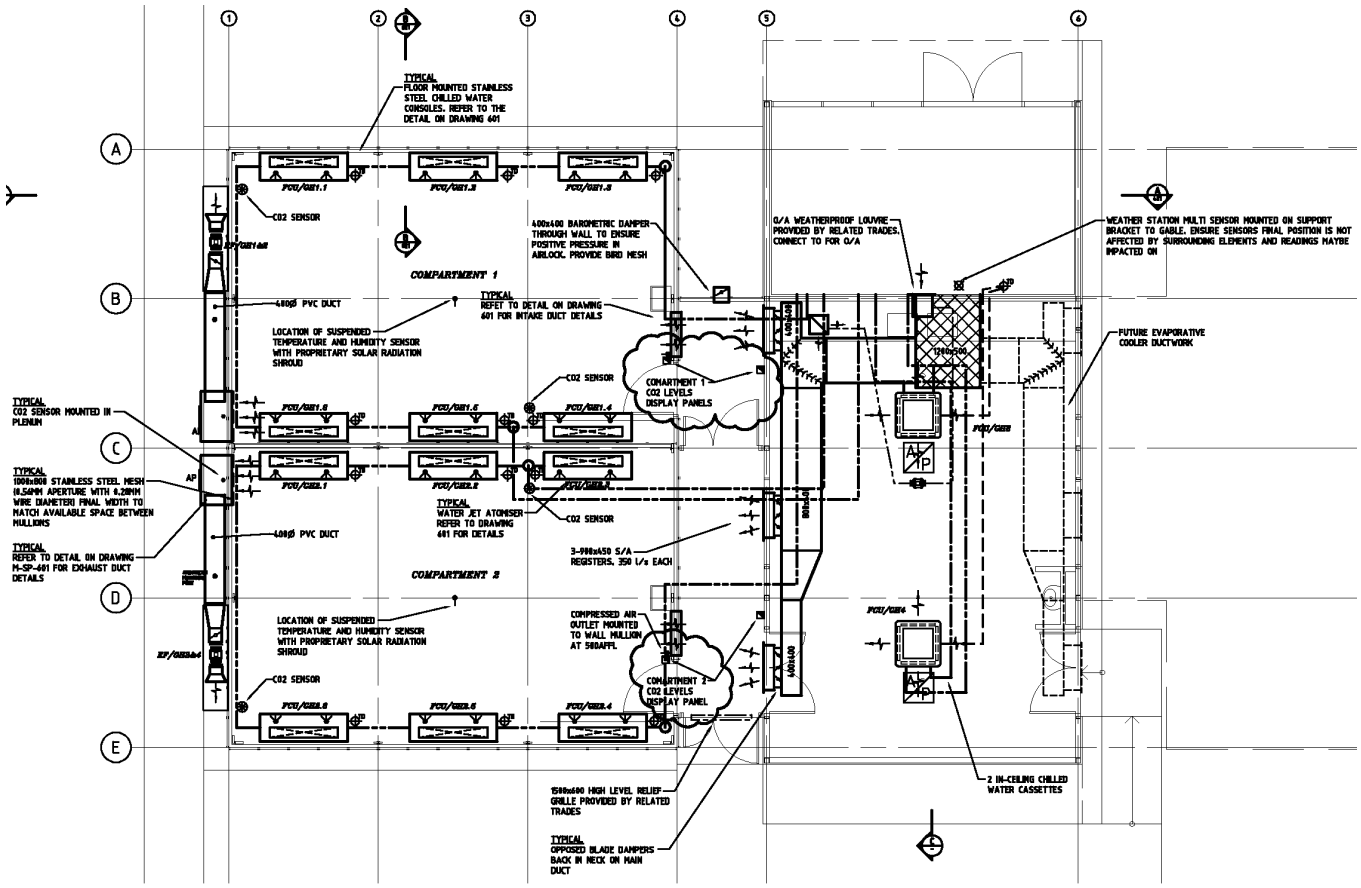

Figure 2. Building plan for the new Tall Plant Facility. The two controlled environment chambers are on the left and the laboratory is on the right.

The original design proposed by the contract engineers was incompatible with the above objectives in a number of regards and required amendments to the design. Some of the more important improvements to the design included:

- moving the point where CO_2 is released from within the chamber itself to the inflow ducts (where the CO_2 can be pre-mixed and measured in the inflow stream);
- using mass-flow controllers to regulate CO_2 release, rather than banks of solenoid valves, to allow more precise and subtle control of CO_2 release (with the added option of being able to directly measure the amount and rate of CO_2 being added to the chambers);
- adjustments to the whole fan and through-flow system to allow higher rates of air flow (which are required to maintain humidity levels);
- adjustments to the fan and through-flow system to allow the chambers to operate under either positive pressure (with fans, on balance, blowing air into the chambers – required to accurately account for the influx of CO_2 into the chambers in this experiment) or negative pressure (with fans, on balance, sucking air out of the chambers – required to meet quarantine specifications); and
- design of a ducting system that would allow the required instrumentation and measurements to be made on the airflows into and out off the chambers.

For the CO_2 supply, we needed an option that would meet our requirements in terms of safety, plant-active contaminants, and cost-effectiveness. After investigating a range of alternative options, we chose a Gasmatic system providing food-grade CO_2 sourced from BOC. The Gasmatic system was installed in October 2010, when the building contractors allowed us to connect it to the new TPF. The Gasmatic system we chose was selected to be just sufficient to meet our flow rate and storage volume requirements (as part of safety considerations to mitigate risks from gas leaks). Food-grade CO_2 is produced by separating the components of normal air (involving molecular sieving), which minimizes contaminants with health risks (e.g. carbon monoxide) and unwanted plant responses (e.g. ethylene) that are associated with other sources of CO_2 (such combustion of naptha or naturally- occurring belowground sources). Even very low levels of contaminants in CO_2 are a concern in enclosed environment experiments, because they can accumulate and become concentrated as plants selectively remove the CO_2 (and ethylene affects plants growth a concentrations as low as parts per billion).

At high concentrations, CO_2 is toxic to humans and potentially lethal. The experiment is designed to run at levels well below that of exhaled breath but, since the gas is being released into an enclosed chamber where it could accumulate, safety considerations have been paramount. To maximize the safety of working in the TPF we undertook extensive risk assessment and controls in modifying the facility hardware, the Building Management System (BMS) software, safety warnings and operating and emergency procedures.

The start of the first experiment was substantially impeded and delayed by a number a major issues involving building defects, contractors not following specifications we had supplied to engineers during the design process, and modifications required to the facility design to meet safety and experimental requirements. The issues we diagnosed and/or resolved included: more than 6 months delay in completion of and access to the new TPF; misspecified mass flow controllers for the controlled injection of CO_2; misconfigured CO_2 sensors that were measuring across too broad a range to have the required precision within the range of CO_2 treatments; airflow that created negative pressure in the chamber ('sucking' air through the chambers, including through leaks) instead of positive pressure (which makes it possible to account for all CO_2 inflows and outflows from the chambers); numerous defects in the Building Management System (BMS) software for triggering safety and caution alarms and defects; lack of a safety cut-off solenoid for CO_2 flow; water and air leaks in the chambers and substantial defects in the BMS control logic software for achieving and maintaining specified CO_2, temperature and humidity settings.

3.1.2 DEMAND-BASED WATERING SYSTEM

A watering system was required to be able to accurately control soil moisture/stress treatments and to be able to measure the amount of water transpired by plants in pot in the experiments. A demand-based watering system was used, to measure and replace transpired water similar the manual approach that had been used in previous sugarcane pot experiments assessing TE. However, in order to prevent feedbacks developing where pots with higher transpirational demand would draw down soil moisture faster and induce greater levels of water stress, the watering needed to be conducted at short-intervals (to limit deviations in moisture treatments between watering – particularly important where CO_2 treatments induce large differences in demand), requiring sophisticated automation. Three methods were investigated for accurately controlling the amount of water delivered. The first involved testing emitters, where the rate of flow could be set and the volume then controlled by the amount of time that water was delivered. However the problems with this approach were that the differences in flow rate between emitters was too variable (requiring individual calibration), accurate flows would require constant control of water pressure to each emitter from the start to the end of delivery, it would take too much maintenance to keep each emitter delivering at its calibrated rate, and it would be difficult to control the exact start and stop time of each emitter. We also considered gravimetric approaches, and investigated the strain gauge approach being used by Hammer and colleagues in Gatton for sorghum. But, with a large plant like sugarcane, it would be difficult to take the mass of the plant into account and the strain gauges were expensive and at the limits of their precision in detecting the relatively small changes in overall mass associated with water use. We therefore settled on a volumetric option (described in detail below), where set volumes of water could be independently delivered to each pot.

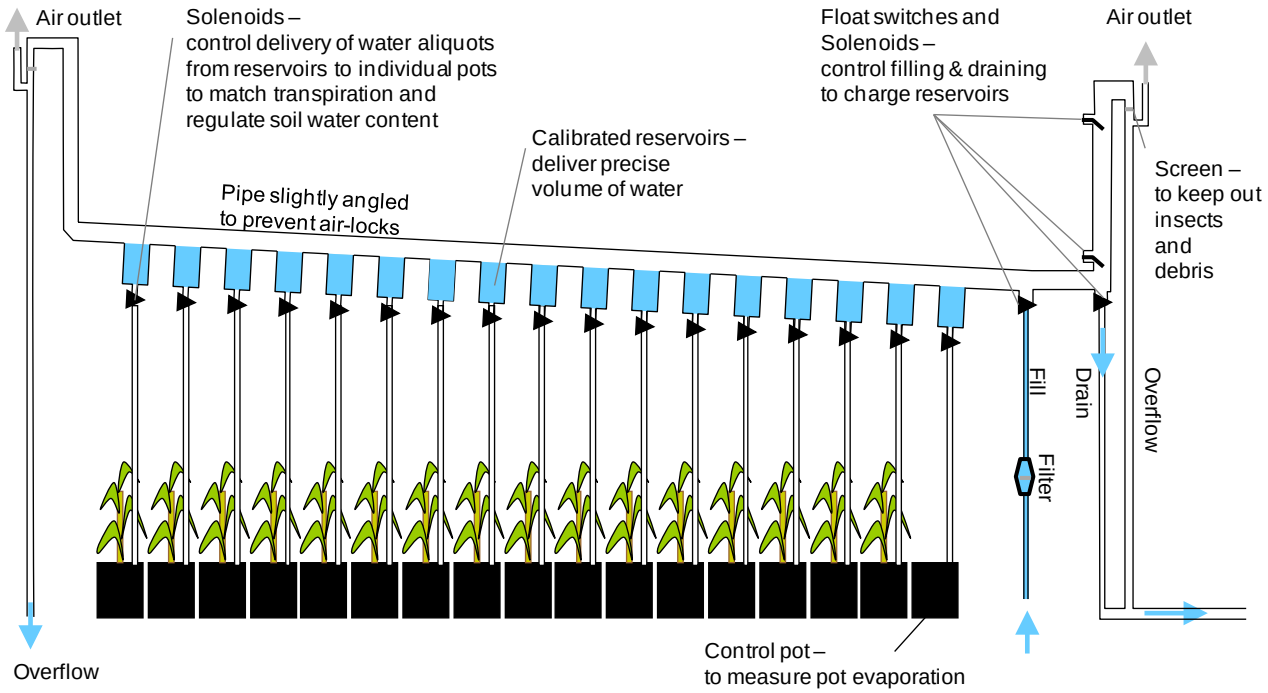

Figure 3. Watering system used to precisely control soil water content and measure plant water use.

The final watering system that was designed and built is one of considerable complexity consisting of over 280 sensors and controllers, networked together through almost 3 km of cables, with over 1600 connections, to a set of data loggers with ethernet connections back to a central computer server (Figure 3). The reservoirs for metering water to each pot were precision-machined (from PVC) in a modular (replaceable/interchangeable) format, allowing them to be calibrated before installation. A narrow

machined collar was fitted to the top filling entrance to each reservoir, and the length of reservoir tube was precision cut to provide a volumetric measure that should remain precise without the need for future calibration adjustments. Following construction and fine-tuning of the system, all reservoir calibrations were again tested again in-situ: reservoirs each delivered 204 ml per drop (within 1 ml).

The operation of the system starts with a charge cycle, initiated by a programmable timing schedule. This opens a tap solenoid to fill the pipes with water until a float switch indicates the system is full. Bubbles are then allowed to leave the system during a short delay before a drain solenoid is triggered. A lower float switch indicates when the charging pipes are empty, and the drain solenoid is then closed. At this point all reservoirs for each individual pot are filled to their calibrated volume.

At 10-minute intervals, the program then measures soil moisture in each pot and, if this falls below its programmed treatment level, then the delivery solenoid for that pot is triggered and the metered volume of water is delivered to the plant. Water use over hourly and daily time intervals is measured by the number of water deliveries and the difference in soil moisture between the start and end of the measurement period. The program automatically recalibrates the soil moisture sensors for each pot based on the step increase in measured water content after each volume of water is delivered.

One of the technical problems we faced with this system was finding solenoid valves that drain and deliver water under gravity alone (without additional pipe pressure). We had intended to use inexpensive, standard low-pressure valves, but discovered that these still require some amount of water pressure to flow and seal reliably. Instead we needed highly specialised 'direct-acting' valves that are designed to work without any pressure. After testing a range of alternative products we were able to find a cost-effective product that met our specifications (but they were so specialized that these had to be manufactured to order).

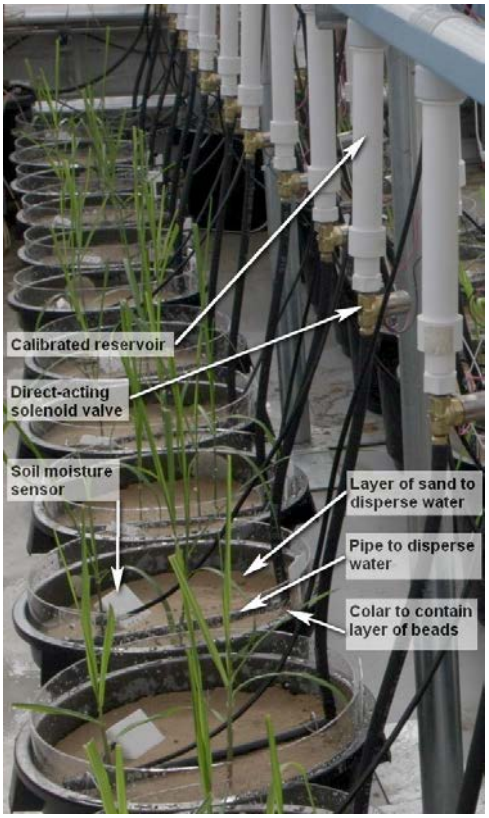

Figure 4. Components of watering control system (prior to covering with a layer of beads to limit

3.1.3 FIRST POT EXPERIMENT

The purpose of the first experiment was to determine how the influence of CO_2 on sugarcane changes through different growth stages of the plant, under both well-watered and water-stressed conditions. This was an important first step to provide a baseline for the effects of prolonged exposure to elevated CO_2 over a complete growth cycle and, through sequential harvesting, testing whether these responses changed through different stages of plant development (e.g., through acclimation). The data gathered was used to identify a short growth window where plant responses to combinations of water stress and CO_2 treatments could be measured so that subsequent experiments could rapidly assess a larger number clones (without needing to grow them for a full year). This first experiment was also used to test and fine-tune various technical and operational aspects of the new systems involved including the newly-built TPF and its environmental control systems, the system for controlling CO_2 levels in the two growth chambers, and the water control and measurement system.

The design of Experiment 1 consisted of full factorial combinations of:

*CO_2 treatments * 2*

A high CO_2 chamber (720 ppm) and an ambient CO_2 chamber (targeted ambient CO2 of ~390 ppm but levels could drop as low as 375ppm when full grown plant were photosynthesising).

*Clones * 2*

The choice of clones was guided by recent and parallel research related to water use efficiency being conducted by the BSES (now SRA) and CSIRO, particularly projects BSS305 and BSS334. The latter project involved a shade house pot trial, adjacent to the TPF, using 5 clones and 4 watering treatments (applied manually), which we coordinated with to run in parallel. We selected two of the clones from that experiment that seemed to have contrasting water use and water stress characteristics in preliminary data: Q208 has high TE (transpiration efficiency) and KQ228 displayed high TE in the well watered, but not in partially stressed, conditions. Both of these are widely-planted, major commercial varieties.

*Water treatments * 2*

A 'wet' treatment, where plants were given ample water (added every 10 minutes if required), was contrasted against a 'dry' treatment, where water stress treatment was imposed by restricting the soil water content (targeting a 50% reduction in leaf extension rate relative to wet treatments).

*Growth windows * 4 (4 sequential sampling dates)*

Batches of plants were sampled progressively at 4 different dates through the trial to determine a smaller growth window that could be used to obtain measurable responses in future trials. One-eyed setts were established in mid-January 2011 and planted into pots in the TPF in mid-February. Experimental treatments were fully implemented in mid-April, and plants were harvested at 2-monthly intervals thereafter (June, August, October and December 2011).

*Replicates * 4*

There were 4 replicate pots for each treatment combination, arranged in a Latin-square experimental layout within the chambers.

Controls

There were an additional four control pots (without plants) for each watering treatment in each chamber to measure evaporation from the sealed pots.

(Total = 136 pots simultaneously)

3.2 Methods

For the first glasshouse pot experiments we used our watering control system to maintain soil water content within a narrow range to limit CO_2 responses mediated by feedbacks on soil moisture while simultaneously measuring the amount of water used. This was accomplished by using a sensor array linked to a control system that could independently control and deliver exact volumes of water to each pot (Figures 3 and 5). Each pot was fitted with a volumetric soil moisture sensor (CS616 reflectometer, Campbell Scientific, Utah) that measured soil moisture every 10 minutes. If the soil moisture for the pot fell below its target treatment level, then a solenoid was opened to deliver an aliquot of water from a precisely-machined reservoir (204 ml) above the pot. The data logger was programmed to automatically calibrate each sensor for the most recent three water deliveries (based on the increase in soil moisture in response to each addition of the known volume of water) so that water use could be interpolated between triggered water deliveries. A horizontal tube with lateral perforations, orientated perpendicularly to the moisture sensors, was used to spread the delivered water evenly in the pots, assisted further by a thin (2 cm) layer of sand between the tube and the soil surface. A 5 cm layer of plastic beads was placed on top of this to limit evaporation.

Figure 5. Setup of first experiment, about two months after transplanting (19 April 2011), showing full arrangement of pots in one of the two TPF chambers.

For the CO_2 treatments we used two large (approximately 6m x 9m by 7m tall) controlled environment chambers, part of a new facility based on the design described in Inman-Bamber et al. (2008) (Figure 2). The chambers included control systems for regulating temperature, humidity and CO_2 levels, with large air handlers to ensure even mixing. To prevent the build-up of plant-active contaminants from the CO_2 supply

in chambers such as ethylene (Morrison and Gifford, 1984a): 1) the system used a continuous flow of air through the chambers, with CO_2 injected into the incoming air stream (diluted in two-stage mixing to within 10 ppm of chamber levels before entering the chamber to eliminate CO_2 gradients); and 2) the source of gas used was produced by separation of atmospheric air, and therefore low in plant active impurities to begin with.

Air temperature and relative humidity (RH) were measured every minute with shielded sensors (HMP45a Vaisala Pty Ltd Melbourne, VIC) placed at the level of the leaves. Solar radiation (350–2500 nm) was measured above the plant canopy at a height of 6 m in each chamber with four one-metre long tube solarimeters (Delta-T Devices Ltd, Cambridge, UK).

The experiment was a complete factorial design of two CO_2 treatments by two water treatments by two sugarcane varieties by four harvest dates, replicated four times (with harvest dates and replicates arranged in a 4x4 Latin Square). For the CO_2 treatments, one growth chamber was supplied with ambient air (approximately 390 ppm CO_2), while the other was elevated to approximately 720 ppm. The two varieties that were used were KQ228 and Q208, two of the mostly widely grown commercial varieties in northern Queensland. The watering control system was used to apply two treatments: in the well-watered treatment the soil moisture in each pot was maintained at 90% of field capacity (watering trigger threshold), while the soil moisture content in the stressed treatment was adjusted until leaf extension rates, as measured with auxanometers, were half of those in the well-watered treatment. The four harvest dates are explained below.

One-eyed setts were germinated and then transplanted, three per pot, into pots (520 mm tall and 380 mm in diameter) containing 25 l of a premium potting mix. Plants were allowed to establish for 2 months at field capacity. During this period, pots were watered to just above field capacity each afternoon, and excess water was allowed to drain through small holes at the bottom of each pot, to individually calibrate the measured field capacity for each moisture sensor-pot combination. Watering and CO_2 treatments were then initiated and the starting mass of plants was determined from destructive harvesting of 10 extra plants of each variety. In each chamber four control pots were set up that were identical to experimental pots except that they contained no plants. These were used to measure pot evaporation (subtracted from pot water use in calculating transpiration).

Plants were harvested in batches at approximately 8-week intervals following the initiation of treatments. Harvested plants were subsampled and separated by plant part into green leaf, dead leaf, sheath and stalk to obtain dry masses following the procedures described in Inman-Bamber et al. (2008). Subsamples of green leaf were processed through a leaf area meter, and the height and number of internodes were measured for stalks. Three stalks, of the same average mass as the full sample, were subsampled for each pot for sugar analysis. Stalks were divided into internodes, the fresh mass of each internode was recorded and internodes numbers (from the top) 3, 5, 7, 10, 13, 16 (and every 3rd internode thereafter, to the end of the stalk) were used for lab analysis. For each of these internodes, sugars were extracted and analysed using HPAE-PAD (High-Performance Anion-Exchange Chromatography with Pulsed Amperometric Detection), as described by Papageorgiou et al. (1997), to determine the concentrations (on a fresh mass basis) of sucrose, glucose and fructose, and the total mass of sucrose stored in each internode. Water use for each pot was calculated for the final seven days before harvest, to calculate the water use per unit green leaf area for each harvest date.

Shortly before the final harvest in November 2011, gas exchange measurements were taken on the youngest fully expanded leaf (leaf number 1) of 24 plants in each glasshouse, using a portable photosynthesis apparatus (Li-6400, Li-Cor Inc., Lincoln, NE, USA). A 6 cm^2 section of the leaf was enclosed in the Li-Cor cuvette and exposed to 2000 $\mu mol/m^2/s$ photosynthetically active radiation and to 375 ppm CO_2.

Readings were later repeated on the same leaves using the same settings but this time with CO_2 set to 720 ppm. Only well-watered treatments were measured since photosynthesis was too low in the stressed treatment.

