

**IMPACTS OF KEY THREATENING PROCESSES ON GRASS-DOMINATED  
ECOSYSTEMS IN A HIGH CO<sub>2</sub> WORLD: A CASE STUDY OF  
CUMBERLAND PLAIN WOODLAND.**

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## ABSTRACT

Rising atmospheric CO<sub>2</sub> concentration is one of the best documented global changes of the past half century and may have a profound effect on the structure and function of ecosystems globally. Grass-dominated ecosystems (hereon referred to as grasslands) may be particularly vulnerable to this increase in atmospheric CO<sub>2</sub> concentration due to their highly dynamic nature. Historically, the major threat to grasslands was their conversion to an agricultural landscape. Although this conversion continues today, grasslands face a number of other threatening processes whose impacts may be exacerbated by rising atmospheric CO<sub>2</sub> concentration. This thesis explores how these threatening processes interact with elevated CO<sub>2</sub> levels and each other to impact the structure and function of grasslands. Four threatening processes to grasslands that may be substantially modified under elevated CO<sub>2</sub> levels were identified and examined in this thesis. These threatening processes were extreme climatic events, exotic plant invasion, woody plant encroachment and changes to fire regime. Data for all chapters were obtained by carrying out manipulative CO<sub>2</sub> glasshouse experiments using Cumberland Plain Woodland, an open grassy woodland community of western Sydney, as the model grassland system. Chapter 2 and 4 test the vulnerability of grasslands to extreme drought and woody plant encroachment under elevated CO<sub>2</sub> levels. Chapter 3 investigates the interaction between extreme drought and elevated CO<sub>2</sub> levels and how this may facilitate exotic plant invasion. Chapter 5 examines the changes in leaf flammability and fuel load accumulation of grassland species under elevated CO<sub>2</sub> levels.



## **CERTIFICATE**

This thesis constitutes an original contribution and has not been submitted, in any form, for a higher degree at any other university or institution.

The work of others has been used to prepare some aspects of the thesis and the extent of their contribution is clearly outlined below. Daniel Sloane (Macquarie University) assisted with running the glasshouse experiment which is presented in Chapter 2. Saskia Grootemaat (Macquarie University) constructed the apparatus used to test leaf flammability in Chapter 5. All other aspects of the work presented in this thesis were done by me.

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## **CHAPTER ONE**

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### **Introduction**



## Background

Rising atmospheric CO<sub>2</sub> concentration is one of the best documented global changes of the past half century (Prentice 2001). Atmospheric CO<sub>2</sub> concentration has been rising steadily since the Industrial Revolution from ~270 ppm to current levels of ~400 ppm and this increase is predicted to continue under a range of emission scenarios (IPCC 2013). Extensive research has been undertaken over the last three decades to understand how plants will respond to rising CO<sub>2</sub> levels (Ainsworth and Long 2005; Leakey et al. 2009). The primary effects of rising CO<sub>2</sub> levels on plants are well documented and include reduction in stomatal conductance and transpiration, improved water and nitrogen use efficiency and higher rates of C<sub>3</sub> photosynthesis (Leakey et al. 2009). Understanding how plants will respond and adapt to changes in CO<sub>2</sub> level is an essential first step in understanding the full impact that rising atmospheric CO<sub>2</sub> concentration will have on the structure and function of global ecosystems.

Rising atmospheric CO<sub>2</sub> concentration may have a profound effect on the structure and function of grass-dominated ecosystems (hereon referred to as grasslands) due to their highly dynamic nature. Grasslands range from open grassland to densely canopied savanna and woodland with up to 80% woody plant cover (Sankaran et al. 2005). They occupy more than 30% of the global terrestrial landscape (Asner et al. 2004) and act as a sink for large amounts of soil carbon, thus playing an important role in the global carbon cycle (Briggs et al. 2005; Grace et al. 2006). Historically, the major threat to grasslands was their conversion to an agricultural landscape (Briggs et al. 2005). Although this conversion continues today, grasslands face a number of other threatening processes whose adverse

impacts on their structure and function may be enhanced by rising atmospheric CO<sub>2</sub> concentration. For example, it is predicted that global grassland structure may shift from C<sub>4</sub> to C<sub>3</sub> dominance in the future as current CO<sub>2</sub> concentrations are sub-optimal for C<sub>3</sub> photosynthesis (Collatz et al. 1998). This thesis examines four threatening processes that may interact with elevated CO<sub>2</sub> levels to profoundly influence the integrity of grasslands. These four processes are: (1) extreme climatic events, (2) exotic plant invasion, (3) woody plant encroachment and (4) changes to fire regime. Understanding the mechanisms by which these threatening processes will interact with rising atmospheric CO<sub>2</sub> concentration and each other will be integral to the successful management of grasslands into the future.

### **Study system: Cumberland Plain Woodland**

To assess the impact of elevated CO<sub>2</sub> levels on the processes that threaten grassland integrity I have selected the Cumberland Plain Woodland (CPW) of western Sydney, Australia as my model study system. The CPW generally occurs on gentle undulating terrain with fertile clayey soils derived from the underlying Wianamatta shale. The area receives approximately 800 mm of rainfall annually (Tozer 2003). The native vegetation of the CPW typically consists of open eucalypt woodland with a highly diverse grass and herbaceous ground-storey (Little 2003). There is believed to be 500–600 native plant species that occur in the remaining CPW remnants across western Sydney (Benson and Howell 2002).

CPW once covered an estimated area of about 125 000 hectares of western Sydney, extending north-west to Kurrajong and south-west to Picton (Tozer 2003). However because of its favourable topography and fertile soils it has been targeted

for agricultural and urban development since European arrival (Benson and Howell 2002). As a result it is now confined to less than 10% of its original extent with most remaining as small remnants (Benson and Howell 2002). Consequently it is now listed as a critically endangered ecological community under both State and Commonwealth threatened species legislation. This dramatic reduction in geographic extent of CPW, coupled with its high species diversity, means that the long-term management of CPW remnants for species conservation is of high priority (Benson and Howell 2002). Although vegetation clearing is the main threatening process in CPW, the threatening processes examined in this thesis (extreme climatic events, exotic plant invasion, woody plant encroachment and changes to fire regime) still pose a major threat.

Since the 1950s there has been an increase in the frequency and intensity of extreme droughts in eastern Australia (Hennessy et al. 2008). Many CPW species are capable of withstanding prolonged drought conditions which may be attributed to the high frequency of fleshy storage roots in CPW species that allow them to recover quickly after drought (Benson and Howell 2002). This suggests that drought tolerance has been a major factor in the long term persistence of many CPW species. Despite this there have been occurrences where drought has caused widespread mortality to particular CPW species. For example, in the early- and mid-90s there was a gradual widespread death of *Eucalyptus crebra* trees, which was believed to be a result of previous stress during the major drought of the early-1980s (Benson and Howell 2002).

Many CPW remnants are heavily invaded and therefore managing invasive exotic plants has become a major priority (Benson and Howell 2002). CPW is particularly vulnerable to exotic plant invasions because of disturbance from clearing and grazing and its highly fragmented nature (Little 2003; Tozer 2003; Hill et al. 2005). The greatest invasive threat to CPW is African olive (*Olea europaea* L. ssp. *cuspidata*) which has had a dramatic recent expansion in its range (Cuneo et al. 2009; Cuneo and Leishman 2012). This may contribute to woody plant encroachment which is also considered a threatening process in CPW.

The CPW mid-storey is characterised by a sparse yet persistent shrub layer with *Bursaria spinosa* being the dominant species (Little 2003). However changes in fire regime may have a profound effect on the composition and structure of the shrub layer. For example, Watson et al. (2009) showed that the frequency and density of *B. spinosa* was considerably higher in low fire frequency sites compared to high fire frequency sites. This example mirrors the encroachment of woody plants into a range of grassland ecosystems around the world when disturbance regimes such as fire are altered (Watson et al. 2009).

Fire is a natural component in the ecology of CPW which is evident from the fact that nearly half of the native CPW species are known to resprout after fire (Hill and French 2004). It influences the plant species composition and vegetation structure of CPW by opening up the ground- and mid-storey, enabling recruitment (Benson and Howell 2002; Watson et al. 2009). For example, competition from dominant grass species, such as *Themeda australis* which occurs in CPW, can cause a decline in native species diversity in grasslands (Lunt et al. 2012). To prevent



these dominant grass species from outcompeting other native species, frequent fire may be required (Lunt et al. 2012).

The high conservation value of the CPW, coupled with its highly fragmented nature and current endangerment, makes it the ideal community to study the impact of rising atmospheric CO<sub>2</sub> concentration on the processes that threaten grasslands. There are four identified major threats to CPW, in addition to habitat clearing: (1) extreme climatic events, (2) exotic plant invasion, (3) woody plant encroachment and (4) changes to fire regimes. While the impacts of these individual threats have been recognised, few studies have considered how these threats may be mitigated or enhanced by elevated CO<sub>2</sub> levels. By understanding the underlying processes that determine the impacts of multiple threats in CPW, this thesis will enable more accurate predictions of likely impacts in grassland ecosystems elsewhere.

### **Thesis scope and structure**

This thesis consists of six chapters: this Introduction, four chapters based on data obtained from manipulative CO<sub>2</sub> glasshouse experiments which have been prepared for submission for publication in relevant scientific journals and a brief Conclusion which integrates the findings from these four data chapters. Although chapters have been prepared for different journals they have been formatted in a consistent style for this thesis. Each chapter was prepared as a stand-alone manuscript, thus this thesis contains some unavoidable repetition of introductory material and methods. A brief outline of the content and major findings of each data chapter is below.