3.3 Results

Compared to previous CO_2 work on sugarcane using open top chambers, the glasshouse experiment provided improvements by: 1) using a demand-driven watering system, with frequent watering, to prevent temporary deviations in soil water levels and plant water stress between CO_2 treatments; 2) including a temperature control system to compensate for the differences in evaporative cooling associated with substantial reductions in transpiration under elevated CO_2; and 3) including a humidity control system to compensate for differences in the rates at which water vapour was added to the air from transpiration. However, as the first experiment in a new facility, there were some initial teething problems in fully regulating and matching conditions between the two chambers. Radiation and temperature conditions were very similar between chambers (Figure 6), but humidity control was insufficient to fully offset the differences in transpiration between chambers and RH was distinctly lower in the high than the low CO_2 chamber (Figure 6).

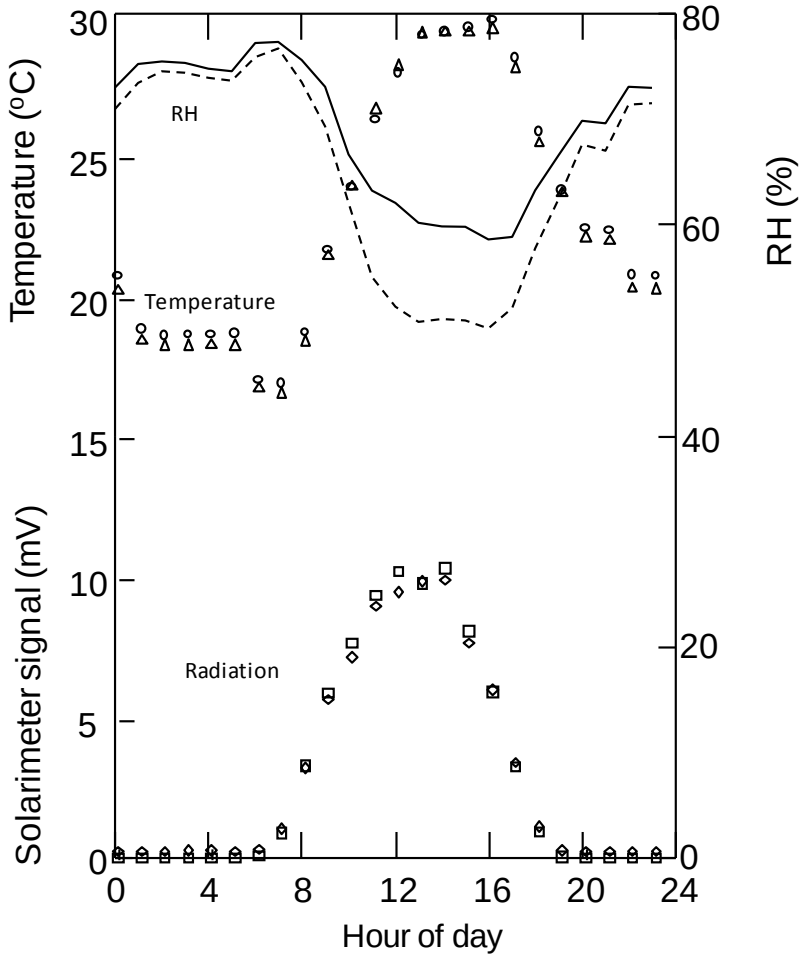

Figure 6. Mean hourly RH (—, - - -), temperature (○,△) and solarimeter voltage (radiation, □,◇) for glasshouse chambers set to ambient (390 ppm; —,○,□) or elevated CO_2 levels (720 ppm; - - -,△,◇).

3.3.1 GAS EXCHANGE

Mite damage was noted on leaves when gas exchange measurements were made shortly before the final harvest. Cuvette (Li-Cor leaf chamber) measurements of gas exchange on leaf #1 indicated that photosynthesis was not affected with mite damage ratings of 1 (no damage) and 2 (slight damage within the range of natural blemishes, <5% of leaf area) (damage scores ranged from 1 to 5). Measurements on leaves where mite damage ratings exceeded natural blemishes (>2) were excluded from the analysis.

Variety, harvest date and CO_2 level all had significant effects ($p=0.022$, $p<0.001$ and $p<0.001$ respectively) on transpiration per unit leaf area over the 7-day period prior to sampling. However no interactions were significant. Elevated CO_2 reduced mean transpiration by 27.5 % (Figure 7) and this, combined with inadequate humidifier-dehumidifier control, was probably the cause of the lower RH in the high than the low CO_2 chamber (Figure 6).

Table 1. Photosynthesis, leaf conductance and internal CO_2 concentration of sugarcane growing with ambient (390 ppm) or elevated (720 ppm) CO_2, supplied temporarily with ambient (375 ppm) or elevated CO_2 (720 ppm) in a small cuvette. Data for two varieties were pooled (and the water-stressed treatment is excluded).

CO_2 concentration (ppm)		Photosynthesis rate	Stomatal conductance	Internal CO_2 concentration
Cuvette	Chamber			
(temporary CO_2 treatment)	(long term CO_2 treatment)	($\mu mol/m^2/s$)	($mmol/m^2/s$)	(ppm)
375	390	25.4	161	66
375	720	28.4	196	81
375	Mean	26.9	179	74
720	390	33.8	120	178
720	720	31.4	116	189
720	Mean	32.6	118	184
p- values				
Cuvette		<0.001***	<0.001***	<0.001***
Chamber		0.818	0.114	0.435
Chamber x Cuvette		0.085	0.054	0.888

Photosynthesis was increased ($p<0.001$) by a temporary increase in CO_2 concentration in the cuvette regardless of whether plants had been growing at ambient or elevated CO_2 levels or not (Table 1). The cuvette x chamber interaction was not significant ($p= 0.085$) but there was a tendency for the response to an increase in cuvette CO_2 level to be greater for plants growing at ambient CO_2 levels than at elevated levels (Table 1). This was probably due to the rather low internal CO_2 concentration (C_i) of leaves in ambient CO_2 (390 ppm) when cuvette CO_2 was 375 ppm (Table 1). For plants growing in elevated CO_2, C_i was also below the level (~100 ppm) thought to be saturating for C_4 species (Ghannoum et al., 2000) when the small cuvette CO_2 concentration was 375 ppm (Table 1).

The mean effect of an increase in cuvette CO_2 concentration on stomatal conductance was substantial (34% reduction, $p<0.001$) and there was a tendency ($p=0.054$) for the reduction to be greater for plants growing at elevated than at ambient CO_2 (Table 1). However the more meaningful response is that between low CO_2 in both cuvette and chamber compared to high CO_2 in both types of chambers (i.e., when gas exchange

measurements are made at the same CO_2 levels as the long term CO_2 treatments plants in which plants have been growing). This is more likely to be a measure of the long-term effect of CO_2 on conductance than any other comparison. In this case, it appears that long-term exposure to elevated CO_2 had reduced stomatal conductance by 28% which is very similar to the reduction in whole-pot transpiration determined for all leaves over a 10-d period (-27.2%, see Table 2). The combined effect of changes in transpiration and photosynthesis was for an increase in instantaneous leaf-level water use efficiency in response to elevated CO_2 (Figure 7).

We would expect that this reduction in transpiration was due largely to decreased stomatal conductance given that air-flow, radiation and temperature conditions as well as the 'canopy' structure in both large chambers were similar. Transpiration in the high CO_2 chamber may have been lower than observed had the relative humidity been the same as in the low CO_2 chamber (Figure 6). However a conservative approach would be to assume that conductance was reduced by 28% by long term exposure to elevated CO_2. This corresponds to a 39% increase in stomatal resistance to gaseous diffusion (r_s) (since r_s is the inverse of conductance) (Table 1) from prolonged exposure to elevated CO_2 (390 vs. 720 ppm). Thus r_s increased 11.8% per 100 ppm increase in CO_2 (expressed in these terms for future in the modelling framework presented in Chapter 5).

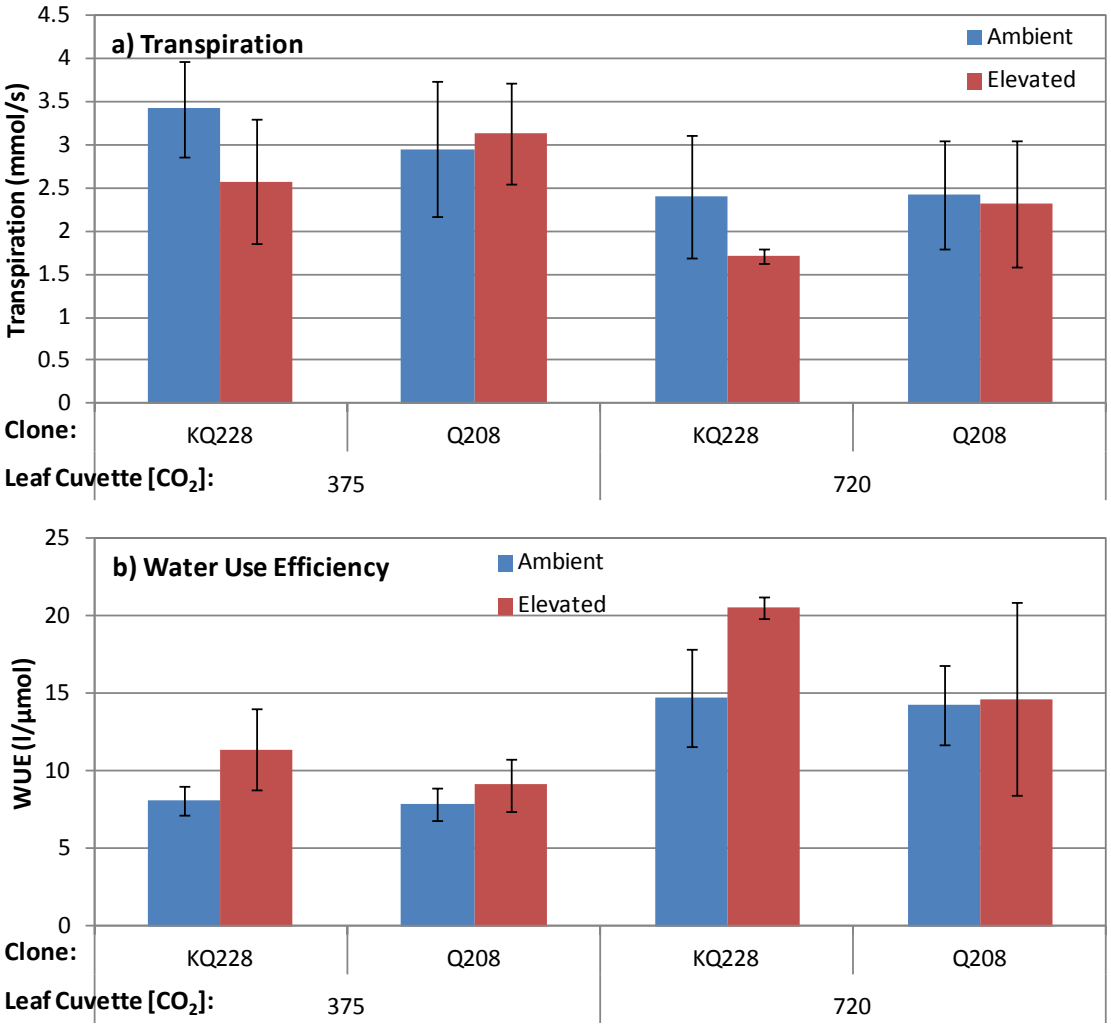

Figure 7. Instantaneous leaf level effects of CO_2 (temporarily set in leaf cuvette of Li-6400) on a) transpiration and b) instantaneous water use efficiency for plants exposed to long-term (~10 mo) chamber CO_2 treatments (ambient and elevated) for each clone. Error bars are for 2 x SE.

3.3.2 WHOLE PLANT HARVEST AND TRANSPIRATION EFFICIENCY

For the whole-plant harvest data it is important to note the water treatments were specifically designed and applied to maintain very tight control of the target soil moisture treatment to allow water-related responses to CO_2 to be separated from other potential effects of CO_2. This is unlike typical experiments where the AMOUNT of water supplied is fixed and the enhanced water use efficiency under elevated CO_2 allows plants to draw down soil moisture more slowly, benefiting plant growth by delaying the onset of water stress. The enhanced water use efficiency is being measured (and can be used to model the well-documented water-mediated mechanisms of plant response), but the indirect feedbacks on growth were eliminated, allowing us to test whether additional direct physiological processes were affecting plants responses to elevated CO_2. Measured variables were therefore divided into two groups for analysis. Those variables related to growth would not be expected to respond positively to elevated CO_2 under these controlled experimental conditions unless such direct physiological mechanisms were operating. Those variables related to water use were expected to respond to CO_2 and, for these measurements, the main interest was in quantifying the size of the CO_2 effect (more so than statistical testing) and how the size of this effect might change through interactions with other treatments.

Statistical analysis of the whole plant harvest data considered eight variables related to plant growth and four related to whole plant (pot) water use (Table 2). Water use data included a measurement of average daily water use over ten days just before harvest, and this was also used to calculate the water use per leaf area as a measure of whole-plant canopy conductance. Analyses of variance were conducted on all twelve variables, log-transforming data for all variables that were not ratios (given that, for most growth processes, variances and treatment effects in absolute terms increase with plant size). The statistical model used the four main treatments in the experiment (CO_2, Age of harvest, Water treatment and Clone) the first order interactions among these treatments and row the replicate was growing in. The treatment effects that were of most interest were the main effects of CO_2 and the first order interactions of CO_2 with other treatments. In most cases the main effects of age, water treatment and clone are trivial, and not discussed.

Table 2. Summary of ANOVA p-values for responses of harvest data (at ages 4, 6 and 8 mo) to experimental treatments. The CO_2 main effect is expressed as the percentage increase in each untransformed variable under elevated relative to ambient CO_2. For simplicity, terms in the statistical model that were not of relevance to research questions have not been presented. The first eight variables are related to plant growth, and the bottom four to whole plant (pot) water use.

Variable	CO_2 Effect	CO_2	Age	Water	Clone	CO_2*Age	CO_2*Water	CO_2*Clone
Aboveground Growth	-14.4	0.101	<0.001 ***	<0.001 ***	0.050 ·	0.593	0.680	0.342
Stalk Mass	-21.6	0.024 *	<0.001 ***	<0.001 ***	0.003 **	0.770	0.493	0.260
Height	-8.6	0.105	<0.001 ***	<0.001 ***	<0.001 ***	0.071 ·	0.221	0.636
No. Internodes/stem	-7.8	0.048 *	<0.001 ***	0.054 ·	0.059 ·	0.028 *	0.127	0.703
Leaf Area	-4.0	0.151	<0.001 ***	<0.001 ***	0.943	0.514	0.385	0.372
Specific Leaf Area	-0.3	0.907	<0.001 ***	0.008 **	0.027 *	0.187	0.576	0.040 *
Leaf Width	-0.6	0.522	0.149	0.001 **	<0.001 ***	0.468	0.747	0.011 *
Leaf N (NIRS)	+1.5	0.459	<0.001 ***	0.764	0.136	0.698	0.311	0.912
Total Water Use	-31.2	<0.001 ***	<0.001 ***	<0.001 ***	0.070 ·	0.933	0.756	0.652
Transpiration Efficiency	+34.3	<0.001 ***	0.004 **	0.000 ***	0.825	0.407	0.054 .	0.448
Final Water Use (10d)	-25.0	<0.001 ***	<0.001 ***	<0.001 ***	0.107	0.870	0.420	0.466
Water Use/Leaf Area	-27.2	<0.001 ***	<0.001 ***	0.000 ***	0.006 **	0.440	0.817	0.304

The first analysis showed a very strong contrast between clones on the fourth harvest date ($p << 0.0001$), possibly related to the fact that mites that infected the high CO_2 chamber at the end of the experiment caused more damage to KQ228 than Q208 (evidenced from the mite damage scores referenced earlier).

The statistical analysis was therefore repeated using only the first three harvests (Table 2), although descriptive summaries, as used in charts and tables, include data for all four harvests.

In each of the bar charts below, the paired bars show the direct comparison between responses in the ambient (blue) and elevated (red) CO_2 chambers for groups of plants sharing the same treatment. The set of sequential harvest batches (1a, 2a, 3a, 4a) are then arranged together for comparison of each combination of variety and water treatment. Interspersed sample batches 1b (June- October) and 2b (August-December), are omitted for simplicity of presenting the data. The data that is presented follows a simpler serial sequence of increasing duration of growth period, and duration of CO_2 and water treatments, in two-monthly increments.

The effects of elevated CO_2 were most pronounced for variables related to water use, and tended to be much weaker for measurements of plant growth (Table 2). There was no evidence of a direct benefit of elevated on any of the growth variables. However two growth variables showed significant negative responses in the elevated CO_2 chambers (stalk mass and, closely related, the number of internodes per stem). All of the CO_2 effects on growth were negative and could have been linked to problems with humidity control, where plants in the ambient CO_2 treatment were benefiting from higher humidity in that chamber. Plants were watered on demand using the same soil moisture criteria, with frequent checks and watering, in both chambers so it is not likely that the lower relative humidity in the high CO_2 chamber would have reduced yield through water stress. There may instead have been some other chamber effect that we could not distinguish from the CO_2 treatment.

In contrast, all the water use-related variables showed strong benefits of elevated CO_2 in reducing water use and improving transpiration efficiency (Table 2). These were all of the order of a 30% benefit for an increase from 390 to 720 ppm CO_2. None of the key water use variables of interest showed an interaction with age of harvest, indicating results obtained in at the initial harvest were representative of those at the final harvest date, and that a shorter treatment period would be sufficient for measuring these effects in subsequent experiments. Neither were CO_2 interactions with water or clone treatments significant for any of the water use variables, although for TE the interaction with water treatment was almost significant. Even though there were no main effects of CO_2 on leaf width and specific leaf area and the effect sizes were small (<1% combined across clones), both these variables showed significant CO_2 by clone interactions. This suggests that CO_2 may be affecting leaf processes in clone-specific ways, possibly indirectly through increases in leaf temperature associated with reduced transpiration cooling. Chamber effects could not be ruled as the contributing to this response in this experiment (but see Chapter 4 for supporting evidence).

Measurements of growth variables all increased linearly over time in wet treatments, suggesting it would be possible to interpolate these variables over time between harvest dates, or backwards from a single final harvest (Figures 8, 9 and 10). For dry treatments however, the responses were more variable between pots both within a harvest batch and between batches. This was related to the difficulty we had in achieving a uniform level of stress between pots with the organic potting and watering arrangement used.

Biomass data showed plants in the elevated CO_2 chamber had slightly lower growth (-12.0%, -3.6%, –17.4% and -8.9% averaged for each of the four harvest dates respectively relative to the ambient CO_2 chamber) (Figure 8). This corresponded with differences in the number of internodes, although the differences between CO_2 treatments were smaller (+7.3%, -7.6%, -13.5% and +1.5% respectively for the elevated CO_2 chamber vs the ambient chamber) (Figure 9). In contrast, leaf area responses started off negative in the first harvest and became slightly positive by the final harvest (-14.9%, -5.8%, +5.5% and +15.4% respectively) (Figure 10).

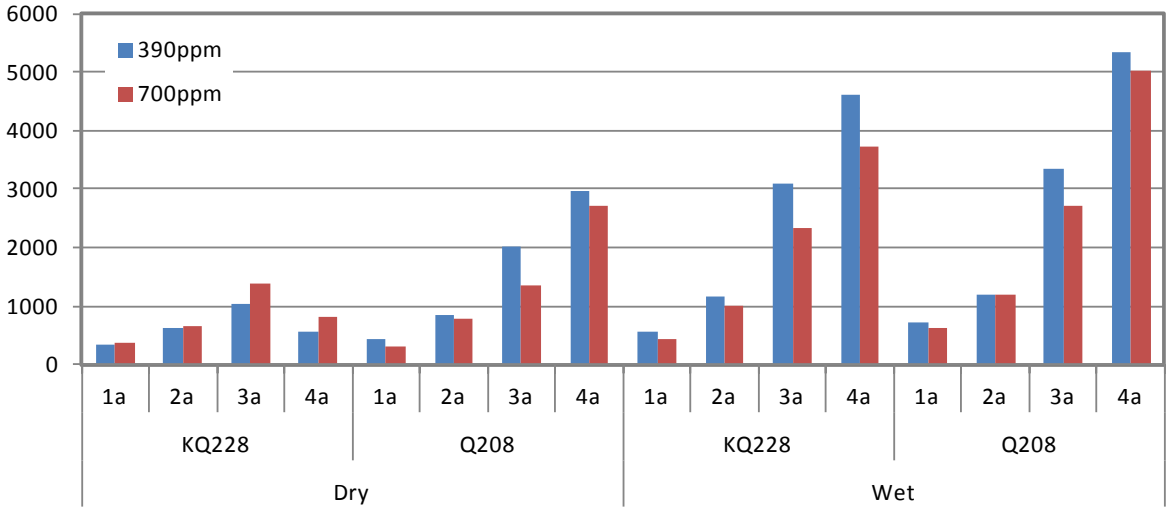

Figure 8. Biomass of total harvested aboveground plant material (g, averaged per pot) for each of four growth windows (1a = Feb-Jun, 2a = Feb-Aug, 3a = Feb- Oct, 4a = Feb-Dec) comparing elevated and ambient CO_2 (paired bars) for each combination of water treatment (Wet, Dry) and sugarcane clone (KQ228, Q208).

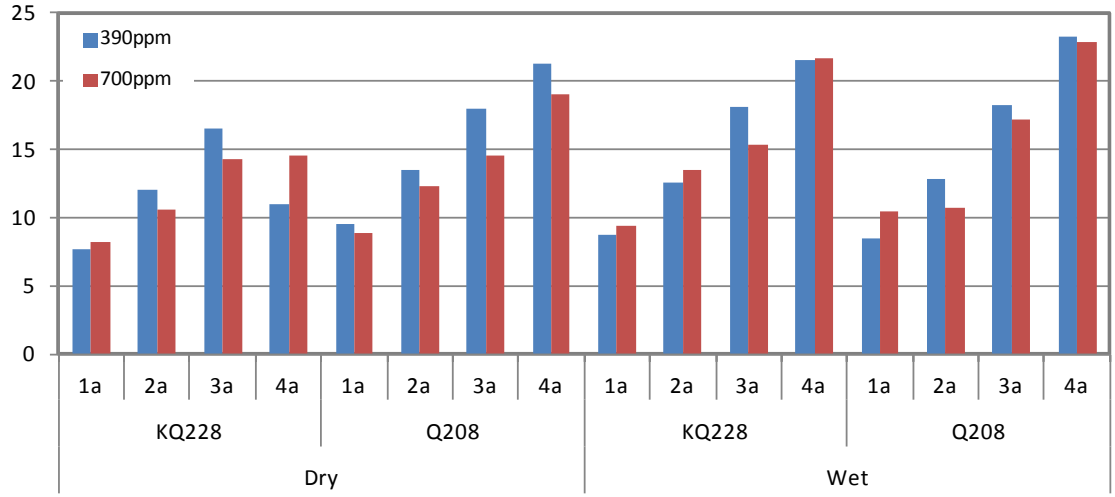

Figure 9. Numbers of internodes per stem (averaged across pots) for each of four growth windows (1a = Feb-Jun, 2a = Feb-Aug, 3a = Feb- Oct, 4a = Feb-Dec) comparing elevated and ambient CO_2 (paired bars) for each combination of water treatment (Wet, Dry) and sugarcane clone (KQ228, Q208).

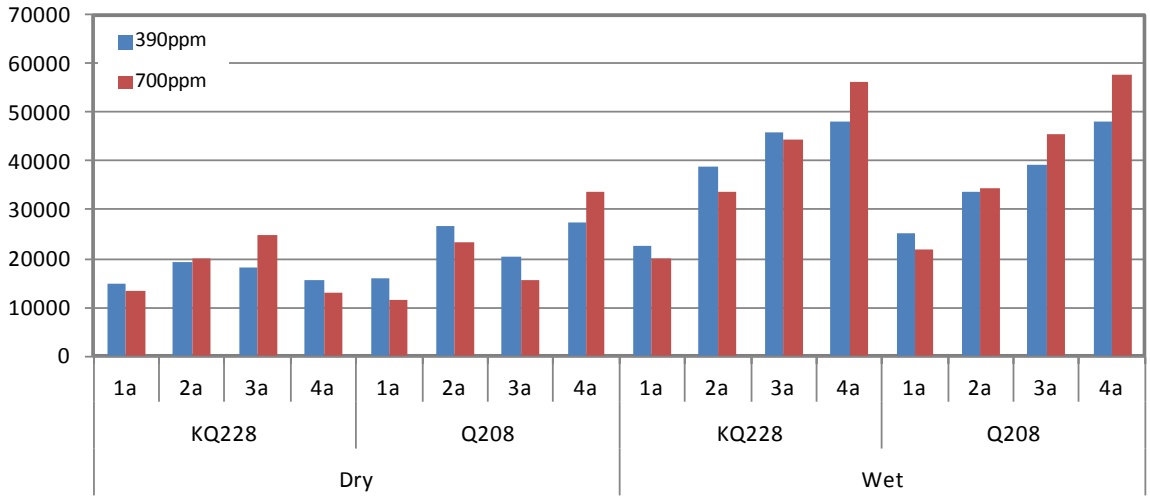

Figure 10. Green leaf area (cm^2, averaged per pot) for each of four growth windows (1a = Feb-Jun, 2a = Feb-Aug, 3a = Feb- Oct, 4a = Feb-Dec) comparing elevated and ambient CO_2 (paired bars) for each combination of water treatment (Wet, Dry) and sugarcane clone (KQ228, Q208).

As a ratio, the transpiration efficiency data (TE, the primary response of interest in this project), compensates for some of the unevenness within the water stressed treatment, and hence showed much more consistent responses over time (Figure 11) with an overall net benefit of CO_2 in improving TE by 32.5% (Table 3). As expected the improvement in TE under elevated CO_2 was greater under water-stress conditions (+43.9%) than well-watered conditions (+21.3%) (Table 3), although this interaction was not quite significant (Table 2). The responses of the two varieties were indistinguishable under well-watered conditions but, under water-stressed conditions, KQ228 received a greater benefit from elevated CO_2 (Figure 12, Table 3). In the field, KQ228 is generally irrigated and performs poorly under dry conditions. Initial results indicate this disadvantage may be reduced under higher CO_2 conditions.

Table 3. Percentage increase in water use efficiency under elevated CO_2 vs ambient conditions.

	Dry	Wet	Combined
KQ228	57.0%	21.1%	38.6%
Q208	32.8%	21.2%	27.0%
(Combined	43.9%	21.3%	32.5%

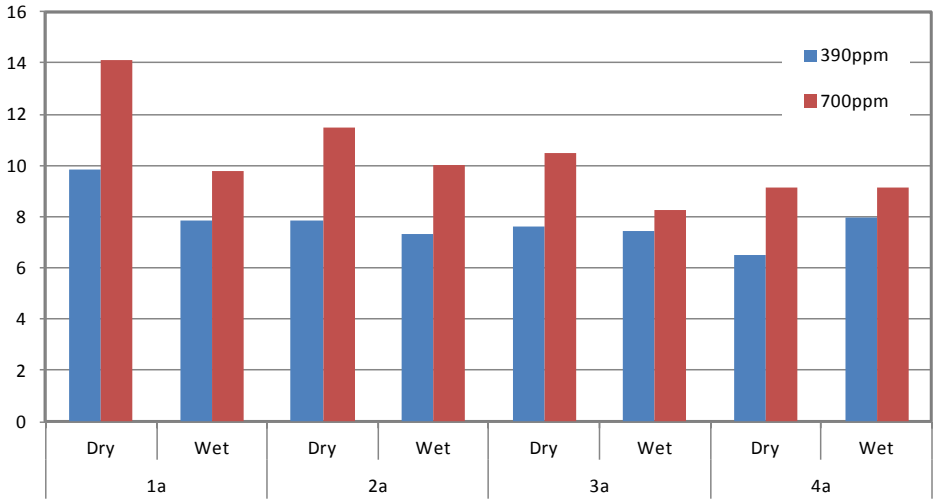

Figure 11. Transpiration efficiency (g dry matter produced per l water transpired, averaged across pots) for each of four growth windows (1a = Feb-Jun, 2a = Feb-Aug, 3a = Feb- Oct, 4a = Feb-Dec) comparing elevated and ambient CO_2 (paired bars) across water treatment (Wet, Dry) (sugarcane clones are lumped together).

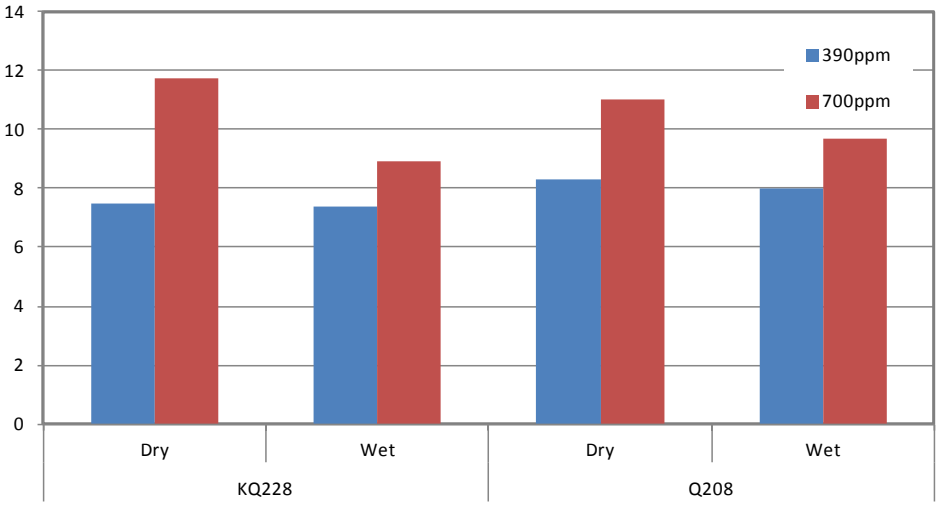

Figure 12. Transpiration efficiency (g dry matter produced per l water transpired, averaged across pots) for each combination of water treatment (Wet, Dry) and sugarcane variety (KQ228, Q208) (the four growth windows are lumped together).

3.3.3 SUGAR ACCUMULATION

An extensive set of sequential harvest data was collected on patterns of sucrose accumulation in internodes down the stem at each of the four growth stages where harvests occurred through the experiment. These were used to summarise the main effects of CO_2, watering and clone treatments for total sucrose per internode, sucrose concentration, glucose concentration and fructose concentration. All concentrations are in terms of the mass of sugars per fresh weight of sample. There was no consistent difference in patterns of sugar accumulation in response to elevated CO_2 (Figure 13). Any observed deviations were not consistent between harvest dates and within the bounds of sample variation indicating that the effect of CO_2 on accumulation of sucrose and other sugars (and the growth and metabolic processes associated with these), if any, is small.

The stress treatment, as expected, increased the concentration of sucrose (on a fresh weight basis), but the total amount of sucrose per internode was reduced. The biggest differences in patterns of sugar accumulation were between clones (Figure 14).

Q208 had higher concentrations of glucose and fructose than KQ228 for the first three harvests (up to 8 months old) although differences in sucrose, in both concentration and total amount, only became evident in the final harvest (Figure 15). This was not a consequence of mite damage at the end of the experiment: when the data from the mite-affected chamber were excluded, the same differences between clones were observed.

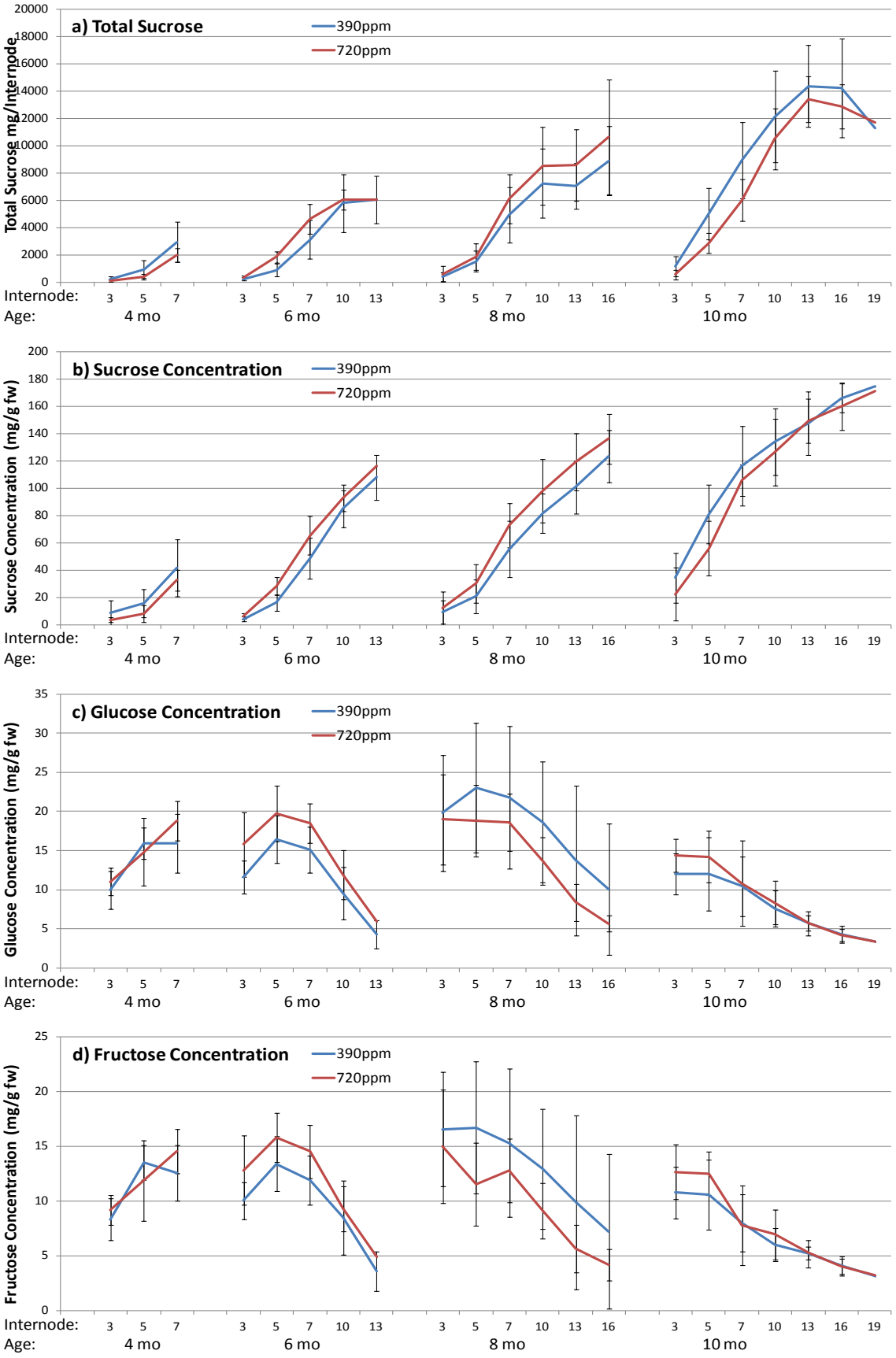

Figure 13. Sugar accumulation patterns in internodes down the stalk at four harvest dates, as affected by CO_2 treatments. Clones and water treatments are averaged within each CO_2 treatment.

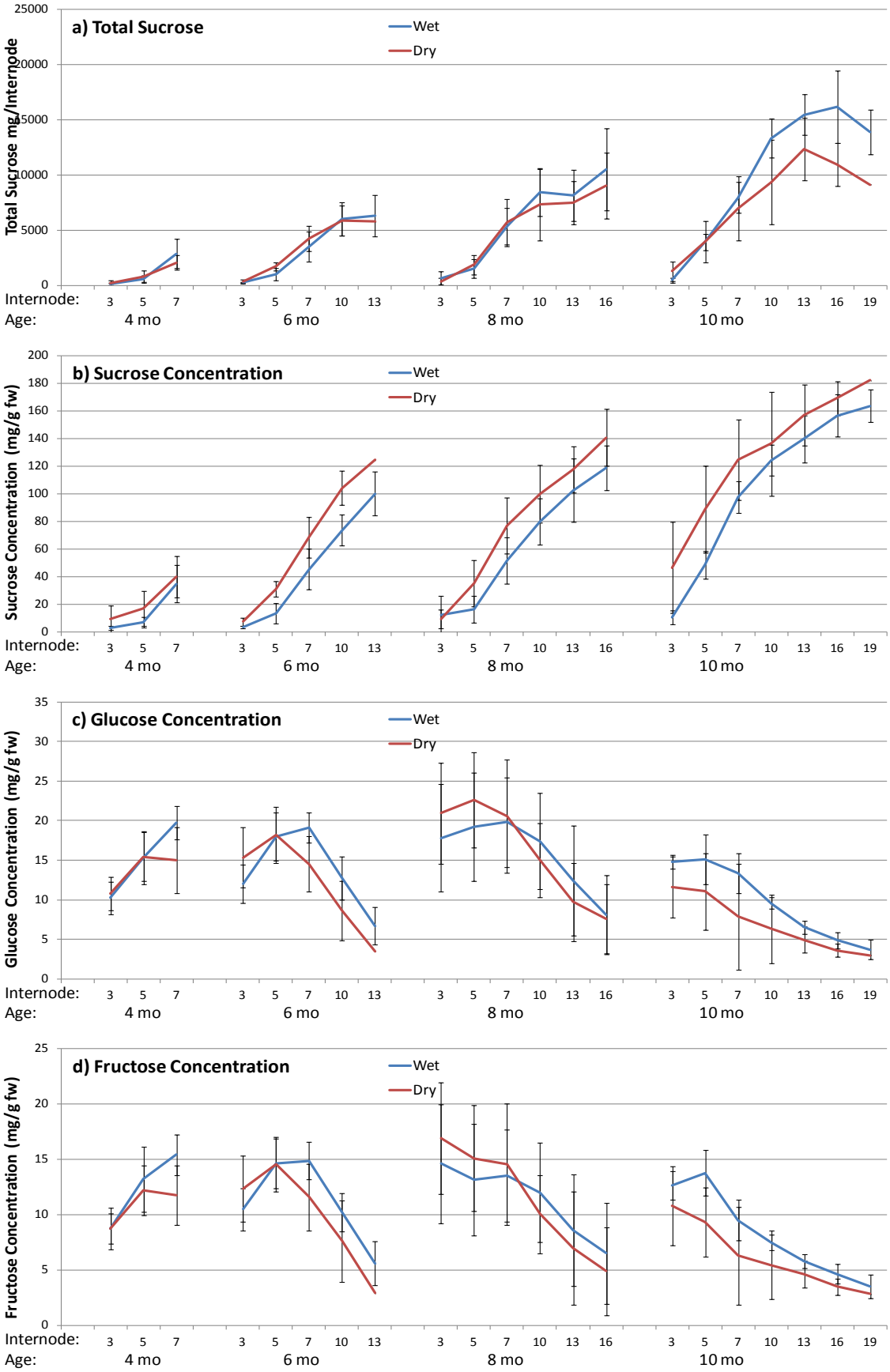

Figure 14. Sugar accumulation patterns in internodes down the stalk at four harvest dates, as affected by water treatments. Clone and CO_2 treatments are averaged within each water treatment.

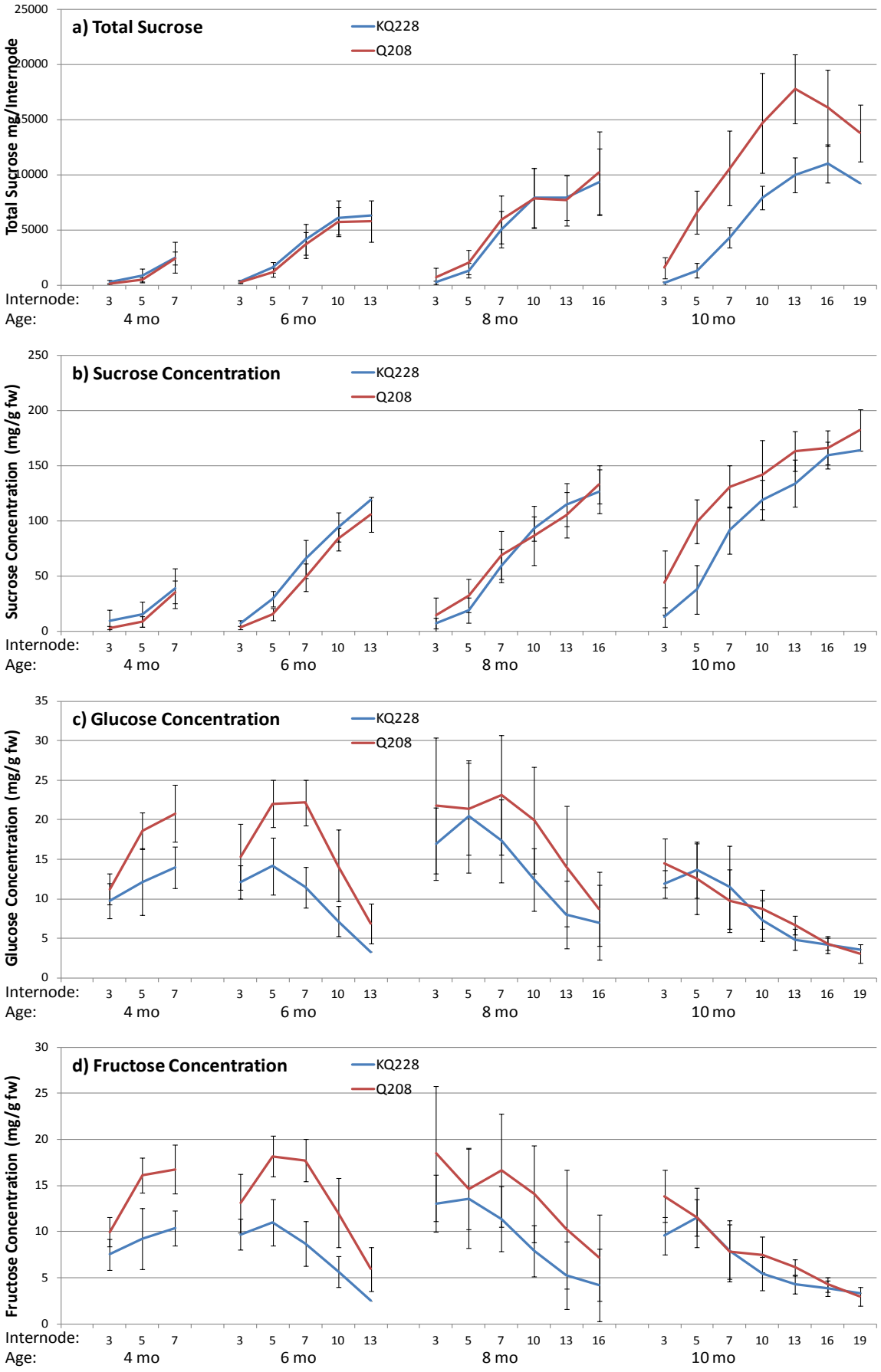

Figure 15. Sugar accumulation patterns in internodes down the stalk at four harvest dates for two clones. CO_2 and water treatments are averaged for each clone.

3.4 Discussion

Notwithstanding the fact that treatment control issues compromised some of the results, this first experiment provided evidence that any direct stimulation of sugarcane growth by elevated CO_2, from direct leaf-level mechanisms, is likely to be small. This suggests that reported increases in yield from previously published CO_2 experiments (Vu et al., 2006; de Souza et al., 2008) are more likely to be largely due to indirect mechanisms related to improved water relations (the alleviation of water stress and prolonged soil water availability) even if water was thought to have been non-limiting during the experiments. Better water relations could have explained the results of Vu et al. (2006) where photosynthesis of small sugarcane leaf segments of young leaves was 10 to 20% greater in 720 ppm than ambient ppm CO_2 because elevated CO_2 reduced stomatal conductance by 51% and transpiration by 39%. Vu et al. (2005) suggested that the increase in leaf area (31%) and stalk yield (55%) with elevated CO_2, could have been partly through enhanced water use efficiency and therefore stress alleviation and prolonged water availability. de Souza et al. (2008) clarified their efforts to ensure adequate irrigation by maintaining soil water tension below 20 kPa and yet the 40% increase in biomass yield under elevated CO_2 was thought to be at least partly due water stress alleviation.

Our results isolated direct from indirect effects of CO_2 and showed no signs of any direct benefit of CO_2 to sugarcane growth. Instead, growth in ambient CO_2 chambers was actually slightly higher than in the elevated CO_2 chambers but this was likely related to the higher humidity in the ambient CO_2 chamber or some other chamber effect that could not be isolated from the CO_2 treatment. Instead, as expected from basic leaf physiology of C_4 plants (Matsuoka et al., 2001), CO_2 effects were strongest amongst measurements related to water use, including transpiration efficiency. Both leaf and whole-plant measurements showed similar results in terms of an approximate 20-30% increase in water use efficiency, or an equivalent decline in leaf/plant conductance while maintaining a similar rate of photosynthesis /growth for an increase in CO_2 levels from 390 to 720ppm.