## Chapter 2: Extreme climatic events

It is predicted that the magnitude and frequency of climatic extremes are likely to increase under future climate change conditions (IPCC 2011). There is increasing evidence of extreme drought causing regional-scale mortality of woody plants that has resulted in major structural and functional changes in these woody plant-dominated ecosystems (Bigler et al. 2007; Adams et al. 2009; Allen et al. 2010). However the vulnerability of non-woody plant dominated ecosystems, such as grasslands, to extreme drought is poorly understood (see examples Kreyling et al. 2008; Beierkuhnlein et al. 2011; Jentsch et al. 2011). Vulnerability of grasslands to extreme drought will depend on soil water content prior to the drought event. A major driver of soil water content is canopy transpiration rate which can be altered by elevated CO<sub>2</sub> levels through reductions in stomatal conductance and changes in leaf area index.

In this chapter (published in *PLoS One*) I asked whether the response of grasslands to repeat extreme drought events is modified by elevated CO<sub>2</sub> levels, and if so, what are the underlying mechanisms? Experimental mesocosms containing both native and exotic CPW grass species were established and subjected to repeated extreme drought/recovery cycles under ambient and elevated CO<sub>2</sub> levels. At the end of each cycle the mortality of the grasses was recorded.

The major finding of this study was that the grasses grown under elevated CO<sub>2</sub> levels had higher leaf area index which increased canopy transpiration rates

and rainfall interception. This resulted in lower soil water content and higher extreme drought-related mortality in the elevated CO<sub>2</sub> mesocosms.

### Chapter 3: Exotic plant invasion

Biological invasions of exotic species have been identified as a major threat to biodiversity (Mack et al. 2000; van der Wal et al. 2008; Lambertini et al. 2011). Invasive exotic plants are generally most successful in environments where there is high resource availability (Davis et al. 2000; Daehler et al. 2003; Leishman and Thomson 2005; Blumenthal et al. 2006). Climate change may alter the invasion success of exotic plants in ecosystems by changing resource availability and creating novel environments for them to invade (Bradley et al. 2010). Rising atmospheric CO<sub>2</sub> concentration and altered precipitation patterns (e.g. extreme drought) are two aspects of climate change that will directly and indirectly affect resource availability in ecosystems (Bradley et al. 2010).

The main objective of this chapter was to determine the mechanism by which the invasion of exotic plants into grasslands occurs when there are climate change-associated changes in resource availability (CO<sub>2</sub> levels and soil water availability as a result of extreme drought). Experimental mesocosms containing native CPW grass species were established under ambient and elevated CO<sub>2</sub> levels and then exposed to an extreme drought event. Seeds of an exotic grass, *Ehrharta erecta*, were then added to each mesocosm and its invasion success (biomass production and reproductive output) was assessed.

The key finding of this work was that reduced biomass production of the native grasses in response to the extreme drought treatment (i.e. reduced soil water availability) enhanced the invasion success of *E. erecta* by creating resource pulses in light and space. Surprisingly CO<sub>2</sub> level did not affect the invasion success of *E. erecta*. These results suggest that changes to the invasion vulnerability of grasslands in the future will be context-dependent, with vulnerability being strongly coupled to soil water availability and the subsequent response of resident native grass species.

#### Chapter 4: Woody plant encroachment

It is being increasingly recognised that grasslands globally face a new threat from the increased cover and abundance of woody plants (Briggs et al. 2005; Ward et al. 2005). Woody plant encroachment into grasslands is generally attributed to changes in climate, grazing intensity and fire regimes (Archer et al. 1995; Scholes and Archer 1997). Recently, it has been proposed that increases in woody plant cover are linked to the rise in atmospheric CO<sub>2</sub> concentration that has occurred over the last 200 years (Polley et al. 1997; Bond and Midgley 2000; Buitenwerf et al. 2012). Rising atmospheric CO<sub>2</sub> concentration may favour woody plant encroachment by increasing the photosynthesis of C<sub>3</sub> woody plants relative to C<sub>4</sub> plants (Bond and Midgley 2000) and/or by reducing canopy transpiration rates of grasses thus increasing soil water availability which may enhance woody plant seedling establishment and growth (Polley et al. 1997).

The aim of this chapter (published in *Oecologia*) was to examine the effect of CO<sub>2</sub> level on the competitive interactions between established C<sub>4</sub> grasses and

woody plant seedlings. This was done by growing woody plant seedlings in mesocosms together with established C<sub>4</sub> grasses in three competition treatments (root competition, shoot competition and root plus shoot competition) under ambient and elevated CO<sub>2</sub> levels.

I found that woody plant seedling growth was suppressed by competition from grasses, with root and shoot competition having similar competitive effects on growth. Surprisingly, woody plant seedling growth was reduced under elevated CO<sub>2</sub> levels across all competition treatments. The most plausible explanation for this was the reduced light and soil water availability in the elevated CO<sub>2</sub> mesocosms which was associated with increased leaf area index of the grasses. These results suggest that the expansion of woody plants into grasslands in the future will likely be context-dependent, with establishment success of woody plant seedlings being strongly coupled to the CO<sub>2</sub> response of competing grasses and to soil water availability.