There were no interactions of CO_2 effects with the duration of treatments or growth phases for any of the water use metrics of interest. The magnitude of the CO_2 effect on transpiration efficiency remained constant across time for well-watered plants, but may have been slightly higher in the initial stage of applying water stress treatments (more likely a consequence of water levels gradually declining, and stress gradually increasing, at the start of the dry treatment than an effect of growth stage per se). There was no evidence of acclimation or any form of decline in CO_2 responses over time. In some plants, and C_3 plants in particular, there can be a down-regulation of photosynthesis with prolonged exposure to elevated CO_2 as the concentration of the nitrogen-rich enzymes involved in photosynthesis declines. This can occur either through active shifts in nitrogen allocation, shifting nitrogen away from photosynthesis enzymes as their efficiency increases under elevated CO_2, or a passive even dilution of nitrogen throughout the plant, as increases in carbon fixation are not matched by increases in nitrogen uptake. The leaf nitrogen did not show any evidence of responses to elevated CO_2. Neither did patterns of sugar accumulation in stems show any evidence that carbon assimilation and subsequent metabolism was being affected by elevated CO_2 at any stage of plant growth. In addition, all the growth variables we measured increased linearly over time, indicating that measurements could easily be interpolated between sampling dates or backwards from the final harvest. Collectively these findings indicate that a shorter growth period is sufficient evaluating CO_2 responses, which allowed more rapid screening of a wider number of clones in subsequent experiments (next Chapter).

There was no statistically significant interaction between CO_2 and clone for any water use variables, and the size of TE responses to CO_2 under well-watered conditions was similar for the two varieties. However water stress increased the strength of CO_2 response and showed potential for possible genetic variation in CO_2 response, warranting further investigation in the following experiments.

Data from the first experiment showed slight negative effects of CO_2 (elevated vs ambient CO_2 chamber) on plant growth (~-12%) and number of internodes produced (~-5%) but a slight overall benefit for leaf area

(~+3%). Because of early teething problems with environmental controls (particularly occasional lapses in temperature and humidity control that resulted in deviations between chambers), we cannot rule out these differences between chambers, rather than CO_2, as contributing to the measured responses. TE provides a more robust indicator of response because it is a ratio that normalises growth responses against the amount of water transpired (with both numerator and denominator subject to some of the same components of statistical variation). TE responses were more consistent over time, showed a clear benefit of CO_2, and indicated differences between clone x water stress combinations (indicating there may be some potential to be able to screen and select clones for future climates on this basis).

As the first experiment using a novel, purpose-built watering system in a new controlled environment there were some initial teething problems that meant some treatments were not applied with the level of control we ultimately hoped to achieve. However, the experiment was effective at meeting its main goals of providing the background data necessary to 1) develop a rapid screening system for identifying sugarcane varieties that are most benefited by rising CO_2, and 2) test and fine-tune the performance of a new controlled environment facility, a new CO_2 control system, and a new water control system. In dealing with these initial challenges, we have developed a system that is more capable and ambitious than we had originally planned, opening new opportunities for exploring mechanisms underlying water stress and water use responses in sugarcane. The next chapter documents the technical improvements that were implemented, based on the lessons learned in this first experiment, to the refine the methodology. In doing so, it resolves some of the issues with findings from this experiment where CO_2 responses could not conclusively be distinguished from chamber effects.

4 Genetic variation in sugarcane responses to elevated CO_2

4.1 Introduction

Following on from the initial pot experiment, two additional pot trials were conducted to assess CO_2 responses across a broader number clones. The main aim of these experiments was to test for genetic variation in responses to CO_2 that might represent an opportunity for selection in breeding. Both experiments used a very similar approach, using a shorter (~3 month) growth window and a range of methodological improvements based on the findings of Experiment 1 in the previous Chapter. As before, the selection of clones was based on preliminary findings from related water use and stress work in other SRDC/SRA projects (particularly BSS305: 'More-Crop-per-Drop'), selecting a range of clones with contrasting characteristics, focussing mainly on clones with strong vigour and good biomass production.

The design of the Experiment 2 consisted of full factorial combinations of:

*CO_2 treatments * 2*

A high CO_2 chamber (720 ppm) and an ambient CO_2 chamber (~390 ppm).

*Clones * 8 (7 useful)*

Eight clones, matching those being used in projects BSS305 and BSS334 and related breeding work to assess field, pot and physiological performance of sugarcane under water-stressed and unstressed conditions (Table 4). After two attempts, CT05-645 established too poorly to generate useable data, and was excluded from the experiment.

*Water treatments * 2*

A treatment where plants are given ample water (watering triggered at 90% field capacity), contrasted against a treatment where a water stress treatment was imposed by restricting the soil water content (watering triggered at about 25% field capacity).

*Replicates * 4*

There were 4 replicate pots for each treatment combination, arranged in a Latin-square experimental layout within the chambers.

Controls

There were an additional four guard row pots in each chamber.

(Total = 136 pots simultaneously)

Table 4. Clones selected for Experiment 2 and for setting up a local nursery for Experiment 3. All clones are currently being used in local sugar breeding research and the rationale for their inclusion is provided based on existing/ongoing measurements and results in related work. '(PS)' indicates photosynthesis measurements have been made.

Clone	Rationale for inclusion
Experiment 2 Clones	
1) CT05-645	Clones with NMM tubes (PS) (Note: grew poorly, excluded from results)
2) QBYN05-20735	Clones with NMM tubes (PS)
3) KQ228	Clones with NMM tubes (PS)
	Commercially-important clone, used in Experiment 1
4) QN66-2008	High reduction in Home Hill, low in Dalbeg - Interaction (PS). Fairly good interaction across 2 water stress conditions with low conductance
5) Q208	Add Q208 from year 1 (PS)
	Commercially-important clone, used in Experiment 1
6) Q183	Clones with NMM tubes (PS)
7) QBYN05-20583	Clones with NMM tubes (PS)
8) QC91-580	Clones with NMM tubes (PS)

Experiment 3 used a very similar design to experiment 2 except that it used 6 clones instead of 8. This allowed doubling the replication for two clones, KQ228 and Q208 (from 4 to 8 replicates for each treatment combination). This was to more fully test the apparent differences in these clones from previous results, which appeared to be the strongest contrast to date. It was also intended to provide a data set to better quantify the statistical variation in response, which we would be able to use in future to plan the amount of replication required to detect specified effect sizes in screening. The design of the Experiment 3 consisted of full factorial combinations of:

*CO_2 treatments * 2*

An elevated CO_2 chamber (720 ppm) and an ambient CO_2 chamber (~390 ppm).

*Clones * 6*

Six clones, matching those being used in SRDC projects BSS305 and BSS334 and related breeding work that is assessing field, pot and physiological performance of sugarcane under water-stressed and unstressed conditions (Table 5). With Q208, the initial one-eyed setts established unevenly, so this may have contributed to resulting higher variation in yields and, as a consequence, lower confidence in the results for this clone.

*Water treatments * 2*

A treatment where plants are given ample water (maintained at about 90% of field capacity), contrasted against a treatment where a water stress treatment was imposed by restricting the soil water content to about 25% of field capacity.

*Replicates * 4 or 8*

There were 4 replicate pots for each treatment combination, arranged in a Latin-square experimental layout within the chambers. For KQ228 and Q208, the replication was doubled to 8 pots for each treatment combination.

Guard row

There were an additional four guard row pots in each chamber.

(Total = 136 pots simultaneously)

Each individual pot contained about 25l of potting medium (and about 6l of plant-available water) within which three sugarcane plants were established.

Table 5. Clones selected for Experiment 3. All clones are currently being used in local sugarcane breeding research and the rationale for their inclusion is provided based on existing/ongoing measurements and results in related work. '(PS)' indicates photosynthesis measurements have been made. There are four replicate pots of each clone for each treatment, except for clones marked 'double reps' where eight replicates were used.

Clones	Rationale for inclusion
Experiment 3 Clones	
KQ228 (double reps)	Clones with NMM tubes (PS)
	Commercially-important clone, used in Experiments 1 & 2
Q208 (double reps)	Commercially-important clone, used in Experiments 1 & 2 (PS)
QBYN04-10951	Clones with NMM tubes
Q183	Clones with NMM tubes (PS)
QB01-10005	Clones with NMM tubes
Q190	Clones with NMM tubes

4.2 Methods

The second and third experiments used mainly the same approach as the first experiment, conducted as pot trials in the Tall Plant Facility (TPF) (Figure 2) with the automated demand-driven watering system (Figure 3). However, based on the findings of Experiment 1, these trials were reduced to a shorter, 3 month, growing window to allow more rapid screening. Treatments were initiated as soon as one-eye setts had established in the pots.

A number of technical and methodological refinements were also made, based on the experiences from Experiment 1:

- The potting medium was changed from a commercial premium potting mix to a 50:50 (m/m) mix of peat moss and fine sand. This provided a more uniform medium for measuring and applying water stress treatments. Peat-sand has good drainage and water dispersal properties allowing more uniform distribution of moisture. The medium itself is also very uniform, making it easier to get consistent readings with soil moisture probes (reducing variability both within and between pots), allowing more precise control of watering and water stress treatments. In addition, dry patches of the potting mix used in Experiment 1 tended to become strongly hydrophobic, resisting rewetting (the field capacity of the potting mix dropped markedly after long-term application of stress treatments in the first trial and could not be wet again to the level it could at the start), which was much less of an issue with sand-peat. Each pot contained about 25 l of sand-peat mix which stored about 6 l of plant-available water.
- The shorter growth period, and smaller plant size, allowed us to switch the plants and CO_2 treatments between the two chambers half way through the treatment period. (In Experiment 1, plants became too tall to move through chamber doorways.) This reduced the potential for differences between chambers to become confounded with the influence of the CO_2 treatments.

- We partially shaded some of the side-walls of the chambers to provide a more even lighting environment between the two chambers (particularly lateral diffuse light).
- These experiments were conducted using a warmer diurnal temperature cycle. The previous experiment had to use a temperature pattern representing slightly more southern (cooler) locations (particularly lower night-time temperatures) to restrict plant growth and prevent plants from outgrowing the chambers (over the 10-month period of the previous trial). In Experiments 2 and 3, we used a warmer temperature regime, representing summer growing conditions in northern Queensland (cycling form a daily minimum of 24 to a maximum of 32°C). With the shorter experiment duration, the warmer temperatures had the added advantage of speeding up the time to harvestable results, and will be more relevant to the warmer climates that our climate-ready screening is targeting.
- Temperature and humidity sensors in each chamber that were independent of environmental control system were continually monitored, and the environmental control settings were adjusted manually to keep the conditions in two chambers as similar as possible. This overcame the problem with the faulty TPF environmental control software that was unable to reach set points.
- Similarly, improvements were made to CO_2 control by getting the TPF environmental control software to make more gradual adjustments to CO_2 injection rates. As with the temperature and humidity control, independent sensors were used to check chamber CO_2 levels and make manual adjustments where the TPF was not automatically achieving specified set points.
- The completion of the CO_2 control system in the second chamber allowed us to also control the CO_2 level in the ambient chamber. During peak periods of photosynthesis in the first experiment, plants could draw down the CO_2 level in the ambient chamber (to about 375ppm). In these following two experiments CO_2 was 'topped up' slightly during such periods to maintain CO_2 at 390ppm.
- The watering control software was modified to allow more than one aliquot (exactly 204 ml) of water to be delivered each time a watering event was triggered by soil moisture falling below the treatment level. Increasing this to two drops per event in the dry treatment increased the depth of initial wetting and allowed more uniform moisture levels in the pots.
- The location of soil moisture probes was changed from being inserted diagonally from the top to being inserted horizontally, through holes in the side of the pot. This again helped to ensure that water in the stressed treatments was not just restricted to wetting the soil surface.

There were two further improvements made in Experiment 3 after further investigations and fine-tuning in Experiment 2:

- In the second experiment small drainage holes were left open in the bottom of the pots so that issues with the water system could be investigated and resolved. There were indications from Experiment 2 that water-holding capacity might decline overtime, either from the medium becoming hydrophobic (particularly in stressed treatments) or from increasing root content. If the field capacity drops below the trigger point for watering, then the set point cannot be maintained and watering would continually be added to pots until they became waterlogged to the level of water sensors. Dips in measured soil moisture at night-time seemed to suggest that water was draining down below the set point and that field capacities were therefore declining. But this was resolved to be result of a subtle temperature influence on the soil moisture sensors. With the drainage holes open in Experiment 2, water occasionally seeped from some pots and would have slightly reduced the quality of the water use measurements. With the issue resolved by Experiment 3, we sealed the drainage holes in pots once field capacities had been calibrated and before treatments began. Without concerns of unaccounted water leaks, we have greater confidence in the water use data from this final experiment.

- Related to the above, in Experiment 2 the field capacity of each pot was recalibrated after the pots and CO_2 treatments were swapped between chambers half way through the experiment. This required rewetting all pots, including those in dry treatments, which took about a week for moisture to return to the target stress level. In Experiment 3 a constant conservative field capacity calibration was applied to all pots after the change-over, without recalibration, so stress treatments could be maintained. This meant that the stress treatment was more uniform, and more severe on average, in Experiment 3.

4.3 Results

There were three aspects to how we analysed the results from the two screening experiments. First, we assessed the main effects of CO_2, in particular aiming to resolve the distinction between direct and indirect mechanisms of CO_2 responses. Second, we assessed genetic variation in water-related response to CO_2. Last, we briefly assessed what water use variables could be collected non-destructively while plants were growing that might be useable as indicators of final harvested growth.

With the progressive methodological improvements through the project, we have more confidence in the results of these experiments than Experiment 1, and most confidence in the results of Experiment 3. Where there are discrepancies between the experiments that can be accounted for by these methodological improvements, we would therefore place stronger emphasis on the results from the later experiments.

4.3.1 MAIN EFFECTS OF CO_2

Statistical analysis of Experiments 2 and 3 followed closely what was done in Experiment 1. Seven variables related to plant growth and four related to whole plant (pot) water use were subjected to analyses of variance, log-transforming data for all variables that were not ratios as before (Table 6 and Table 7). 'Above-ground growth' was measured as the difference in above-ground biomass between the final harvest and the start of the experiments, when treatments and water use measurements began. The statistical model used the three main treatments in the experiments (CO_2, Water treatment and Clone) together with a blocking variable, and all first order interactions. The treatment effects that were of most interest were the main effects of CO_2 and the first order interactions of CO_2 with other treatments. In most cases the main effects of water treatment and clone are to be expected, and not discussed.

In neither experiment was there any evidence of CO_2 directly stimulating growth of total plant biomass or stalks (the first four variables in Tables 6 and 7). The estimates of the magnitude of the CO_2 effect for all of these plant attributes were very small, and averaged only +0.5% for the overall combined effect on aboveground biomass growth (Table 8 and Figure 16). However there was evidence of CO_2 affects leaf attributes. There were significant changes in specific leaf area (area per unit leaf mass) and leaf width in both experiments (Tables 6 and 7). The direction of change in leaf width was opposite in the two trials, decreasing in Experiment 2 but increasing in Experiment 3. However, the change in specific leaf area was consistent. In both experiments specific leaf area increased under elevated CO_2, in both wet and dry watering treatments, increasing on average by 9.3% overall (Table 9 and Figure 17). While increases in leaf area were of a similar magnitude in both experiments, these changes were not significant (Tables 6 and 7). Neither were these changes in leaves associated with any detectable change in stalk or whole-plant growth.

The biggest CO_2 responses were all related to water use (last four variables in Tables 6 and 7). All measures of transpiration declined significantly and transpiration efficiency (TE) increased significantly under elevated CO_2, with an overall average increase in TE of 48.9% (Table 10 and Figure 18). In Experiment 3 there was a significant interaction between CO_2 and water treatments on TE, with a much larger effect under dry (+82.2%) versus wet (+55.2%) conditions. This interaction was detected only in Experiment 3, possibly due to improvements in methodology between experiments. Any leaks from pots in Experiment 2 could have made up a larger proportion of measured water use in dry treatments (where double the amount of water was added for each triggered event, increasing the potential for leaks), lowering TE measurements more in dry than wet treatments. In addition, water stress was alleviated in Experiment 2 when field capacities were recalibrated at the chamber swap in the middle of the experiment. These factors could also account for the slightly lower TE measurements in Experiment 2 overall.

Table 6. Summary of ANOVA p-values for responses of harvest data to treatments in Experiment 2. The CO_2 main effect is expressed as the percentage increase in each untransformed variable under elevated relative to ambient CO_2. For simplicity, terms in the statistical model that were not of relevance to research questions have not been presented. The first seven variables are related to plant growth, and the bottom four to whole plant (pot) water use.

Variable	CO_2 Effect	CO_2	Water	Clone	CO_2*Water	CO_2*Clone
Aboveground Growth	-0.9	0.361	<0.001 ***	<0.001 ***	0.525	0.005 **
Stalk Mass	-1.3	0.518	<0.001 ***	<0.001 ***	0.745	0.007 **
Height	+1.3	0.482	<0.001 ***	<0.001 ***	0.057 ·	0.122
No. Internodes/stem	+5.2	0.069 ·	<0.001 ***	0.003 **	0.094 ·	0.230
Leaf Area	+10.0	0.086 ·	<0.001 ***	0.005 **	0.287	0.196
Specific Leaf Area	+11.5	<0.001 ***	0.315	<0.001 ***	0.058 ·	0.015 *
Leaf Width	-7.3	<0.001 ***	<0.001 ***	<0.001 ***	<0.001 ***	0.020 *
Total Water Use	-22.1	<0.001 ***	<0.001 ***	<0.001 ***	0.835	0.022 *
Transpiration Efficiency	+24.1	<0.001 ***	<0.001 ***	0.004 **	0.115	0.171
Final Water Use (10d)	-19.3	0.007 **	<0.001 ***	0.165	0.512	0.031 *
Water Use/Leaf Area	-27.3	<0.001 ***	<0.001 ***	<0.001 ***	<0.001 ***	0.055 ·

Table 7. Summary of ANOVA p-values for responses of harvest data to treatments in Experiment 3. The CO_2 main effect is expressed as the percentage increase in each untransformed variable under elevated relative to ambient CO_2. For simplicity, terms in the statistical model that were not of relevance to research questions have not been presented. The first seven variables are related to plant growth, and the bottom four to whole plant (pot) water use.

Variable	CO_2 Effect	CO_2	Water	Clone	CO_2*Water	CO_2*Clone
Aboveground Growth	+1.4	0.177	<0.001 ***	<0.001 ***	0.059 ·	0.252
Stalk Mass	+0.0	0.087 ·	<0.001 ***	<0.001 ***	0.027 *	0.334
Height	-4.6	0.714	<0.001 ***	<0.001 ***	0.003 **	0.396
No. Internodes/stem	-4.6	0.810	<0.001 ***	0.012 *	0.094 ·	0.077 ·
Leaf Area	+8.3	0.201	<0.001 ***	<0.001 ***	0.708	0.703
Specific Leaf Area	+7.5	0.002 **	<0.001 ***	0.002 **	0.086 ·	0.184
Leaf Width	+5.4	0.011 *	<0.001 ***	<0.001 ***	0.040 *	0.056 ·
Total Water Use	-34.4	<0.001 ***	<0.001 ***	<0.001 ***	0.616	0.586
Transpiration Efficiency	+69.5	<0.001 ***	<0.001 ***	<0.001 ***	<0.001 ***	0.552
Final Water Use (10d)	-33.3	<0.001 ***	<0.001 ***	<0.001 ***	0.330	0.499
Water Use/Leaf Area	-56.6	<0.001 ***	0.153	0.002 **	0.019 *	0.006 **

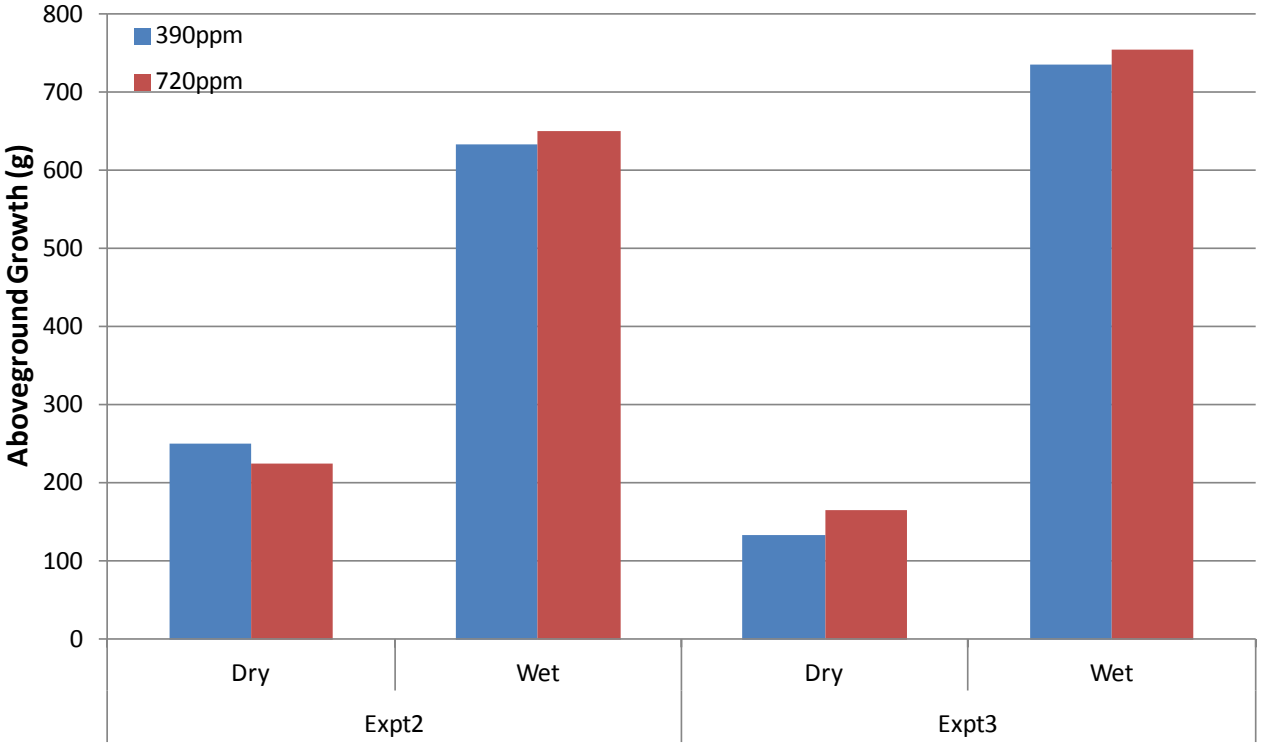

Figure 16. Overall aboveground biomass responses to water and CO_2 treatments, averaged across clones in Experiments 2 and 3.

Table 8. Total aboveground biomass (g dry matter per pot, averaged across clones) for each combination of CO_2 and watering treatment in Experiments 2 and 3. The magnitudes of the effects of these treatments are shown as percentage increases for elevated, relative to ambient, CO_2 conditions.