## Chapter 5: Changes to fire regime

Fire is a common process that shapes the structure of many of the world's grasslands (Bond et al. 2005; Bond and Midgley 2012; Higgins et al. 2012). It is projected that increases in extreme drought and temperatures associated with climate change may increase the risk of wildfire (IPCC 2011). In addition to this, rising atmospheric CO<sub>2</sub> concentration (IPCC 2013) may increase fire frequency in grasslands by increasing leaf flammability and fuel load accumulation of grassland species.

In this chapter (in press at *International Journal of Wildland Fire*) I asked if CO<sub>2</sub> and/or soil P availability alters leaf flammability, fuel load accumulation and resprouting ability of grassland species. I grew native CPW grass and woody plant species individually in pots in a full factorial design with CO<sub>2</sub> and soil P availability being the factors. Using leaf tissue chemistry traits and biomass production as proxies I measured leaf flammability, fuel load accumulation and resprouting ability of the selected species.

The major findings of this study were that leaf flammability may increase under elevated CO<sub>2</sub> levels due to decreased leaf moisture content and foliar N (both flame retardants) while fuel load accumulation may increase due to increased aboveground biomass production and slower litter decomposition rates (foliar N), suggesting fire frequency may increase in grasslands. However the enhanced resprouting ability of grasses under elevated CO<sub>2</sub> levels may result in a shift in the vegetation structure of grasslands. Plant CO<sub>2</sub> responses were not modified by soil P availability.

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## CHAPTER TWO

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**Leaf area index drives soil water availability and extreme drought-related mortality under elevated CO<sub>2</sub> levels in a model temperate grassland system.**

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My contribution to the research and paper: Concept – 90%; Data collection – 100%; Analysis – 100%; Writing – 90%.



**Abstract**

The magnitude and frequency of climatic extremes, such as drought, are predicted to increase under future climate change conditions. However little is known about how factors such as atmospheric CO<sub>2</sub> concentration will modify plant community responses to these extreme climatic events, even though such modifications are highly likely. We asked whether the response of grasslands to repeat extreme drought events is modified by elevated CO<sub>2</sub> levels, and if so, what are the underlying mechanisms? We grew grassland mesocosms consisting of 10 co-occurring grass species common to the Cumberland Plain Woodland of western Sydney under ambient and elevated CO<sub>2</sub> levels and subjected them to repeated extreme drought treatments. The 10 species included a mix of C<sub>3</sub>, C<sub>4</sub>, native and exotic species. We hypothesized that a reduction in the stomatal conductance of the grasses under elevated CO<sub>2</sub> levels would be offset by increases in the leaf area index thus the retention of soil water and the consequent vulnerability of the grasses to extreme drought would not differ between the CO<sub>2</sub> treatments. Our results did not support this hypothesis: soil water content was significantly lower in the elevated CO<sub>2</sub> mesocosms and extreme drought-related mortality of the grasses was greater. The C<sub>4</sub> and native grasses had significantly higher leaf area index under elevated CO<sub>2</sub> levels. This offset the reduction in the stomatal conductance of the exotic grasses as well as increased rainfall interception, resulting in reduced soil water content in the elevated CO<sub>2</sub> mesocosms. Our results suggest that projected increases in net primary productivity globally of grasslands in a high CO<sub>2</sub> world may be limited by reduced soil water availability in the future.

## Introduction

A major driver that shapes the physiology, ecology and evolution of terrestrial plants is climatic extremes (Gutschick and BassiriRad 2003). It is widely acknowledged that the magnitude and frequency of climatic extremes, such as extreme drought, are likely to increase under future climate change conditions (IPCC 2011). The IPCC (2011) defines a climatic extreme as an event that occurs once every 20 years, on average. The potential for climatic extremes to alter the structural and functional dynamics of ecological communities (Easterling et al. 2000; IPCC 2011), coupled with their increasing magnitude and frequency in the future, suggests research on climatic extremes should be a high priority.

Grass-dominated ecosystems (hereon referred to as grasslands) occupy more than 30% of the global terrestrial landscape (Asner et al. 2004) and play an important role in the global carbon cycle (Briggs et al. 2005). The productivity of grasslands is strongly mediated by soil water availability (Asner et al. 2004; Morgan et al. 2004; Morgan et al. 2011). For example, Fay et al. (2008) increased the mean annual rainfall to an experimental grassland community in Kansas by 250% and found that both soil water content (SWC) and aboveground net primary productivity significantly increased (see exception Jentsch et al. 2011). Consequently it is likely that soil water availability prior to an extreme drought event will be a major driver in the response of the grassland to the event.

It has rarely been considered how CO<sub>2</sub> concentration will alter soil water availability in grasslands and thus modify grassland responses to extreme drought. One of the major drivers of soil water availability in grasslands is canopy



transpiration (Morgan et al. 2004). The amount of water that is lost through canopy transpiration depends on the stomatal conductance and leaf area index (LAI) of the grasses (Woodward 1990). Reduction in stomatal conductance of grasses under elevated CO<sub>2</sub> levels has been well-documented (Anderson et al. 2001; Morgan et al. 2001; Morgan et al. 2004). For example, Morgan et al. (2011) found that in the Wyoming mixed-grass prairies in the United States, the annual SWC increased on average by 17.3% over a three year period due partly to reductions in stomatal conductance under elevated CO<sub>2</sub> levels. The physiology (C<sub>3</sub> or C<sub>4</sub>) and origin (native or exotic) of the grasses play an important role in this reduction of stomatal conductance under elevated CO<sub>2</sub> levels. C<sub>4</sub> plants evolved in a low CO<sub>2</sub> environment, allowing high rates of photosynthesis at low stomatal conductance (Osborne and Sack 2012). This suggests that C<sub>4</sub> plants should have greater reductions in stomatal conductance compared to C<sub>3</sub> plants under elevated CO<sub>2</sub> levels. In addition, meta-analysis studies have shown that natives tend to have lower stomatal conductance than exotics (Cavaleri and Sack 2010).

In contrast to reductions in stomatal conductance, grasses often have higher LAI under elevated CO<sub>2</sub> levels, resulting in increased canopy transpiration. For example, the LAI of the C<sub>4</sub> bunchgrass *Pleuraphis rigida* significantly increased under elevated CO<sub>2</sub> levels in a 10 year Mojave Desert FACE experiment (Newingham et al. 2013). In addition, a meta-analysis study of semi-wild and wild C<sub>3</sub> and C<sub>4</sub> grasses found that leaf area (related to LAI) increased under elevated CO<sub>2</sub> levels by 15% and 25% respectively (Wand et al. 1999). Therefore reductions in the stomatal conductance of grasses under elevated CO<sub>2</sub> levels may be offset by increases in their LAI (Piao et al. 2007).

In this study we asked whether the response of grasslands to repeat extreme drought events is modified by elevated CO<sub>2</sub> levels, and if so, what are the underlying mechanisms? Experimental mesocosms containing common co-occurring native and exotic grass species of the Cumberland Plain Woodland (CPW) of western Sydney, Australia were grown under ambient and elevated CO<sub>2</sub> levels and exposed to repeated one in 20 year extreme drought events. We hypothesised that a reduction in the stomatal conductance of the grasses, particularly the C<sub>4</sub> and native grasses, grown under elevated CO<sub>2</sub> levels would be offset by increases in the LAI of the grasses. That is, the canopy transpiration rate of the grasses would not differ between the CO<sub>2</sub> treatments. Therefore the retention of soil water in the mesocosms and consequently the vulnerability of the grasses to extreme drought would not differ between the CO<sub>2</sub> treatments.

## **Material and methods**

### Species selection

We selected five native and five exotic perennial grass species which commonly co-occur in CPW. All the exotic species are considered to be invasive rather than simply exotics that have become naturalised in CPW (Little 2003). Within both the native and exotic groups we included two C<sub>3</sub> and three C<sub>4</sub> species. Seeds of the exotic species were collected from a number of individual plants from sites on the Cumberland Plain while seeds of the native species were obtained from a commercial supplier (Nindethana Seed Service, Albany, WA, Australia). Information on the biology of each grass species is provided in Table 1. Once collected, the seeds for each of the 10 grass species were germinated on moist

paper towels within covered aluminium trays. To spread the risk of germination failure, each grass species was germinated in a number of different aluminium trays.

Species	Origin	Seed mass (mg)	Photosynthetic pathway
<i>Chloris gayana</i> Kunth	Exotic	0.4	C <sub>4</sub>
<i>Eragostis curvula</i> (Schrud.) Nees	Exotic	0.2	C <sub>4</sub>
<i>Pennisetum clandestinum</i> Hochst. Ex Chiov	Exotic	7.0	C <sub>4</sub>
<i>Bromus catharticus</i> Vahl	Exotic	10.7	C <sub>3</sub>
<i>Ehrharta erecta</i> Lam.	Exotic	2.0	C <sub>3</sub>
<i>Bothriochloa macra</i> (Steud.) S.T.Blake	Native	1.2	C <sub>4</sub>
<i>Chloris truncata</i> R.Br.	Native	0.3	C <sub>4</sub>
<i>Themeda australis</i> (R.Br.) Stapf	Native	2.6	C <sub>4</sub>
<i>Austrodanthonia racemosa</i> (R.Br.) H.P.Linder	Native	0.7	C <sub>3</sub>
<i>Microlaena stipoides</i> (Labill.) R.Br.	Native	4.3	C <sub>3</sub>

Table 1: Selected characteristics of grass species used in this study. Seed mass was obtained by oven-drying 50 seeds from each species at 60°C for 48 hours and then weighing them. Other data obtained from PlantNET (The Royal Botanic Gardens and Domain Trust, Sydney, NSW, Australia, <http://www.plantnet.com>).