	390ppm	720ppm	CO2 effects (Δ%)
Experiment 2			
Dry	250.0	224.1	-10.4%
Wet	634.0	650.7	+2.6%
Combined	449.4	445.6	-0.8%
Experiment 3			
Dry	133.5	166.3	+24.6%
Wet	736.7	754.3	+2.4%
Combined	452.2	460.3	+1.8%
Both Experiments Combined			
Dry	191.7	195.2	+1.8%
Wet	685.4	702.5	+2.5%
Combined	450.8	453.0	+0.5%

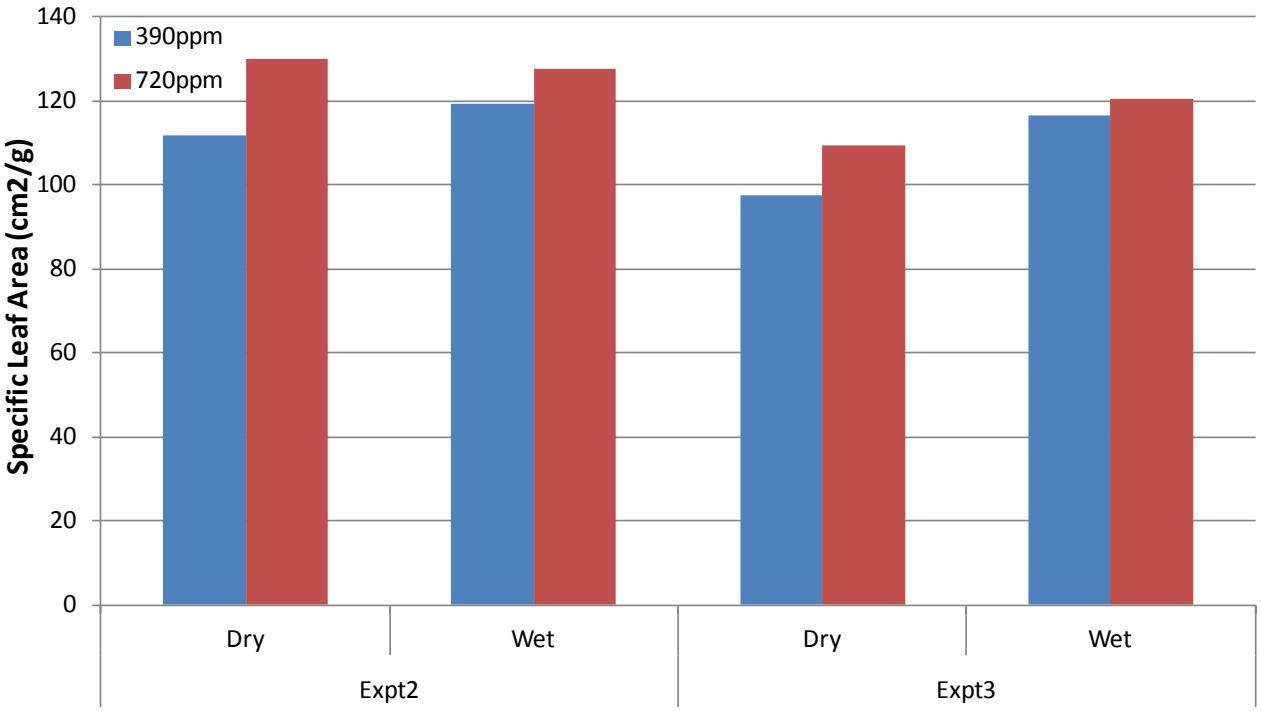

Figure 17. Overall changes in specific leaf area in response to water and CO_2 treatments, averaged across clones in Experiments 2 and 3.

Table 9. Specific leaf area (g dry matter per cm^2 of leaf, averaged across clones) for each combination of CO_2 and watering treatment in Experiments 2 and 3. The magnitudes of the effects of these treatments are shown as percentage increases for elevated, relative to ambient, CO_2 conditions.

	390ppm	720ppm	CO2 effects (Δ%)
Experiment 2			
Dry	111.7	130.1	+16.5%
Wet	119.2	127.6	+7.1%
Combined	115.6	128.8	+11.5%
Experiment 3			
Dry	97.6	109.5	+12.2%
Wet	116.5	120.5	+3.4%
Combined	107.6	115.0	+6.9%
Both Experiments Combined			
Dry	104.6	119.8	+14.5%
Wet	117.9	124.1	+5.3%
Combined	111.6	121.9	+9.3%

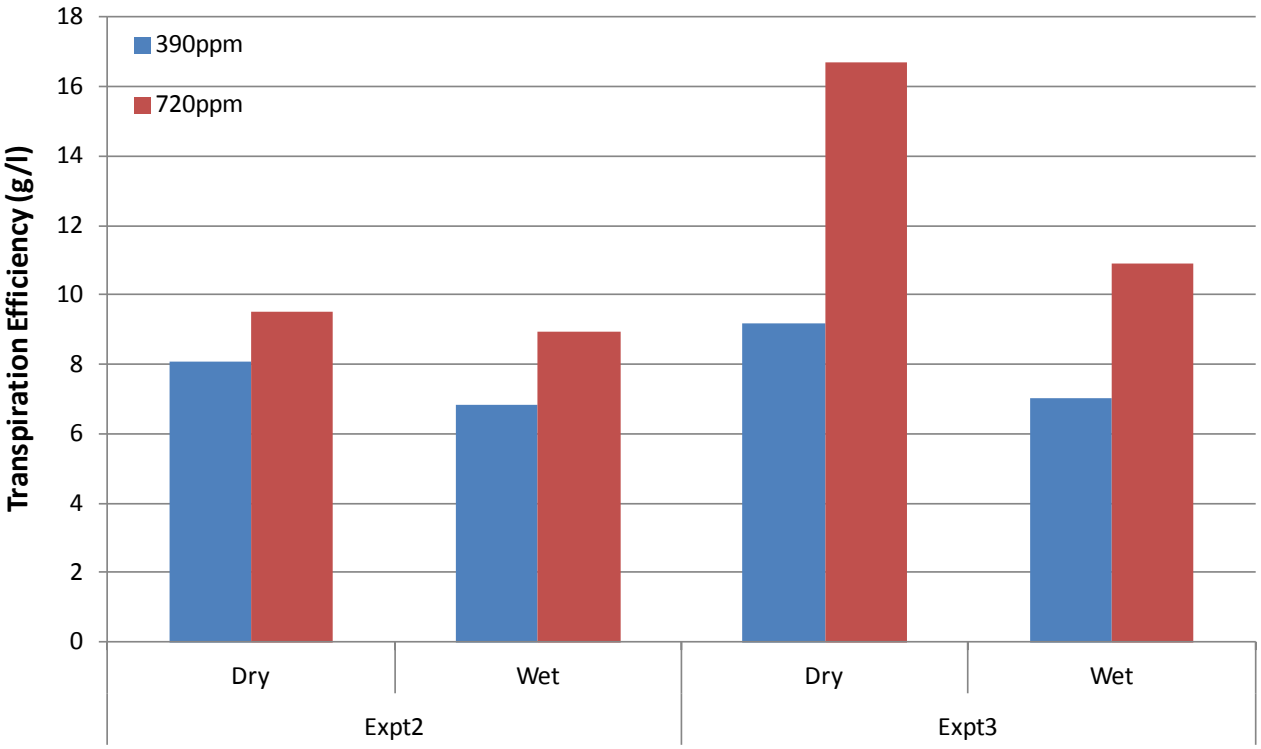

Figure 18. Overall transpiration efficiency responses to water and CO_2 treatments, averaged across clones.

Table 10. Transpiration efficiency (g dry biomass produced per litre water transpired, averaged across clones) for each combination of CO_2 and watering treatment combinations. The magnitudes of the effects of these CO_2 treatments are shown as percentage increases for elevated, relative to ambient, CO_2 conditions.

	390ppm	720ppm	CO2 effects (Δ%)
Experiment 2			
Dry	8.1	9.5	+18.0%
Wet	6.8	8.9	+30.6%
Combined	7.4	9.2	+24.0%
Experiment 3			
Dry	9.2	16.7	+82.2%
Wet	7.0	10.9	+55.2%
Combined	8.0	13.8	+71.8%
Both Experiments Combined			
Dry	8.6	13.1	+52.2%
Wet	6.9	9.9	+43.1%
Combined	7.7	11.5	+48.9%

4.3.2 GENETIC VARIATION IN RESPONSE TO CO_2 AND WATER STRESS

There were significant interactions between clone and CO_2 treatment for water use variables in both trials (Tables 6 and 7). The most consistent interaction between the two experiments was for differences in the decline of water use per leaf area (expressed as the total water transpired divided by the final leaf area at harvest). The measurement of greater interest for this line of research however is transpiration efficiency. While this did not show a significant interaction in the ANOVA (which tests for absolute differences in TE), in relative terms (expressing CO_2 effects as percentage changes that consider both the absolute change in TE and the original starting TE) there were large differences in CO_2 effects among clones with increases in TE varying between +7.3% and +126.2% (Table 11 and Figure 19). It is these relative changes that are more important for parameterizing models which translate more directly into relative changes in plant growth when water is limiting.

The genetic variation in TE among clones varied among CO_2 x water treatment combinations (quantified as standard errors in Table 11). The variation in TE amongst clones was lowest when clones were grown under wet conditions at ambient CO_2, increased with the both elevated CO_2 and water stress treatments, and was greatest under water stressed conditions *and* elevated CO_2. This suggests that efforts to enhance TE through selective breeding of sugarcane will become more important and provide greater benefit under future elevated CO_2 conditions, particularly if exposure to water stress becomes more frequent.

From a practical perspective for breeding, the magnitude of CO_2 response of a given clone is of less importance than the ultimate ranking in TE and performance of that clone under elevated CO_2 conditions. The clones that ranked the highest in the size of the CO_2 effect were often those that had lower TEs to begin with, particularly in Experiment 3 (Table 11). If a given treatment does not change the ranking much (even if there is substantial genetic variation in the relative or absolute magnitude of responses), then this would make breeding easier because less consideration would have to be placed on accounting for these effects when screening and selecting clones.

Plotting the TEs of clones under ambient versus elevated CO_2 conditions demonstrates more clearly how CO_2 is affecting the ranking of clones (Figure 20). In Experiment 3 there were remarkably strong correlations, with TE under ambient conditions accounting for over 80% of the genetic variation in TE under elevated CO_2 conditions (Figure 20b). The size of the CO_2 effect is indicated by the vertical distance of each point above the marked 1:1 line. This figure also shows clearly the greater variation in TE among clones under water-stressed (red) relative to well-watered (blue) conditions, and the larger average effect of CO_2 under water stress (red arrow). However, these relationships were not as strongly expressed in Experiment 2, possibly as a result of less accurate measurements of water use and, hence, TE.

A similar pair of charts to those above were used to show how water stress was affecting the relative ranking of TE among clones (Figure 21). In Experiment 3 TE under wet conditions accounted for 33% (for elevated CO_2) to 47% (for ambient CO_2) of the variation in TE under water-stressed conditions, and the average overall effect of water stress in improving TE was stronger under elevated CO_2 conditions (red arrow). In Experiment 2, the relationships were slightly stronger with TE under wet conditions accounting for 40% and 72% of the variation in TE under stressed conditions, for elevated and ambient CO_2 treatments respectively. This suggests that while TE under well-watered conditions accounts for a substantial proportion of variation in TE under stressed conditions, other sources of variation are also influencing the results. This source of variation could either be through clone x water treatment interactions associated with genetic variation in responses to water stress, or experimental error.

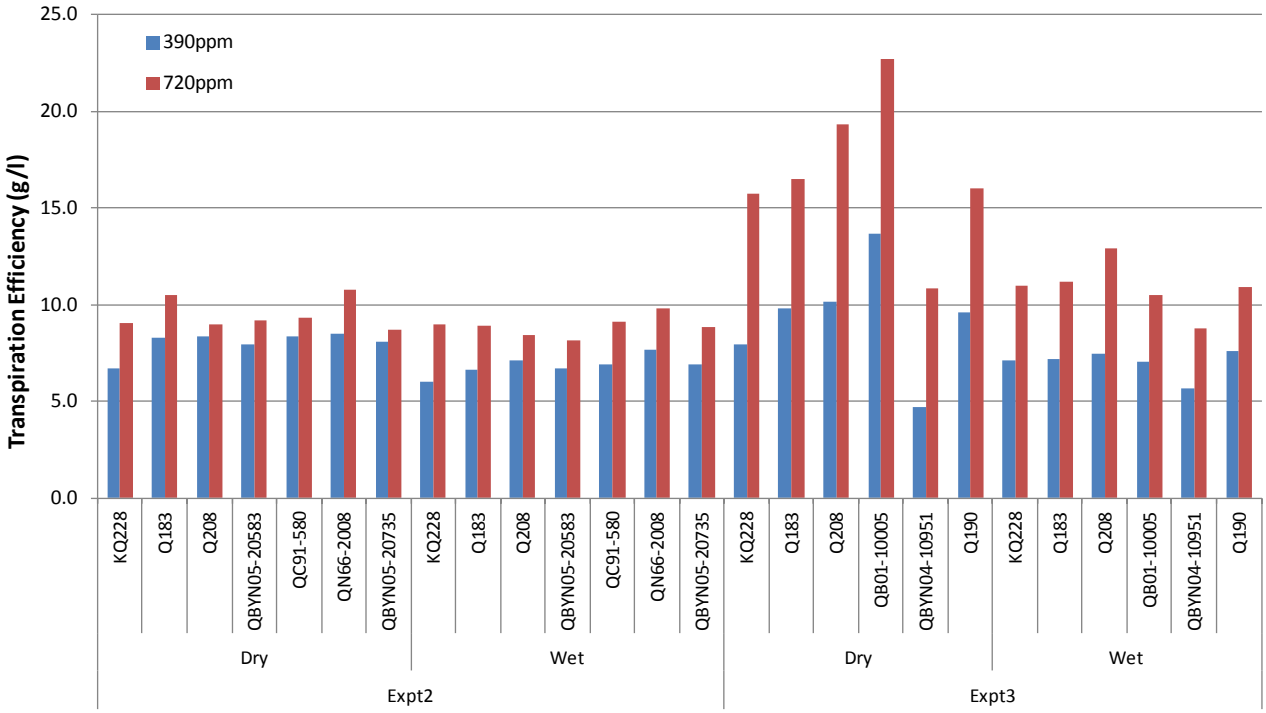

Figure 19. Transpiration efficiency responses to water and CO_2 treatments combinations for each clone in Experiments 2 and 3.

Table 11. Transpiration efficiencies (g dry matter per litre of water transpired) for each water x CO_2 x clone combination in Experiments 2 and 3. The responses of TE to elevated CO_2 are contrasted between well-watered and water-stressed treatments. The standard error (SE) quantifies the genetic variation in TE within each CO_2 x water combination. The top two ranked clones in each category are marked by '+' and the bottom two by '—'.

	Dry			Wet		
Clone	390ppm	720ppm	CO_2 effects (Δ%)	390ppm	720ppm	CO_2 effects (Δ%)
Experiment 2						
QBYN05-20735	8.11	8.74 --	+7.8% --	6.91	8.86	+28.3%
KQ228	6.68 --	9.08	+35.9% +	6.01 --	8.98	+49.4% +
QN66-2008	8.53 +	10.78 +	+26.4% +	7.69 +	9.82 +	+27.8%
Q208	8.36 +	8.97 --	+7.3% --	7.15 +	8.42 --	+17.7% --
Q183	8.32	10.49 +	+26.1%	6.64 --	8.88	+33.7% +
QBYN05-20583	7.93 --	9.21	+16.1%	6.68	8.18 --	+22.5% --
QC91-580	8.33	9.35	+12.2%	6.94	9.12 +	+31.4%
Combined	8.06	9.51	+18.0%	6.83	8.92	+30.6%
SE among clones	0.63	0.79	+26.2%	0.51	0.52	+2.2%
Experiment 3						
Q208	10.90 +	18.99 +	+74.2%	7.70 +	12.61 +	+63.9% +
KQ228	8.21 --	16.21 --	+97.3% +	7.14	11.05	+54.9%
QBYN04-10951	4.83 --	10.92 --	+126.2% +	5.66 --	8.78 --	+55.2% +
Q183	9.97	16.74	+68.0%	7.25	11.22 +	+54.7%
QB01-10005	13.80 +	22.90 +	+65.9% --	7.05 --	10.54 --	+49.6% --
Q190	9.76	16.22	+66.1% --	7.60 +	10.91	+43.6% --
Combined	9.64	17.15	+77.9%	7.15	11.10	+55.2%
SE among clones	2.97	3.92	+32.0%	0.73	1.24	+68.6%

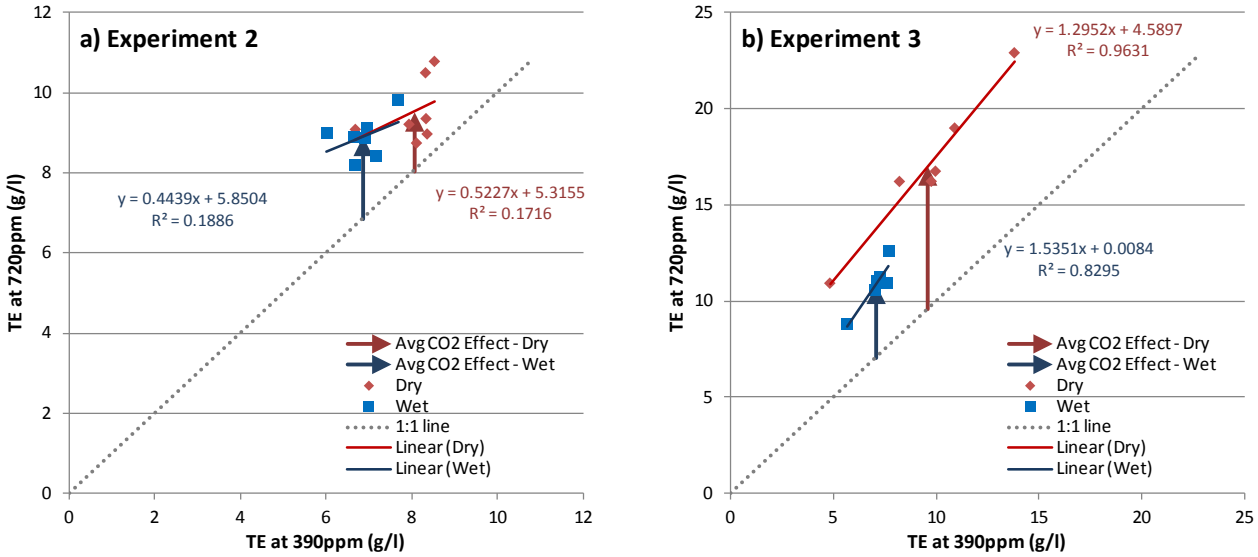

Figure 20. Relationship between transpiration efficiency (TE) at elevated CO_2 (720ppm) and TE at current concentrations (390ppm) for a) Experiment 2 and b) Experiment 3. Each point is the average for a clone (4-8 reps), with the relationships for the two water treatments separated out. Arrows indicate average TE responses to elevated CO_2 (from the 1:1 line) under well-watered (blue) and water-stressed (red) conditions.

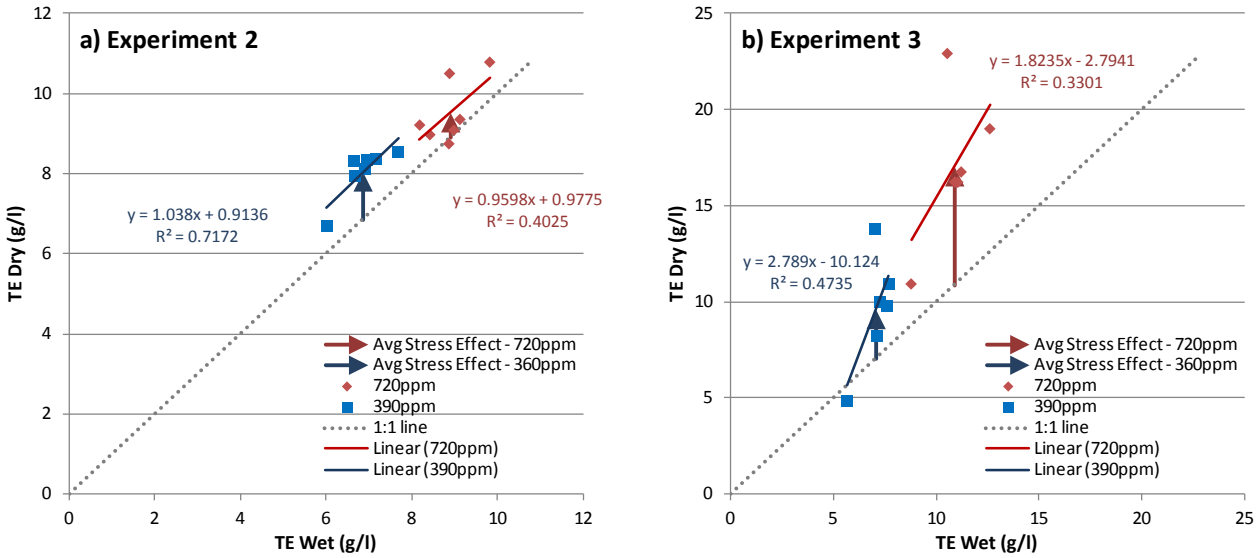

Figure 21. Relationship between transpiration efficiency (TE) under water-stressed conditions and TE under well-watered conditions for a) Experiment 2 and b) Experiment 3. Each point is the average for a clone (4-8 reps), with the relationships for the two CO_2 treatments separated out. Arrows indicate average improvement in TE in response to water stress (from the 1:1 line) under ambient (blue) and elevated CO_2 (red) conditions.

4.3.3 INDICATORS OF FINAL BIOMASS

The final part of the analysis assessed how well final harvest above-ground growth in pots could be related to water use characteristics that could be non-destructively measured while plants were growing. The rationale for this was first that any such non-destructive measurements that could account for variation in final harvest performance could provide a basis for more rapidly screening clones. The other rationale is that identifying characteristics of clones that contribute to final performance would assist in investigating the mechanisms for the genetic variation in response, particularly if these responses could be dynamically monitored, in relation to changing factors influencing water supply and demand, throughout the plant's growth. That in turn could assist in identifying more practically useful functional traits for breeding.

As an integrated measure of plant canopy conductance throughout the trial we calculated the total water transpired in each pot through the trial divided by the final green-leaf area. Results from Experiment 1 showed that leaf area increased uniformly over time for wet treatments, but that in dry treatments leaf area trends could be more erratic (Figure 10). This was probably because green-leaf area depended on the stress level and extent of leaf senescence at the time of measurement, and it was more difficult to apply uniform treatments in the stressed than the well-watered treatments. Also, since plants were watered on demand, whole-plant conductance was likely to be a limiting factor for growth in wet treatments, but less so in dry treatments where water supply was restricted. For both these reasons, water use per unit leaf area was expected to be a better indicator of final growth in the wet treatment. The results show that this expectation was met for well-watered plants in the ambient CO_2 treatment in Experiment 2, with water use per leaf area accounting for 86% of variation in final aboveground growth (Figure 22a). The relationship was weaker in the well-watered treatments in Experiment 3, accounting for 31% of variation in biomass growth in the ambient CO_2 treatment and 52% of variation in the elevated CO_2 treatment (Figure 23). However, in the wet elevated CO_2 treatment in Experiment 2 the relationship was very weak, accounting for only 10% of variation in final aboveground growth (Figure 22b).

In the water-stressed plants, where the supply of water was limiting, TE, rather than conductance, was expected to play a greater role in influencing final harvested aboveground growth. In Experiment 2 there were reasonably strong relationships for plants growing under dry conditions, with TE accounting for 36% and 49% of variation in growth for the ambient and elevated CO_2 treatments respectively (Figure 24). However the relationships were much weaker in the dry treatments of Experiment 3, accounting for less than a quarter of variation in biomass in both CO_2 treatments (Figure 25). It is worth noting the demand-driven watering system was designed to maintain similar levels of stress between treatments and prevent differences in TE being expressed through soil moisture feedbacks. Under conditions where all clones were to receive the same limited supply of water, such as drought conditions in the field, the influence of TE on plant growth would be more fully expressed.

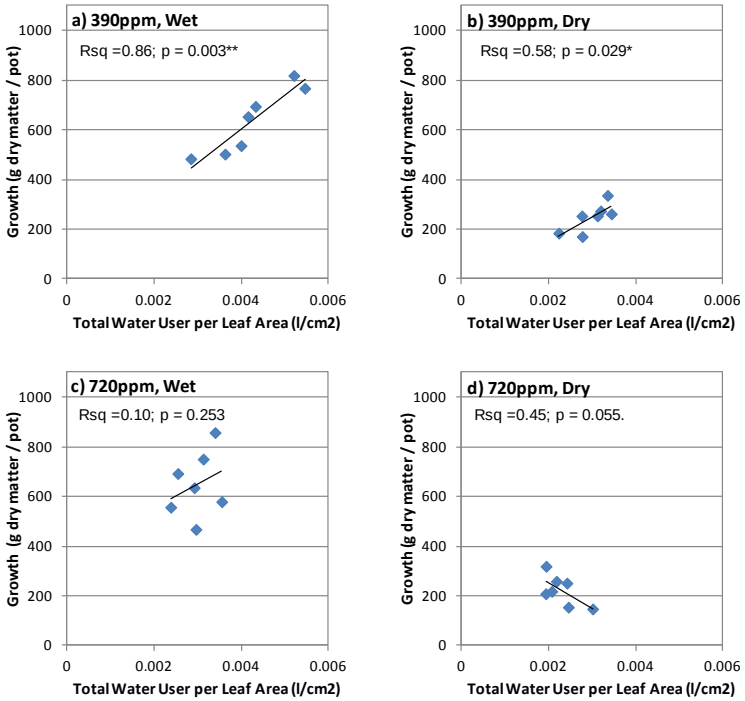

Figure 22. Relationships between water use per leaf area (expressed as the total water transpired during growth divided by the final leaf area at harvest) and aboveground growth for Experiment 2. Each point is the average for an individual clone, and panels separate each of the CO_2 by water treatment combinations.

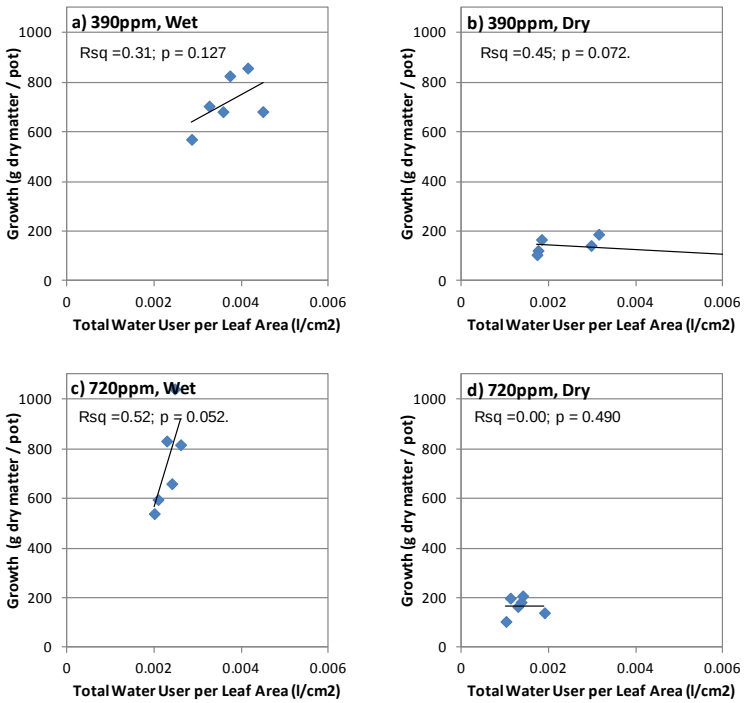

Figure 23. Relationships between water use per leaf area (expressed as the total water transpired during growth divided by the final leaf area at harvest) and aboveground growth for Experiment 3. Each point is the average for an individual clone, and panels separate each of the CO_2 by water treatment combinations.

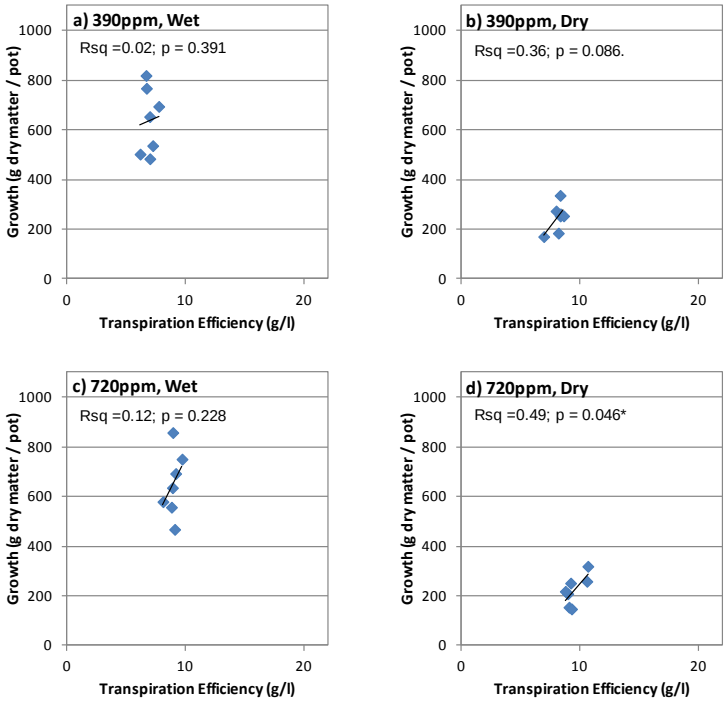

Figure 24. Relationships between transpiration efficiency (TE, expressed as total aboveground biomass growth divided by the total water transpired during growth) and final biomass for Experiment 2. Each point is the average for an individual clone, and panels separate each of the CO_2 by water treatment combinations.

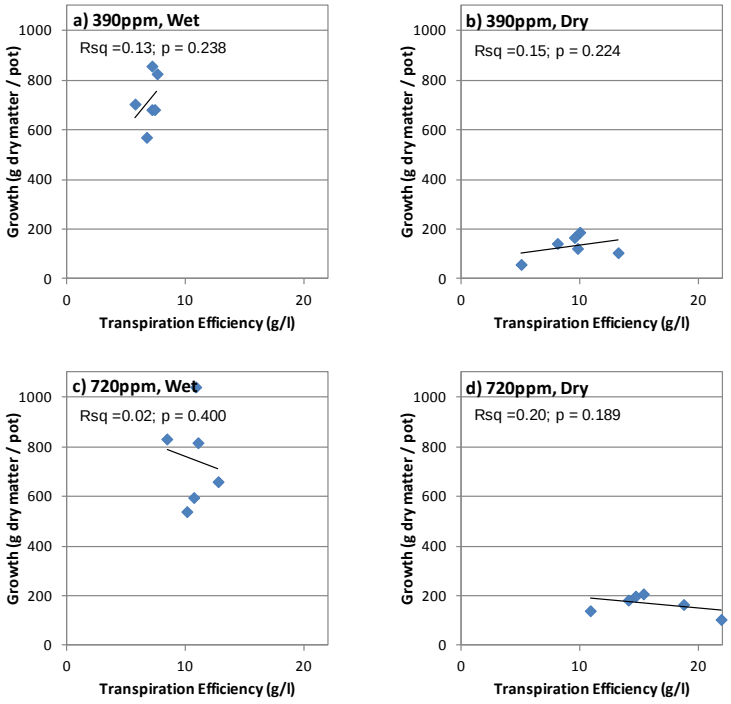

Figure 25. Relationships between transpiration efficiency (TE, expressed as total aboveground biomass growth divided by the total water transpired during growth) and final biomass for Experiment 3. Each point is the average for an individual clone, and panels separate each of the CO_2 by water treatment combinations.

4.4 Discussion

The results of Experiments 2 and 3 provide clear evidence that the predominant influence of CO_2 on sugarcane is through changing patterns of water use, and associated indirect mechanisms of influencing growth. The effects of CO_2 in directly stimulating carbon fixation and growth, if any, are small; less than 0.5% on average across the two experiments (Table 8). This is in line with conventional understanding of basic leaf physiology in C_4 plants (Ghannoum et al, 2000, 2003), but is the first time this has been clearly demonstrated for sugarcane, under both water-satiated and water-stressed conditions. These findings contrast with the strong growth responses observed by de Souza et al. (2008), and would reinforce these authors suggestion that watering frequency was insufficient in these open-top chamber experiments to suppress transient water stress and exclude indirect effects of CO_2.

The demand-driven irrigation system in this project was specifically designed to prevent indirect moisture-mediated responses from occurring. But under water limited conditions in the field the mechanism for these indirect responses would be expected to operate as follows. The proximate effect of CO_2 on sugarcane at the leaf level is primarily to reduce water use (relative to carbon fixation), more so than directly stimulate carbon fixation in the leaf. Instead, reduced transpiration reduces the rate at which water is extracted from the soil, so soils take longer to dry down. This extends the period over which plants grow (and fix carbon) before soils dry to the extent that plants can no longer extract water (wilting point) and photosynthesis ceases. These indirect mechanisms therefore involve moisture-mediated feedbacks between the whole plant and soils linked by water tension/stress and changes in use and availability of soil moisture. This project has also for the first time quantified the improvement in whole-plant TE for sugarcane. This averaged from 31% (under wet conditions) to 55% (under water-stressed conditions) in Experiment 3 for an increase from 390 to 720 ppm CO_2 (Table 10).

In addition, there was evidence that leaves were also influenced by elevated CO_2 (Tables 6, 7 and 9). These effects were most strongly expressed under water-stressed conditions. It is likely that these effects were indirect and we suggest three possible mechanisms (not mutually exclusive) by which leaves might be responding. Lower rates of transpiration under elevated CO_2 reduce evaporative cooling, raising canopy temperatures which could influence leaf growth. Lower stomatal conductance could improve water potentials in leaves and hence the conditions under which cells in leaves grow and expand. Or, alternatively, elevated CO_2 could alleviate stress, altering process of leaf senescence and processes of reallocation of resources between senescing and growing leaves. The most consistent leaf response observed was a 7.5 – 11.5% increase in specific leaf area under elevated CO_2 which, conversely, corresponds with a reduction in leaf dry matter per unit leaf area. Further work would be required to identify the mechanisms for these responses (which was beyond the scope of this project). Changes in leaf morphology have often been observed for the responses of other grasses to elevated CO_2, but these have been associated with overall changes in plant growth.

The main responses of sugarcane to elevated CO_2, as evidenced in these experiments, is primarily through influencing plant water use and, to a lesser extent, by influencing attributes of leaves (leaf width and the mass per unit leaf area). Changes in leaf area were not statistically significant, but increased by 10% ($p = 0.081$, close to significant) in Experiment 2 and 8.3% ($p = 0.201$) in Experiment 3, so the possibility that leaf responses translate into overall changes in canopy cannot be ruled out and warrants further investigation. Genetic variation in water use and canopy development have been identified as two of the most important factors affecting genetic variation in sugarcane performance (More-Crop-Per-Drop). The fact that both are potentially affected by CO_2 suggests that genetic variation in CO_2 response could have important consequences in determining the relative performance among clones in the future.

The results showed clear evidence of genetic variation in water-related responses to CO_2. Results focused on genetic variation in TE, because this was the water use metric that translates most directly into differences among clones in final growth performance under water-limited conditions. There were key aspects to these interactions. The first was how CO_2 influenced the magnitude of genetic variation and amount of separation between TEs of different clones. The results showed that both elevated CO_2 and

drought increased the genetic variation in TE, suggesting that the value of this trait will increase in future CO_2-enriched climates, particularly if water stress conditions are experienced more frequently.

The second aspect to genetic variation in TE was how this affected relative rankings among clones. In Experiment 3, in which we had most confidence in water-use related measurements, the amount of correspondence in TEs between current and future CO_2 conditions was remarkably strong: TEs under ambient CO_2 accounted for 83% (wet conditions) and 96% (water-stressed) of genetic variation under elevated CO_2. This is too small a number of clones (6) to extrapolate across the full genetic diversity of sugarcane, or even that within the Australian sugarcane breeding program, at this stage. But if the result were to hold up generally it would have the important implication that screening for TE at present, would be sufficient to ensure that this trait was still expressed in the future, at which time it would be of greater benefit (from preceding paragraph). The strong correlation may be an inadvertent consequence of screening approaches within the Australian breeding program and the specific sub-set of genetic variation this has selected. Many traits are highly correlated with each other so it is common that breeding approaches that favour one target trait can simultaneously alters other non-target traits, favourably or unfavourably, within the selected population. There is therefore no guarantee that varieties that are selected for current climates will continue to be the best varieties in future climates, and inadvertent selection at present for traits that affect future performance could just as easily have negative consequences as positive ones. If the results from Experiment 3 are a consequence of the particular pool of genetic variation that has been selected in Australian breeding, then this indicates, for TE at least, that such potential maladaptation has not occurred and that selecting high TE clones within this breeding population will continue to have benefits in the future. The results for Experiment 2 showed much weaker correspondence, with TEs at ambient CO_2 accounting for less than 20% of genetic variation in TEs under elevated CO_2. It is not clear whether the unexplained variation was because of genetic interactions in CO_2 in responses of the different set of clones used (where differing CO_2 responses were changing the ranking of TE among clones), or because of additional experimental error associated with the lower accuracy of measuring water use in Experiment 2 (relative to Experiment 3). Whatever the case, it would be prudent to expand these experiments to a broader range of clones before extrapolating this finding too far.

Similar analyses to those for CO_2 interactions above were also applied to water treatments, testing whether TEs under well-watered conditions were a good indicator of TEs under water-stressed conditions. In this case the relationships were stronger in Experiment 2, accounting for 72% (under ambient CO_2) and 96% (under elevated CO_2) of genetic variation in TE under stressed conditions. The corresponding amounts of genetic variation explained in Experiment 3 were 47 and 33%. This again would suggest that screening for high TE under well-watered conditions, at least in the first instance, would also select for high TE under stressed conditions. The same condition as before would apply: these results would have to be replicated over a broader range of clones to have greater confidence in the findings and their application.

The experiments in this project have provided some novel results with important implications for breeding programs now and in the future. They have clearly demonstrated that direct effects of elevated CO_2 on sugarcane growth, if any, are small, and that predominant effects are on TE, an important trait for breeding. They have also shown that leaves are affected by elevated CO_2, and there are potential implications for canopy development, another important trait, that needs further investigation. While there were large differences in relative responses of clones to CO_2 in terms of the percentage increase in TE (from +7% to +126%), these may follow a predictable pattern that does not alter the relative ranking among clones. In the process of designing and building the equipment to conduct these experiments, we have developed a technical resource that is far more capable than originally intended and will be able to assist in further exploring the mechanisms that account for differences in performance between clones, particularly for water-related traits. In particular, it has often proved difficult to translate differences in leaf-level responses among clones to differences in their final harvest performance. Being able to dynamically monitor whole-plant patterns of water use and relate these functionally to experimentally-controlled changes in factors controlling the supply and demand for water (e.g., water stress, CO_2, VPD), may provide an intervening step that links leaf-level mechanisms, through whole-plant processes, to final plant performance.

5 Incorporating CO_2 effects into sugarcane modelling

Note: this chapter is based on Stokes et al. (2014) (listed under 6.7), with only slight modification.

5.1 Introduction

One of the key aims of our experiments has been to determine the mechanisms by which rising atmospheric CO_2 is affecting sugarcane. In particular, the experiments have been designed to separate the direct (proximate leaf-level) responses from indirect responses (involving whole plant-soil interactions mediated by water use, stress and availability of soil moisture). Brazilian work using open top chambers found a 40% increase in sugarcane biomass for a doubling of CO_2 under well watered conditions (de Souza et al. 2008). This implied that there could be a direct leaf-level stimulation of photosynthesis, a result that even the authors found hard to explain for a C_4 plant. An important finding from our experiments has been to resolve this issue and show direct effects of elevated CO_2 on sugarcane are very small. Most of the effects of CO_2 are indirect, mediated by changes in stomatal conductance, and the consequent changes in how whole plants interact with soils through changes in water use, availability and tension/stress.

However, to date, most modelling approaches to representing the effects of rising CO_2 on sugarcane have included both direct effects (represented as increases in radiation use efficiency: RUE) and indirect effects (represented by changes in transpiration efficiency (TE), where these changes would have subsequent flow on effects through changing patterns of water use). Webster et al. (2009) assumed that intrinsic transpiration efficiency (TE) as defined by Sinclair et al. (2012) and Keating et al. (1999) would increase by 8% for every 100 ppm increase in CO_2 when estimating sugarcane yields for future climates in Australia, using the APSIM-Sugarcane model (Keating et al., 1999). Weber et al. (2009) also assumed that radiation use efficiency (RUE) would increase by 1.43% for every 100 ppm increase in CO_2 concentration. The assumptions about TE and RUE for sugarcane came from an internal report by Park et al. (2007). Biggs et al. (2012) used the same model and assumptions to predict yield and off-site impacts in future climates, for one sugarcane region. Responses of intrinsic water use efficiency (WUE) to twice normal CO_2 measured in small cuvettes, supported the Weber et al. (2008) assumption about WUE in one case (Vu et al., 2009) but not in another where WUE increased 62% in twice normal CO_2 (de Souza et al., 2008). The modest increase in RUE assumed by Webster et al. (2009) would not account for the 40 % increase in biomass for well watered plants in twice normal CO_2 (de Souza et al., 2008). de Souza et al. (2008) also found difficulty in explaining this result and suggested that even though plants were irrigated when soil water was at a low tension of 20 kPa, plants in normal CO_2 must have experienced water stress which those in elevated CO_2 did not. One would need to provide a different water regime for plants growing in high levels of CO_2 for them to experience the same degree of water stress to plants growing in normal CO_2 levels, as we have now done in the experiments presented in this report.

Many studies on the effect of elevated CO_2 on biomass yield have been conducted on potted plants in glasshouses (GH) or on plants growing in open top chambers (OTC). Biomass yield responses in these conditions were about twice the responses measured in free-air CO_2 enrichment (FACE) experiments, for C_3 species (Ainsworth and McGrath, 2010). The most common elevated CO_2 treatment used in enclosed environment research is 720ppm (double ambient levels at the time this was initially set), but this is too expensive to maintain in unenclosed conditions so FACE experiments usually use a level of 550ppm (about

half the step increase from ambient levels). Tubiello et al. (2007) reviewed the literature on GH, OTC and FACE experiments and concluded there was broad agreement between the different techniques for estimates of the impact of elevated CO_2 on crop yield. They found that the results from most crop model simulations were consistent with the results from FACE experiments, when measurements from different types of experiments were appropriately incorporated into crop models. To date no FACE experiments with sugarcane have been published and, while the GH and OTC experiments with sugarcane indicate there may be substantial benefits for this crop in a future enriched CO_2 environments (de Souza et al., 2008, Ainsworth and McGrath, 2010), Ghannoum et al. (2000) suggested more research was required to find out how this could be achieved with a C_4 photosynthetic pathway already saturated with CO_2. The experiments described in the previous sections of this report provide an important evidence base for improving how CO_2 effects are simulated in sugarcane models.

Two sugarcane modelling platforms are available internationally for sugarcane, Canegro in the DSSAT platform (Inman-Bamber et al., 1993; Kiker et al., 2002) and 'sugar' in the APSIM platform (Keating et al., 1999). Both have been used to predict the impact of climate change, including CO_2 enrichment, on sugarcane yield (Park et al., 2007; Webster et al., 2009; Marin et al., 2013; Knox et al., 2010 and Biggs et al., 2013). None of these modelling studies appeared to use experimental evidence for assumptions about the effects of CO_2 on sugarcane yield-building processes. The experimental components of this project specifically involved decoupling the direct leaf-level effects of increased atmospheric CO_2 concentration on net photosynthesis (and hence biomass accumulation), from the indirect effects mediated through altered transpiration and improved plant water status, so that these processes could be better represented in sugarcane models. FACE experiments in sugarcane would be expensive to do, and do not allow separating proximate leaf-level CO_2 responses from those mediated by whole-plant-soil hydrological feedbacks, so we used an approach that is better suited to testing and measuring the mechanisms that should be represented in crop models (Chapters 3 and 4).

In this part of the project we made advancements in improving ways to simulate the effects of CO_2 enrichment on sugarcane growth and water use. We developed a model (from existing ones) that could make use of the leaf level results obtained in Experiment 1, to test what would happen in a field of sugarcane subjected to rising CO_2, based on the simulated linked, flow through effects of elevated CO_2 on conductance, water use, soil moisture and plant growth. Results from the TPF experiments on the effects of CO_2 on biomass yield, transpiration and stomatal conductance were used to decide the mechanisms of response, how these mechanisms would be represented in the model, and, together with other published sources of information, the magnitudes of the effects used to parameterise the model. (Experiments 2 and 3 had not been fully analysed when this modelling work took place, so parameterization of the magnitude of CO_2 effects only used results from Experiment 1.) Unlike previous approaches, we do not assume any direct stimulation of RUE by elevated CO_2, but represent the CO_2 effect entirely in terms of improved TE, using a Penman-Monteith approach to account for subsequent changes in gas and energy fluxes. The model was then applied to simulate the responses of a sugarcane crop under field conditions. These crop simulations used a previous field experiment, the Bowen ratio energy balance (BREB) experiment by Inman-Bamber and McGlinchey (2003), to parameterise the model for current climate conditions. The results were then compared to simulations where the effects of elevated CO_2 were added.