### Experimental design and treatments

The native and exotic grass species were grown together in mesocosms under ambient and elevated CO<sub>2</sub> levels. The mesocosms consisted of 65 L tubs (60 cm long × 40 cm wide × 28 cm deep), each tub containing 55 L of soil mixture

consisting of field-collected CPW soil and coarse river sand in a ratio of 3:1. The CPW soil was obtained from Mt Annan (34.07°S, 150.76°E) and Luddenham (33.88°S, 150.69°E) in western Sydney and was homogenized into one batch using a concrete mixer. The river sand was obtained from a commercial supplier (Australian Native Landscapes, North Ryde, NSW, Australia). Seedlings were transplanted from the germination trays into the CO<sub>2</sub> treatment mesocosms at the stage of second true leaf emergence. All seedlings were planted within 24 hours of each other. The seedlings were planted in two rows of five with each species allocated a position randomly within each mesocosm. For each grass species multiple seedlings were transplanted into each mesocosm as insurance against seedling mortality. After six days, the excess seedlings were removed from the mesocosms, leaving one individual per species per mesocosm.

CO<sub>2</sub> treatments were set to two levels: ambient (380-420 ppm) and elevated (530-570 ppm). These CO<sub>2</sub> concentration ranges were maintained and monitored continuously by a CO<sub>2</sub> dosing and monitoring system (Canary Company Pty Ltd, Lane Cove, NSW, Australia). The elevated CO<sub>2</sub> treatment represents the predicted atmospheric CO<sub>2</sub> concentration by 2060 under the RCP 4.5 emissions scenario (IPCC 2013).

We defined a drought as the number of consecutive days with <1mm of rainfall. This definition is a part of the ETCCDI/CRD climate change indices (CLIVAR, Laoshan, QIN, China, <http://www.etccdi.pacificclimate.org>). We used the IPCC (2011) definition of climatic extreme which is an extreme that occurs once every 20 years, on average. Gumbel I distributions were fitted to the annual

drought extremes of the Cumberland Plain for each year from 1867-2010. Rainfall on the Cumberland Plain is not seasonal so the times of year that extreme droughts occur vary. The data used were obtained from the Australian Bureau of Meteorology historical records of Brownlow Hill (34.03°S, 150.65°E, 1867-1969), Kentlyn (34.05°S, 155.88°E; 1970-1971), Camden Airport (34.04°S, 150.69°E; 1972-1992, 1998-2001), Ruse (34.06°S, 150.85°E; 1993-1997) and Mt Annan Botanical Gardens (34.07°S, 150.76°E; 2002-2010). A one in 20 year extreme drought event for the Cumberland Plain was calculated to last for a period of 53 days. The extreme drought was simulated by turning off the watering system for the treatment period.

The extreme drought treatment mesocosms were replicated five times at each CO<sub>2</sub> level. These were called the 'drought treatment' mesocosms. In addition, five extra mesocosms were grown under each CO<sub>2</sub> level. These were called the 'before treatment' mesocosms. All mesocosms were mist watered for one minute twice daily which is representative of the average daily amount of rainfall (828 mm annually) on the Cumberland Plain. This daily rainfall average was based on the same Australian Bureau of Meteorology historical records from Camden airport (1943-2004) as described in the above paragraph. This design resulted in a total of 20 mesocosms each containing 10 grass species (i.e. [2 CO<sub>2</sub> treatments × 5 replicates] + 10 extra mesocosms). The mesocosms within each CO<sub>2</sub> treatment were split between two glasshouses. The mesocosms were switched between glasshouses within each CO<sub>2</sub> treatment once during the growth period and once during the treatment period to reduce any glasshouse effect.

The temperature of the glasshouses was set for a minimum of 16°C and a maximum of 24°C. Relative humidity of the glasshouses was monitored daily at 9am and 3pm using a HOBO temperature/RH/2 external channel data logger (OneTemp, Parramatta, NSW, Australia). Using a paired t-test with a Bonferroni adjustment we found no significant difference in the relative humidity between the ambient and elevated CO<sub>2</sub> glasshouses ( $t_{1,38}=1.00$ ,  $p=0.326$ ).

The grasses were grown for 12 weeks at which stage they were mature (i.e. had several tillers or were seeding). After the growth period the 'before treatment' mesocosms were harvested, washed free of soil and separated into their following components: leaf biomass, stem biomass and root biomass. The total leaf area of the leaf biomass was measured using a LI-3100C Area Meter (Li-Cor, Lincoln, NE, United States). The plant components were then oven-dried at 60°C for 72 hours and weighed using an analytical electronic balance (Mettler Toledo, Port Melbourne, VIC, Australia).

The extreme drought treatment was then applied to the five 'drought treatment' mesocosms grown under each CO<sub>2</sub> level. After the 53 day treatment period the mesocosms were mist-watered twice daily as previously for four weeks to allow a recovery period. In field conditions on the Cumberland Plain four weeks would be ample time for the grasses to recover (i.e. resprout) from a drought period. After the four week recovery period, the mortality of the grasses was recorded. Each grass was classified as dead or alive depending on if it showed signs of regeneration by the end of the recovery period. The grasses were then exposed to two more cycles of drought and recovery with mortality of the grasses

recorded after each cycle. At the end of the recovery period of the final cycle, every mesocosm was dug up to ensure that the grasses classified as dead had no living root material. The repeated extreme drought treatments are not realistic of field conditions. The purpose of the repeated treatments was to ensure that the grasses were driven to death so possible mechanisms for mortality could be identified.

From 30 days prior to and during the drought/recovery cycles the SWC of each mesocosm was measured at a depth of 15 cm every 10 days using a Hydrosense II Portable Soil Moisture System (Campbell Scientific Australia Pty Ltd, Garbutt, QLD, Australia). In addition, for the 30 days prior to the extreme drought treatment the stomatal conductance of each grass was measured every five days using a Porometer AP4 (Delta-T Devices, Burwell, CB, United Kingdom). Measurements would begin at 8.30 am and would take approximately three and a half hours to complete. Stomatal conductance was measured at the widest part of the newest fully developed leaf.

### Data analysis

We calculated root to shoot ratio (R:S) and LAI based on the harvested biomass data of the 'before treatment' mesocosms. R:S for each individual grass was calculated as root biomass divided by shoot biomass (leaf and stem biomass). LAI for each individual grass was calculated as their total leaf area divided by ground area in the mesocosm ( $0.24 \text{ m}^2$ ). We used two-way mixed model nested ANOVAs to test for a  $\text{CO}_2$  and plant type (physiology and origin) effect on biomass, R:S and LAI across all grass species in the 'before treatment' mesocosms.  $\text{CO}_2$  level

and plant type were fixed factors and species which was nested within plant type was a random factor.

Kaplan-Meier survival curves were generated to determine the survival function across all the grass species in relation to (1) CO<sub>2</sub> level, (2) physiology, (3) origin and (4) CO<sub>2</sub> level combined with each plant type (origin and physiology). If there was a significant difference between the Kaplan-Meier survival curves within each CO<sub>2</sub> level/plant type combination then the survival distributions within the combination were tested for a significant difference. All survival distributions were compared using log rank tests.

We used General Estimating Equation (GEE) models with a Bonferroni adjustment to determine if there was a difference in the stomatal conductance between CO<sub>2</sub> treatments and each plant type (origin and physiology) combination during the 30 days prior to the extreme drought treatment. The same analyses were performed for SWC with the addition of testing for a difference between the CO<sub>2</sub> treatments for the three drought/recovery cycles. For all GEE models we specified a gamma distribution with log-link model using an exchangeable correlation matrix for the continuous variables (stomatal conductance and SWC).

The canopy transpiration rate of each species was calculated as their average stomatal conductance multiplied by their average LAI. We carried out species-pair comparisons using paired t-tests with a Bonferroni adjustment to test for a CO<sub>2</sub> effect on canopy transpiration rate.



All data analyses were performed using IBM SPSS statistical software, Version 21.0.0 (SPSS Inc., IL, United States, <http://www.spss.com>) with the significance level set at 0.05. Data were  $\log_{10}$  transformed (except for R:S which was arcsine transformed) when necessary to satisfy requirement for normality and fulfil the assumptions of ANOVA.

## Results

There were no significant interactions between CO<sub>2</sub> and plant type for biomass, R:S and LAI. Biomass ( $F_{1,88}=19.13$ ,  $p<0.001$ ) and LAI ( $F_{1,88}=14.08$ ,  $p<0.001$ ) were significantly greater (55% and 30% on average respectively) under elevated CO<sub>2</sub> levels across all the grass species while R:S ( $F_{1,88}=0.21$ ,  $p=0.645$ ) did not significantly differ between CO<sub>2</sub> treatments. The invasive exotic grasses had significantly greater biomass ( $F_{1,88}=15.48$ ,  $p=0.004$ ; 683% on average) and LAI ( $F_{1,88}=19.23$ ,  $p=0.002$ ; 1021% on average). There was a significant species effect for biomass, R:S and LAI (Table S1).

The average survival rate across all grass species was significantly higher under ambient CO<sub>2</sub> levels ( $\chi^2=8.58$ ,  $df=1$ ,  $p=0.003$ ). The physiology ( $\chi^2=3.24$ ,  $df=1$ ,  $p=0.072$ ) or origin ( $\chi^2=0.03$ ,  $df=1$ ,  $p=0.855$ ) of the grasses did not significantly influence their survival rates.

There was a significant difference in survival between the different CO<sub>2</sub> × physiology combinations ( $\chi^2=13.29$ ,  $df=3$ ,  $p=0.004$ ; Fig. 1a). Both the C<sub>3</sub> ( $\chi^2=10.01$ ,  $df=1$ ,  $p=0.002$ ) and C<sub>4</sub> ( $\chi^2=7.91$ ,  $df=1$ ,  $p=0.005$ ) grasses grown under ambient CO<sub>2</sub>

levels had significantly higher survival rates than the C<sub>4</sub> grasses grown under elevated CO<sub>2</sub> levels.