5.2 Methods

5.2.1 FIELD EXPERIMENT

The Bowen ratio energy balance (BREB) field experiment (Inman-Bamber and McGlinchey, 2003) was not part of the current project, but is briefly described here because it was used to set up the model for baseline conditions under current CO_2 levels.

A BREB system was set up in a 10.3 ha commercial block of sugarcane (cv. Q127, first ratoon) at Kalamia estate (19.6 °S, 147.4 °E), near Ayr in the Burdekin district, north-east Australia. The details and results of BREB system were provided by Inman-Bamber and McGlinchey (2003) and only brief details are repeated here. The crop was ratooned (allowed to regrow) after harvesting the plant crop on 23 August 2000 and was irrigated and fertilized according to industry recommendations for achieving potential yields. On 22 October 2000, four recently calibrated tube solarimeters (1 m long) were placed on the soil surface in two places near the BREB installation, to span the 1.8 m dual crop row configuration exactly. Two more tube solarimeters were mounted above the canopy so that fraction of intercepted radiation could be determined.

On 17 September 1998, an automatic weather station (AWS) was installed in an open grassed area about 1 km from the BREB system at Kalamia. All components were described by Inman-Bamber and McGlinchey (2003). AWS data were used to determine daily reference evapotranspiration (ET_0) from Allen et al. (1998).

Total above ground biomass was determined on seven occasions during the development of the crop at Kalamia. All plant material was removed from one 18 m^2 quadrat in each of four sampling sites on each occasion. Shoots and stalks were counted and then weighed altogether. A sub sample of stalks was also weighed and then partitioned into green leaf, sheath plus immature stem, mature stem and dead leaf components. A sub sample of each of these components was weighed and then dried to constant mass in a forced draught oven set at 80°C. Stalk and crop heights were estimated based on a stalk diameter of 23 mm, and leaves extending an additional 2 m above the stalks.

5.2.2 MODELLING THE FIELD EXPERIMENT AT CURRENT AND FUTURE CO_2 LEVELS

Canegro and APSIM-sugar differ considerably in regard to the transpiration process. Potential transpiration (T_O) in APSIM is determined by the amount of radiation intercepted (R_I), RUE, TE and the vapour pressure deficit (VPD) (Eq. 1). Actual transpiration (T_A) is limited either by T_O or the rate of root water supply to the crop (W_R) (Eq. 2). The ratio of T_A to T_O (0 to 1) is the measure of water stress affecting biomass gain directly and leaf expansion proportionally (Keating et al. 1999).

$$T_O = RUE * R_I * VPD / TE \quad (1)$$

$$T_A = \min(T_O, W_R) \quad (2)$$

Older versions of Canegro use the Penman-Monteith (PM) equation in a procedure which includes a daily estimate of the canopy height (Z_c) and leaf area index (LAI) (Inman-Bamber et al., 1993) and newer versions

use the FAO56 approach (Allen et al., 1998) based on crop factors (Singels et al., 2008). WaterSense is a web-based irrigation scheduling service based on a model using the most appropriate components of APSIM and Canegro for the purpose of helping sugarcane farmers to manage irrigation (Haines et al., 2008). For this study we used WaterSense logic (Armour et al., 2012) but we replaced the crop coefficient approach with the one in which evapotranspiration estimates vary with Z_c and leaf area index (LAI) as reported by Inman-Bamber and McGlinchey (2003) and Inman-Bamber et al. (1993; 2005). In this procedure latent heat flux is derived from the Penman-Monteith equation (Eq. 4) and functions for wind speed, canopy and aerodynamic resistance (Eqs. 3, 5 and 6). This was done to allow estimates of leaf conductance obtained from the glasshouse study to be scaled up to an estimate of canopy conductance of a field crop.

Wind speeds were adjusted for the height of the canopy using the wind profile equation (Monteith and Unsworth, 1990; Inman-Bamber and McGlinchey, 2003):

$$u_2 = u_1\left[\frac{\ln((z_2 - d_r)/z_{0r})}{\ln((z_1 - d_r)/z_{0r})}\right] \quad (3)$$

where:

u_1 and u_2 = wind speed at 2 and 10 m (m s^{-1})

z_1 and z_2 = heights 2 and 10 m above the ground

d_r = zero plane displacement of reference surface = 0.07 m

z_{or} = roughness length of reference surface = 0.013 m

Latent heat flux from sugarcane transpiration is:

$$\lambda T_{cane} = \frac{\Delta(R_n - G) + \rho c_p VPD_2 / r_a}{\Delta + \gamma(1 + r_c / r_a)} \quad (4)$$

and canopy resistance is:

$r_c = r_s/(0.5LAI)$ (5)

and aerodynamic resistance is:

$r_a = (\ln((z_2-0.7z_c)/0.026z_c))^2 / (u\kappa^2)$ (6)

The factor (F) for transpiration at future, relative to current, levels of CO_2 is (Tubiello et al. 2000):

$$F = \frac{(\Delta + \gamma(r_{c0} + r_a)/r_a)}{(\Delta + \gamma(r_{cf} + r_a)/r_a)} \quad (7)$$

where:

T_{cane} = Potential transpiration for sugarcane (mm d^{-1})

Cp = specific heat of air at constant pressure (J kg^{-1} K^{-1})

Δ = slope of the vapour pressure curve (kPa°C^{-1})

G = soil heat flux density (MJ m^{-2} day^{-1})

γ = psychrometric constant (kPa°C^{-1}).

κ = von Karmans's constant = 0.41

λ = latent heat of vaporization of water (J kg^{-1} $^0C^{-1}$)

R_n = net radiation (MJ m^{-2} d^{-1})

ρ = air pressure (kPa)

r_a = aerodynamic resistance (s m^{-1})

r_c = canopy surface resistance (s m^{-1})

r_{c0} = r_c at current CO_2 levels

r_{cf} = r_c at future CO_2 levels

r_s = leaf resistance (s m^{-1})

Z_c = sugarcane canopy height (m)

Thus T_{cane} is responsive to changes in LAI, crop height and leaf (mainly stomatal) resistance (1/conductance). Here we are concerned only with the effect of CO_2 on stomatal resistance (r_s) even though this may influence LAI and height (Z_c) development indirectly through water supply and demand. McGlinchey and Inman-Bamber (1996) standardized LAI at 3.5 and r_s at 100 s m^{-1} to account for daily evapotranspiration as measured in large weighing lysimeters. For these simulations we used the latter value for r_s for CO_2 levels (~325 ppm) at the time of the lysimeter measurements in the late 1960's (Thompson 1986). r_s was allowed to increase at 12 s m^{-1} for every 100 ppm increase in CO_2 based on the glasshouse experiment results (presented below). LAI and Z_c were allowed to vary with crop development but a maximum of 5 m was allowed for Z_c because of the tendency for sugarcane crops to lean or lodge when individual plants are longer than 5 m.

Evaporation from the soil (E_S) was derived as in WaterSense (Armour et al., 2012); based on the depth of water in the top soil layer in excess of the depth remaining after air drying (term 1 in Eq. 7); and the fraction of radiation reaching the soil surface (remaining terms) (Eq. 8).

$$E_S = E_0[(\min((\theta_C - \theta_{AD})/((\theta_S - \theta_{AD}),1.0))^3 (0.05+\exp(-0.38LAI) - c) - 0.1(1-\exp(-0.38LAI)) + 0.1] \quad (8)$$

where E_O is reference evapotranspiration (Allen et al., 1998); θ_C, θ_{AD} and θ_S are water contents for the soil on the day of calculation (θ_C), for air-dried soil (θ_{AD}) and for saturated soil (θ_S) and c is the fraction of the soil surface covered by cane residue (trash); c = 0 for this simulation (burnt cane).

Sugarcane actual evapotranspiration (ET_c in mm d^{-1}) is the sum of soil evaporation and potential transpiration or root water supply whichever is the least (Eq. 9).

$$ET_c = E_s + \min(T_{cane}, W_R) \quad (9)$$

The CO_2 level used in our simulations was 375 ppm (r_s = 106 m s^{-1}) which was the 'current' level at the time of the BREB experiment by Inman-Bamber and McGlinchey (2003). The 'future' CO_2 concentration was chosen as 720 ppm (r_s = 148 m s^{-1}) to correspond with several experiments including ours, where the high CO_2 treatment was set at this level.

LAI was determined as in the APSIM-Sugar model (Keating et al., 1999) with leaf characteristics for the variety Q172 as in Table 12. APSIM interpolates between inflection points defined as 'x' and 'y' parameters (Table 12). For example the maximum area is 50 cm^2 for leaf #1 and it increases linearly to 500 cm^2 for leaf #12 and remaining leaves.

Table 12. Traits parameters for variety Q172 for determining leaf area index (LAI) and biomass gain as in the APSIM-Sugar model (Keating et al., 1999) and maintenance respiration as in Canegro (Singels and Bezuidenhout, 2002).

Trait/APSIM term	Parameters				Units
Shoot population	10				per m^2
Thermal time for sprouting	100				Heat units (base 9°C)
Planting depth	100				(mm)
Maximum green leaf number per stalk	8				
Leaf number / xleaf_size	1	12	40		
Leaf area / yleaf_size	50	500	500		cm^2
Leaf number / xleaf_till	1	4	10	16	
Leaf area multiplier for tillers	1.5	1.5	1.5	1	
Leaf number/ xleaf_pchron	1	20	30	40	
Phyllochron	90	140	150	160	Heat units (base 9°C)
Maintenance respiration fraction (*M*)	0.004				
Assimilation stress temperature /x_ave_temp	5	15	35	50	°C
Assimilation temperature stress factor (S_A)/ y_stress_photo	0	1	1	0	

Dry above ground biomass accumulation (△B) was also based on the APSIM-Sugar model and the Canegro term for maintenance respiration (Eq. 10).

$$\triangle B = \max(((W_R/T_{cane})(R_I.S_A.RUE) - M.B), 0.0) \quad (10)$$

5.3 Results

The simulated fraction of solar radiation (400 to 7000 nm) reaching the soil surface was similar to what was observed using the tube solarimeters (Figure 26). A close approximation of radiation levels at the soil surface was important for the correct partitioning of latent heat flux between evaporation from the soil (Eq. 8) and transpiration from the canopy.

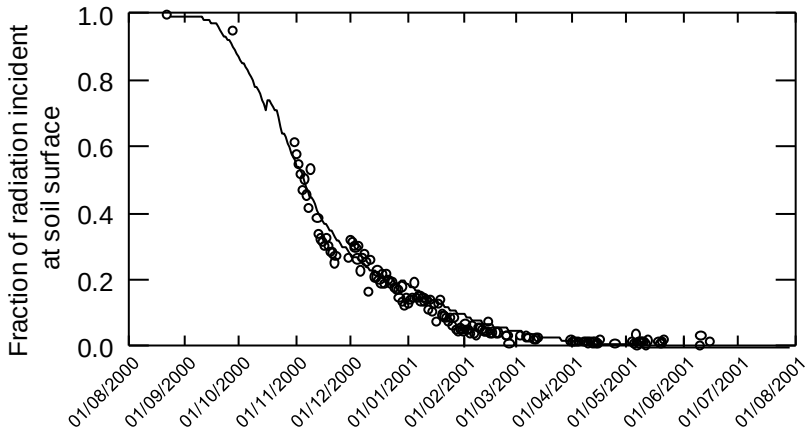

Figure 26. Measured (○) and simulated (—) fraction of solar radiation reaching soil surface.

Simulated ET_c provided a good approximation of ET_c measured with the BREB system (Figure 27). The process for deciding if ET_c measurements logged at 20 min intervals were deemed acceptable or not depending on wind direction and the resolution of the sensors (Inman-Bamber and McGlinchey, 2003). If all 40 ET_c readings between 06:00 and 19:00 on a given day were acceptable, then the acceptance rate for cumulative ET_c on that day was 100%. The correlation between simulated and measured daily ET_c was high (R^2 = 0.65, n=127) when an acceptance rate of 80% was used for measured ET_c and was very high (R^2 = 0.83, n=41) when the acceptance rate was 90%. Thus the more reliable the measurements, the closer they were to those estimated by the model. In this case one would rely more on the model than the measurements when their acceptance rates were low.

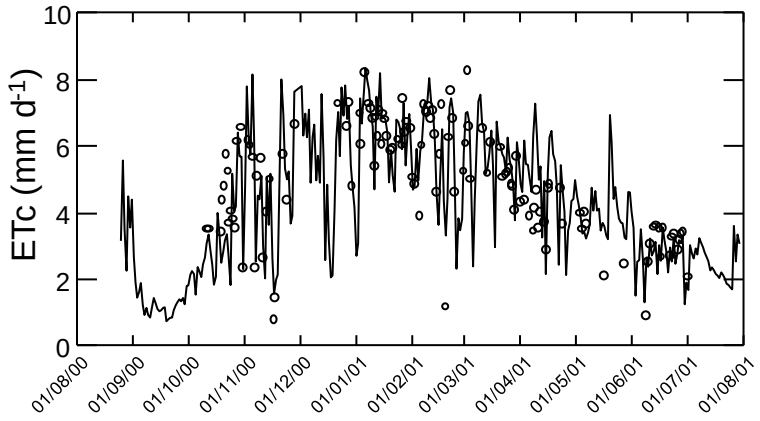

Figure 27. Measured (○) and simulated (—) evapotranspiration (ET_c). Acceptance rate for measurements as defined by Inman-Bamber and McGlinchey (2003) was 80%.

The simulation of biomass yield was close to the yield observed by means of the seven sample harvests conducted during the growth of the crop. Observed biomass yield was similar to simulated biomass yield for three of the harvest samples and was lower than simulated yield for one sample and higher for three of the samples (Figure 28). Although simulations of elevated CO_2 made little difference to biomass yield because this crop was well irrigated, biomass yield was 8% greater with elevated CO_2 than without it when the crop was young due to a short period of water stress in October 2000, and it was 3% greater at harvest (August 2001), also because of water stress caused by the drying off process which limited water availability to the crop. Reduced water use due to elevated CO_2 resulted in more water being available during the stress periods (Figure 29).

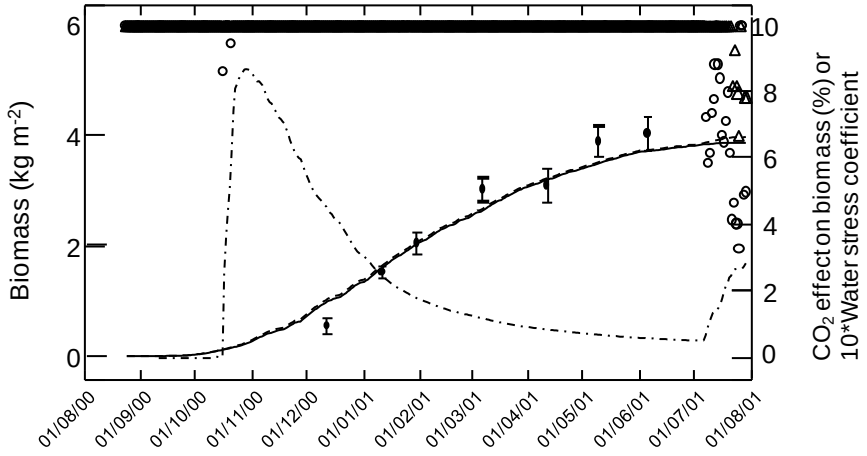

Figure 28. Measured (•) and simulated crop dry biomass at current (—) and future (- - -) CO_2 levels, simulated response (future CO_2/current CO_2 biomass* 100) to increased CO_2 (-·-·-) and the water stress coefficient (W_R/T_{cane}) under normal (○) and elevated CO_2 (△). Bars are 2 x standard error of the mean (measurements are from Inman-Bamber and McGlinchey, 2003).

Simulated LAI corresponded with measured LAI when this was determined for the first time in January 2001 (Figure 29). LAI observed in March and May also supported the assumptions about canopy development

processes in the model. Measurements of LAI in April and June were lower than simulated LAI. The low LAI in April was unexpected but the reduction in LAI in June was possibly due to drying off prior to harvesting – a process not reflected in the simulation until July (Figure 29). Canopy development would have benefitted from elevated CO_2 to only a small extent during the short-lived stress period in October 2000 (Figure 29).

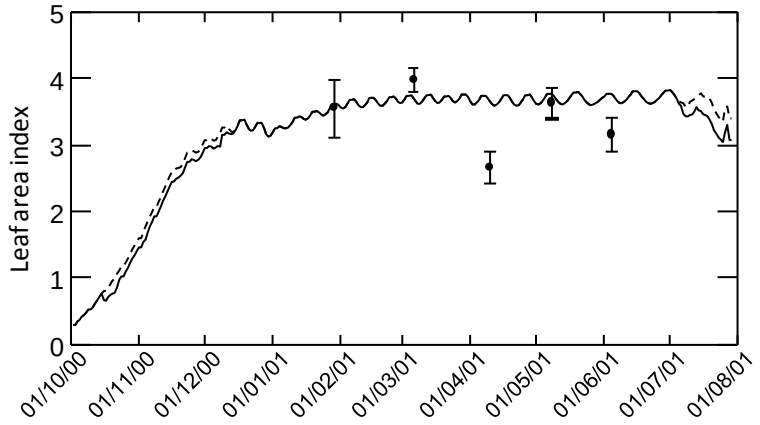

Figure 29. Measured (•) and simulated leaf area index (LAI) at current CO_2 levels (—) and future (- - -) CO_2 levels. Bars are 2 x standard error of the mean (measurements are from Inman-Bamber and McGlinchey, 2003).

The simulation of stalk length was realistic (Figure 30). Crop height (stalk height plus leaf length) was not measured as such but a maximum canopy height of 5 m was realistic given the 4 m height of the scaffolding built to service the BREB sensors and observations that leaves extended only about 1 m above that platform when the crop was at its tallest.

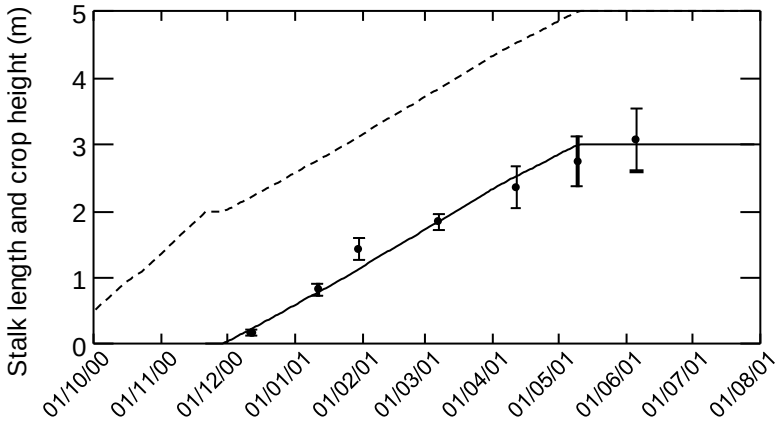

Figure 30. Observed stalk length (•) derived from measured fresh stalk mass and an estimate of stalk diameter and simulated stalk length (—). Simulated crop height (- - -) accounts for the approximate 2 m that leaves extend above the top of the stalks.

The ratio (F) of transpiration at 720 ppm CO_2 relative to transpiration at 375 ppm CO_2 was as low as 0.7 when LAI was small and F increased to a range of 0.8 to 0.95 (Figure 31) as LAI reached 3.5 and crop height 5 m (Figures 29 and 30). F increased to as much as 1.5 during the drying-off period in July because high CO_2 and consequently reduced transpiration, increased the amount of water stored in the soil which could then

be transpired during this period more readily than the crop subjected to low CO_2. When LAI and canopy height (Z_c) are small, r_a is small and the effect of CO_2 on leaf resistance (r_s) has a large flow-on effect on transpiration (Eqs. 4, 5 and 7). When LAI and Z_c are large, r_a is relatively large so the impact of elevated CO_2 on transpiration is reduced when water is not limiting.

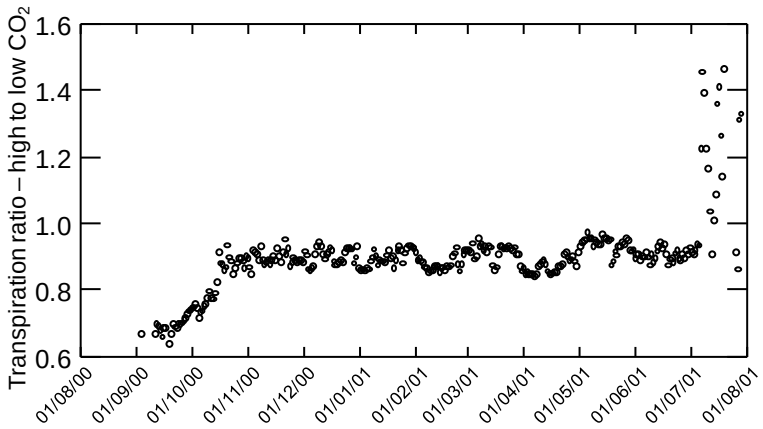

Figure 31. Simulated ratio (*F*) of transpiration at 720 ppm CO_2 relative to transpiration at 375 ppm CO_2 for the duration of the field experiment, as the plants mature and the canopy closes.

The ratio of ET_c to ET_0 is the crop coefficient (K_c) as defined by Allen et al. (1998). Measured and simulated K_c were similar and in agreement with maximum K_c = 1.25 as in Allen et al. (1998) and Inman-Bamber and McGlinchey (2003). However it is clear from the measurements and simulation that K_c for sugarcane is not constant but varies with water content at the soil surface, with crop height and with LAI and with climatic factors (wind speed). Simulated and measured Kc varied mostly between 1.0 and 1.5 indicating that irrigation scheduling based on simple models using constant Kc values could be flawed particularly if irrigation is applied daily in sandy soils. LAI for sugarcane in the BREB experiment was as high as 4.0 compared to an effective LAI=1.44 for grass in the reference ET_0 calculation (Allen et al., 1998). Crop height exceeded 4 m for sugarcane in the BREB experiment compared to a grass height of 0.12 m in the ET_0 calculation (Allen et al., 1998). Stomatal resistance for grass (Allen et al. 1998) and sugarcane was similar (100 and 106 s m^{-1}, respectively) at current CO_2 levels and was 148 s m^{-1} for sugarcane at future CO_2 levels and this increase caused a slight reduction in K_c for sugarcane growing at twice normal CO_2 (Figure 32).

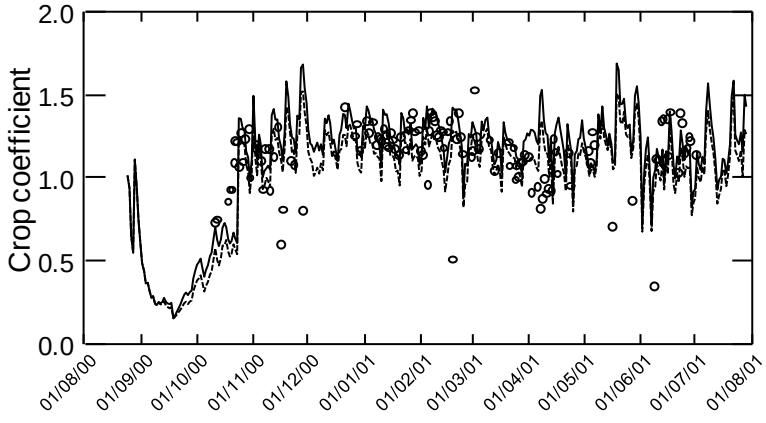

Figure 32. Measured (○) and simulated crop coefficient (K_c) at current (—) and future CO_2 levels (----).

5.4 Discussion

The glasshouse experiments identified the predominant mechanism by which CO_2 influences sugarcane growth by isolating direct from indirect effects. These experiments provided evidence that any direct stimulation of sugarcane growth by elevated CO_2, from direct proximate leaf-level mechanisms, is likely to be small. This suggests that reported increases in yield from previously published CO_2 experiments (Vu et al., 2006; de Souza et al., 2008) are more likely to be largely due to indirect mechanisms related to improved water relations (the alleviation of water stress and prolonged soil water availability) even if water was thought to have been non-limiting during those experiments. The modelling framework presented here therefore used only these indirect, water-related effects, as identified in the Experiments 2 and 3, together with information from the Experiment 1, supplemented with other literature, to quantify and parameterise the strength of this effect.

Interestingly even without any direct effect of CO_2 on photosynthesis, the modelling of the BREB experiment showed a biomass yield advantage as high as 8%, early in the accumulation of biomass by the crop (Figure 28) which we thought at the time was irrigated adequately. However the final yield of the BREB experiment would have increased only by about 3% at 720 ppm CO_2 according to the simulation. Marin et al. (2013) simulated a 10% increase in fresh cane yield with 750 versus 380 ppm CO_2 in their study using climatic conditions in Sao Paulo state, Brazil where irrigation is generally not practiced but rainfall is high. Their model was DSSAT/Canegro which uses a crop factor approach based on the Penman-Monteith formula to determine grass reference evapotranspiration (ET_0) where canopy resistance for ' grass' was allowed to vary with CO_2 (Marin et al., 2013). However sugarcane evapotranspiration in their model could not be influenced by the interaction between CO_2 concentration, crop height and LAI as we suggest it should be. Simulation of the BREB experiment with 375 and 720 ppm CO_2 indicated that transpiration could be reduced by about 30% when the crop is small but only by about 10% when the canopy is closed and the crop is tall (Figure 31). Similar simulations were produced by Morison and Gifford (1984b) who plotted *F* against the ratio of r_a to r_s for different responses of r_s to the doubling of CO_2 concentrations. *F* was as low as 66% (when r_a/r_s was small, 0.04) and was as high as 0.90 (when r_a/r_s was 0.96 and stomatal conductance was reduced by 36%) for a doubling of CO_2 concentration. These authors point out, as we do, that the effect of stomatal closure on transpiration depends on the ratio of stomatal to boundary layer or aerodynamic resistance. Only when stomatal resistance is large relative to aerodynamic resistance is the reduction in stomatal conductance with increased CO_2 concentration reflected as a reduction of similar magnitude in transpiration (Morison and Gifford 1984b). In our case when simulating the BREB experiment, a reduction of 29% in stomatal conductance (a 40% increase in r_s) resulted in a 30% reduction in transpiration only when the crop was small (Figure 31).

If changing levels of CO_2 affect crop growth only through alleviation of water stress then clearly the degree of water stress encountered by the crop under current CO_2 conditions will influence the degree to which the crop responds to CO_2. Inman-Bamber and Smith (2005) show how sensitive sugarcane is to water stress in terms of expansive growth. Inman-Bamber et al. (2009) showed how an increase in temperature for plants growing in a glasshouse can cause a reduction in leaf extension at midday presumably through water stress even when plants have water 'on demand' as was the case in our glasshouse experiment. Depending on the variety, hourly photosynthesis may not be reduced at all or by 50% at most by water stress which is sufficient to stop leaf extension (Inman-Bamber et al. 2008) so it was not surprising that our glasshouse grown plants did not respond to elevated CO_2 in terms of increased biomass. Growth of leaves of *Panicum coloratum* growing in controlled environment chambers was greater when elevated CO_2 (1000 ppm) decreased stomatal conductance and transpiration under high VPD conditions, even though soil water content was maintained at 100 % (Seneweera et al., 1998). These authors concluded that greater CO_2 concentrations allow C_4 grasses to maintain better internal water relations by reducing transpirational water losses and so allow expansion of these species into more arid climates. In a simulation study on possible sugarcane yields in Ghana, Black et al. (2012) found that a doubling of atmospheric CO_2

concentration offset a 20% increase in demand for irrigation associated with a 4°C rise in temperature. The model used a simple approach for representing direct effects of CO_2 on plant water use whereby changes in stomatal conductance were assumed to be inversely proportional to changes in CO_2 level (e.g. a halving of conductance for a doubling of CO_2: stronger than the actual measured responses for sugarcane summarised in the paragraph below).

A comprehensive review on the effects of elevated CO_2 on C_4 species by Leakey (2009) sides with conclusions by Ghannoum et al. (2000) that C_4 photosynthesis could only be stimulated by elevated CO_2 either directly, when internal CO_2 concentration (Ci) is below about 100 ppm or indirectly, when reduced stomatal conductance stimulated photosynthesis via altered water relations or energy balance. Of the 220 Ci measurements (not published) on sugarcane growing at normal CO_2 levels, taken by Inman-Bamber et al. (2008) only seven Ci readings were less than 100 ppm; six of these were for water stressed plants. Mean Ci was significantly lower (120 ppm) for the dry treatments compared to the wet treatment (198 ppm, p=0.014). Thus both direct and indirect mechanisms could be operating when elevated CO_2 alleviates water stress effects on sugarcane and other C_4 species. However the most consistent effect in the sugarcane literature is that on stomatal conductance; a 37% and 34% reduction in twice normal CO_2 (de Souza et al., 2008; Vu et al., 2008) and in our case a 28% reduction for well-watered plants when CO_2 concentration was elevated from 390 to 720 ppm (Table 1). Conductance of maize growing at 550 compared 367 ppm CO_2 was reduced by 34% (Leakey et al., 2006). In the absence of water stress, growth at elevated CO_2 did not stimulate photosynthesis, biomass, or yield. Nor was there any CO_2 effect on the activity of key photosynthetic enzymes, or metabolic markers of carbon and nitrogen status (Leakey et al., 2006).

Long et al. (2006) challenged the optimistic findings from simulation and chamber research on the effects of elevated CO_2 on both C_3 and C_4 species, saying that assumptions used in models were based on misleading chamber experiments rather than free air carbon enrichment (FACE) experiments. Tubiello et al. (2007) countered arguments by Long et al. (2006) demonstrating that model predictions were rather similar to those arising from FACE experiments. Nevertheless it behoves modellers to be careful about assumptions used in their models and experimental evidence has been lacking up to now for simulating the response of sugarcane yields to future CO_2 concentrations. Webster et al. (2009) predicted a sugarcane yield increase of up to 7 % by 2030 in a very high rainfall (>3000 mm pa) region of Australia when considering all aspects of climate change. Biggs et al. (2013) estimated that the effects of rising CO_2 alone would increase yields 10 to 14 % by 2030 in a region of Australia with high rainfall (>1700 mm pa). Using the DSSAT-Canegro model Knox et al. (2010) estimated that yields in Swaziland would increase by 15% by 2050 due only to increased CO_2 and this response was thought to be more from the direct than indirect effect of CO_2 (Ghannoum et al., 2000).

In this project we presented experimental evidence from sugarcane for a largely indirect response to CO_2 elevation for use in modelling future climate scenarios and we also provided a modelling framework which we hope will satisfy the criticisms of those opposed to controlled studies that test and isolate proposed response mechanisms and subsequently reconstruct (scale up) their combined action under a field crop situation, through modelling. Our findings were incorporated into a modelling framework (Stokes et al. 2014) that investigated sugarcane growth under future climate scenarios in Australia (Everingham et al., 2014). While the initial application and testing of our modelling framework only considered CO_2 effects in isolation, the study by Everingham et al. (2014) also considered projections for changes in temperature, rainfall and radiation. The climate and biophysical modelling approaches in these two companion studies could also be used to predict sugarcane yields and irrigation requirements in new climates and production areas as in the study by Black et al. (2012). The findings are also being used in model improvements that will allow better representation of a broader array of functional plant traits. This will allow future applications of trait-based modelling to also include genetic variation in responses to CO_2, and improved application in informing trait-based breeding, particularly in regard to maximizing the benefits of traits related to plant water use, drought stress and water use efficiency.

6 Project reporting

6.1 Outputs:

1) Identified the benefits of elevated $CO_2$to sugarcane, including biomass and sucrose accumulation

Our results have shown clearly that the benefit of rising CO_2 level to sugarcane is almost entirely due to more efficient water use, and that direct effects, if any, are limited (Experiments 2 and 3, Chapter 4). Such benefits would only be expected where water supplies are not fully adequate. However, the modelling showed that even in a crop that was considered to be well irrigated, there could be a benefit from 720ppm of CO_2 of 3 to 8% in growth, at different stages of crop growth. Under experimental conditions where fully sufficient water was artificially maintained, there was no direct benefit to growth or sucrose accumulation.

2) Assessment of benefits for whole plant TE and photosynthesis in response to elevated CO_2 to confirm or revise measurements made on small leaf area

Both leaf and whole-plant measurements showed similar results in terms of an approximate 20-30% increase in water use efficiency, or an equivalent decline in leaf/plant conductance while maintaining a similar rate of photosynthesis/growth for an increase in CO_2 levels from 390 to 720ppm (Experiment 1, Chapter 3). In Experiments 2 and 3 responses to CO_2 were stronger, with an average ~50% in TE (Chapter 4).

3) Knowledge of the growth phases, if any, that are most influenced by elevated CO_2

Sugarcane responses to CO_2 were evaluated across a range of growth stages over a one-year period, and found no evidence of interactions of CO_2 effects with growth phases of plants for any of the water use metrics of interest (Experiment 1, Chapter 3). The magnitude of the CO_2 effect on transpiration efficiency remained constant across time for well-watered plants, but may have been slightly higher in the initial stage of applying water stress treatments (more likely a consequence of water levels gradually declining, and stress gradually increasing, at the start of the dry treatment than an effect of growth stage per se).

Crop modelling showed that the ultimate effects of CO_2 in the field (including numerous changing feedbacks and interactions) are likely to decline as the crop develops and plant-atmosphere coupling weakens.

4) Knowledge of the time required for acclimation to elevated CO_2. This knowledge is essential for designing future experiments with elevated CO_2, for considering traits for varieties suited to new climatic conditions, and for related screening procedures for such traits

A corollary of the previous point is that there was no evidence of any down passive or active regulation of CO_2 responses over time (Experiment 1, Chapter 3). Negative effects of CO_2 on photosynthesis, where they occur, are often associated with a dilution in leaf nitrogen (about half of which is associated with enzymes used in photosynthesis). At no growth stage did we find any evidence that leaf nitrogen levels were lower in the elevated than ambient CO_2 treatment. A shorter 3-month growth period, from transplanting of seedlings, was considered sufficient for evaluating CO_2 responses and was used after the initial experiment. This corresponds with the period when CO_2 effects are likely to be strongest for crops growing in the field, which is an additional rationale for using the growth period for screening.

5) Identification of genetic variation in response to elevated CO_2

Experiment 1 (Chapter 3) indicated a possible interaction in the effects of CO_2 on the two contrasting varieties used, Q208 and KQ228. Subsequent Experiments 2 and 3 (Chapter 4) expanded the number of clones assessed to 7 and 6 respectively. Both these experiments showed statistical evidence of genetic interactions in the water use responses of different clones to elevated CO_2. In relative terms, the percentage increase in transpiration efficiency (TE) varied substantially among clones and treatments (from +7% to +126%). However, in Experiment 3 (but less so in Experiment 2) there was a very predictable pattern to these responses such that genetic variation in TE among clones under current CO_2 was very closely related to variation in TE under elevated (accounting for 80% of the variation). This result needs to be validated across a broader number of clones but, if it holds generally, then screening for TE under current conditions may be sufficient to ensure this benefit continues to be expressed in the future. Results also showed that variation in TE among clones increased under elevated CO_2, indicating that differences among clones for this trait will become more strongly expressed in the future, potentially increasing the relative value of this trait.

6) Assessment of the capability to screen a large number of clones for response to CO_2 in terms of increased TE

Experiment 1 (Chapter 3), which assessed CO_2 responses over different growth stages over a year, found that CO_2 responses in the first 3 months were a reasonable reflection of responses over longer growth periods. Experiments 2 and 3 (Chapter 4) used this shorter (3 mo) growth period to more rapidly assess a wider number of clones (7 and 6 respectively). This methodology is intensive and is limited by the size of the facility to screening 8 – 32 (depending on other treatments and replication/precision) clones at a time, so would unlikely be viable for high-throughput, early stage screening. The approach would be most suitable for handling smaller number of clones: 1) at a later stage of screening, for germplasm that has already some promising potential, including existing commercial varieties; 2) for screening potential parent material, particularly for including novel new material that could contain traits of benefit for future climates that may have been inadvertently selected out of the current breeding pool by historic selection practices; 3) in screening work that includes a mechanistic component, focussing on developing a more functional and targeted basis to trait selection methodologies (where the stream of monitored sensor data provided by this approach could be used to its full potential).

7) Improved modelling capability to simulate responses to increased CO_2 accurately

In addition, we have worked in collaboration with the research team from SRDC project JCU032 (lead by Yvette Everingham at JCU) on their modelling of climate change impacts (particularly CO2 aspects) on Australian sugarcane production. This has provided an early opportunity for results of our project to be applied (while benefiting JCU032 with models that incorporate the latest evidence).

The results from this project provide the first measurements of whole-plant effects of CO_2 on transpiration efficiency, which had not been available for parameterizing and calibrating sugarcane crop models before. We developed a model (from existing ones) that could make use of the results obtained in our glasshouse experiments to predict the effects of rising CO_2 on sugarcane, based on the simulated linked, flow through effects of elevated CO_2 on conductance, water use, soil moisture and plant growth (Chapter 5). Unlike previous approaches, we did not assume any direct stimulation of radiation use efficiency by elevated CO_2, but represented the CO_2 effect entirely in terms of improved transpiration, using a Penman-Monteith approach to account for subsequent changes in gas and energy fluxes. We then tested this model against field observations from a previous experiment, and simulated what effect elevated CO_2 would have had on that crop. The model showed that even in a crop that was considered to be well irrigated, there could be a benefit from 720ppm of CO_2 of 3 to 8% in growth, through different stages of crop growth.

8) Value adding to BSS305 and BSS334 by accurately measuring transpiration efficiency as a sub-trait of WUE in 'More-Crop-per-Drop'

Throughout this project we communicated results at an early stage with researchers from the More-Crop-per-Drop project (BSS305). This included seeking their input on refining methodologies and, particularly, selecting clones from BSS305 trials that showed contrasting responses in the field, for which they were collecting data, and for which they had most interest in obtaining further data for comparison from our experiments, including CO_2 effects.

9) Value adding to sugarcane modelling by producing data that can be used to test a detailed sucrose accumulation model for conditions never experienced by sugarcane

A detailed set of sugar (sucrose, glucose and fructose) parameters were measured in Experiment 1 (Chapter 3) to show the patterns of sugar accumulation down internodes in stalks and across growth stages. This included the effects of CO_2 treatments on sugar accumulation, which have never been measured before. This will provide a valuable resource for parameterizing and testing models of sucrose accumulation in the future.

10) Communications activities

The main target audience for this research is pre-breeders. The main avenue to adoption for this project's outputs and ultimate industry benefit would be through incorporating our findings into ongoing work related to trait-based selection in sugarcane, particularly work related to water use/stress (e.g. More-Crop-per-Drop) by incorporating considerations of future climate and dynamic whole-plant responses to that work.

Informal and formal meetings were held throughout the project with researchers working in similar areas in sugarcane and other crops. These included annual sugarcane physiology workshops in Queensland, including:
Sugarcane Molecule to Mill, Gene to Phenotype workshop, 28 October 2009, St Lucia, Brisbane
Sugarcane Physiology Workshop, 10 November 2010, St Lucia, Brisbane
Sugarcane Drought Review, 6-7 September, 2012, Douglas, Townsville
Sugarcane Modelling Workshop, 16 November 2012, St Lucia, Brisbane
Sugarcane Research Review Meeting, 14-15 November 2014, St Lucia, Brisbane

Several magazine and newspaper articles were produced related to the work in this project including:
"Developing climate ready sugarcane" (March 2013) SRDC magazine and associated media release.
"Sweet-as! Climate-ready sugarcane in Australia" (11 April 2013) Rural Press.
"Developing climate ready crops of the future" (May 2013) prepared with The Linde Group (CO_2 supplier for the research) and was printed in several industry publications including CryoGas, GasWorld, Gases & Instrumentation (USA) and Food Processing and Food (Europe).
Regular contributions to SRDC annual reports.

WIN News interviewed Chris Stokes at the Tall Plant Facility on 4 March 2013, covering the research in this project (following a media release on 21 February 2013).

The paper, Stokes et al. (2014), listed in the publications (section 6.7) was submitted in November 2013.

11) Technical report summarising the findings of the project

This technical report.

6.2 Intellectual Property and Confidentiality:

There are no substantive IP issues related this project or the findings in this report.

6.3 Environmental and Social Impacts:

We do not anticipate any negative environmental or social impacts from this work or its future application. The long-term aims of this work are likely to be neutral to positive. Ultimate outcomes related to improved water use efficiency could be beneficial for the environment by allowing more efficient use of this resource, particularly in places where water is limiting and there is competition between extractive and non-extractive water use and services (including ecosystem services). Ultimate outcomes related to developing and identifying climate-ready varieties would be expected to have positive social outcomes by contributing to the sugar industry being better able to respond to climate change. Outcomes related to developing and identifying more stress tolerant varieties, that could assist with sugarcane production being expanded into new, more marginal areas, could have positive social impacts, but these would need to be balanced against the risks of failure and of environmental impacts of introducing sugarcane to new areas. Any large-scale use of sugarcane for biofuels or biomass energy would likely require such varieties, and supporting management practices, to be able to expand into more marginal areas, if food production from sugarcane is to be maintained at current levels.

6.4 Expected Outcomes:

SRA/SRDC has already invested in the search for drought resistance and improved water use efficiency. Our work has quantified the amount by which TE will increase under higher CO_2 conditions and shown that the value of TE as a trait, and the difference in TE among clones, could well increase in the future. By incorporating the findings from this project and this line of research into similar TE-related work, such as More-Crop-per-Drop, we should be able to ensure that current breeding initiatives and directions continue to deliver benefits under future climate conditions. In particular, it should be able to assist in identifying the traits that will be of most benefit over the coming decades, and in incorporating these into practical screening approaches. The current average cost of water stress to the industry is $230 million (Inman-Bamber, 2007 ASSCT 29, 167-175) and this is likely to increase. If TE can be improved by 10 % we estimate this would reduce this loss by at least 5% or about $12 million annually.

The improved understanding of the mechanisms of sugarcane responses to elevated CO_2, and quantifying the magnitude of these effects, will also allow more accurate assessments of implications of climate change for the sugarcane industry. This information has been incorporated into an improved crop modelling approach. This should contribute to enhanced resilience of the sugar industry to climate change by allowing assessments that better explore the effectiveness of adaptation strategies it may consider employing.

6.5 Future Research Needs:

The findings of this project have highlighted several lines of investigation that would be worth pursuing further:

- The findings of this project, and possibly some of the methodologies that were developed, need to be integrated with existing trait-based breeding work, particularly work related to TE.
- A larger number of clones need to be investigated for CO_2 responses – for example, the findings for Experiment 3 (with only 6 clones) would have important implications for breeding if it held generally than TE rankings of clones under well-watered conditions and ambient CO_2 were good first approximations of TE ranking under elevated CO_2 and/or under water-stressed conditions.
- Dynamic monitoring of whole-plant transpiration responses to manipulated supply and demand factors may assist in being able to better link leaf-scale processes and measurements to genetic differences in final harvest performance (in pot experiments first), something that has often proved difficult in the past. This in turn may help to indentify more functional and effective traits for use in screening and breeding.
- The improved modelling approaches and mechanistic understanding of sugarcane CO_2 responses developed in this project should be included in future trait-based modelling (conducted in conjunction with experimental development of trait-based breeding approaches).
- The preparedness of the sugarcane industry for adapting to climate change should be explored by using the improved crop modelling to evaluate the effectiveness of adaptation options.
- Modelling analyses need to be conducted to test how the timing of water stress through a crops growth cycle affects the potential benefits of CO_2 in reducing that stress. The simulations we conducted showed that the largest effects of CO_2 are likely to occur early on when plants are small and the canopy is open but that, as the canopy closes and plant-atmosphere coupling weakens, the effectives of elevated CO_2 (and reduced stomatal conductance) in improving WUE declines. This suggests that CO_2 may be of less benefit where stress occurs late in the crop's development.

6.6 Recommendations:

There are no immediate recommendations for on ground application of the project findings at this stage, given the project was specifically focussed on mid- to long-term issue of climate change and plant improvement. The project did produce some major findings with important practical implications in this regard. These are summarised in the previous list of future research needs and the recommended pathway to impact to would be through incorporating this project's findings into those existing and new initiatives, as suggested above.

6.7 List of Publications:

Stokes CJ, Inman-Bamber NG, Everingham Y, Sexton J (2014) Measuring and modelling CO_2 effects on sugarcane. Agricultural and Forest Meteorology (in review)

Submitted November 2013
Revised February 2014 – editor's decision still pending (May 2014)
Chapter 5, of this report is based on this paper with only slight modification.
A final copy of this publication can be provided to SRA when/if it is accepted.

Shortened forms

A - assimilation (of carbon, at the leaf level, through photosynthesis)

BREB - Bowen ratio energy balance

ET - evapotranspiration

LAI - leaf area index

TE - transpiration efficiency (integrated whole-plant biomass growth divided by water transpired)

TPF - Tall Plant Facility

WUE - water use efficiency (instantaneous leaf level ratio of photosynthesis to transpiration)

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CONTACT US

t 1300 363 400
+61 3 9545 2176
e enquiries@csiro.au
w www.csiro.au

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Climate Adaptation National Research Flagship
Chris Johnson, Communication Manger
t +61 3 9239 4691
e chris.johnson@csiro.au
w http://www.csiro.au/en/Organisation-Structure/Flagships/Climate-Adaptation-Flagship.aspx