There was a significant difference in survival between the different CO<sub>2</sub> × origin combinations ( $\chi^2=16.64$ ,  $df=3$ ,  $p=0.001$ ; Fig. 1b). The native grasses grown under ambient CO<sub>2</sub> levels had a significantly higher survival rate than the native ( $\chi^2=17.45$ ,  $df=1$ ,  $p<0.001$ ) and exotic ( $\chi^2=6.52$ ,  $df=1$ ,  $p=0.011$ ) grasses grown under elevated CO<sub>2</sub> levels. The exotic grasses grown under ambient CO<sub>2</sub> levels had a significantly higher survival rate than the native grasses grown under elevated CO<sub>2</sub> levels ( $\chi^2=4.13$ ,  $df=1$ ,  $p=0.042$ ).

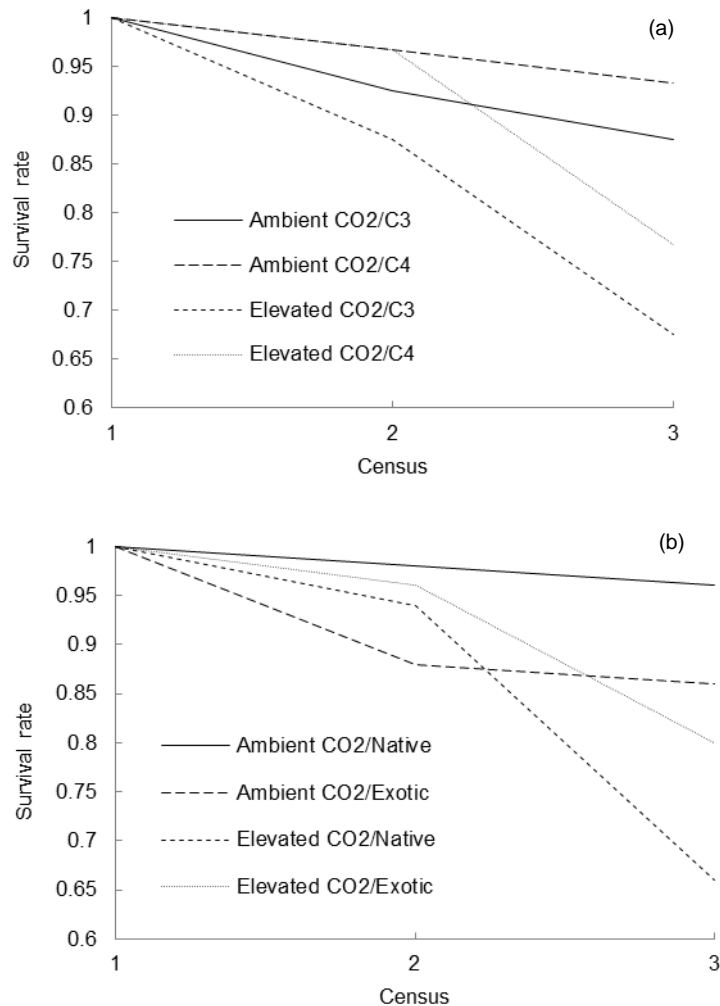


Figure 1: The average survival rate across all grass species of each (a) CO<sub>2</sub> × physiology and (b) CO<sub>2</sub> × origin combination, at each survival census. A survival census was carried out after each cycle which consisted of a 53 extreme drought period and 28 day recovery period.

There was no significant interaction between CO<sub>2</sub> and physiology for stomatal conductance (Wald  $\chi^2=1.786$ , df=1, p=0.181). The C<sub>3</sub> grasses had significantly higher stomatal conductance than the C<sub>4</sub> grasses (Wald  $\chi^2=46.00$ , df=1, p<0.001; figure 2a). There was a significant interaction between CO<sub>2</sub> and origin for stomatal conductance (Wald  $\chi^2=7.369$ , df=1, p=0.007; figure 2b). The exotics grown under ambient CO<sub>2</sub> had significantly higher stomatal conductance than all of the other CO<sub>2</sub> × origin combinations. Stomatal conductance across all grass species was significantly lower under elevated CO<sub>2</sub> levels (Wald  $\chi^2=7.254$ , df=1, p<0.007).

There was no significant difference in the canopy transpiration rate of the grasses between the CO<sub>2</sub> treatments ( $t_8=-0.93$ , p=0.375).

The SWC during the 30 days prior to the drought/recovery cycles was significantly higher under ambient CO<sub>2</sub> compared to elevated CO<sub>2</sub> (Wald  $\chi^2=5.91$ , df=1, p=0.015). The overall SWC of the mesocosms during the experiment drought/recovery cycles was significantly higher under ambient CO<sub>2</sub> compared to elevated CO<sub>2</sub> (Wald  $\chi^2=11.48$ , df=1, p=0.001; figure 3). However SWC differences varied between drought/recovery cycles. During the first drought/recovery cycle SWC was significantly higher in the ambient compared with elevated CO<sub>2</sub> treatment (Wald  $\chi^2=8.98$ , df=1, p=0.003). During the second and third drought/recovery cycles SWC did not significantly differ between CO<sub>2</sub> treatments (second cycle Wald  $\chi^2=0.71$ , df=1, p=0.400; third cycle Wald  $\chi^2=2.504$ , df=1, p=0.114).

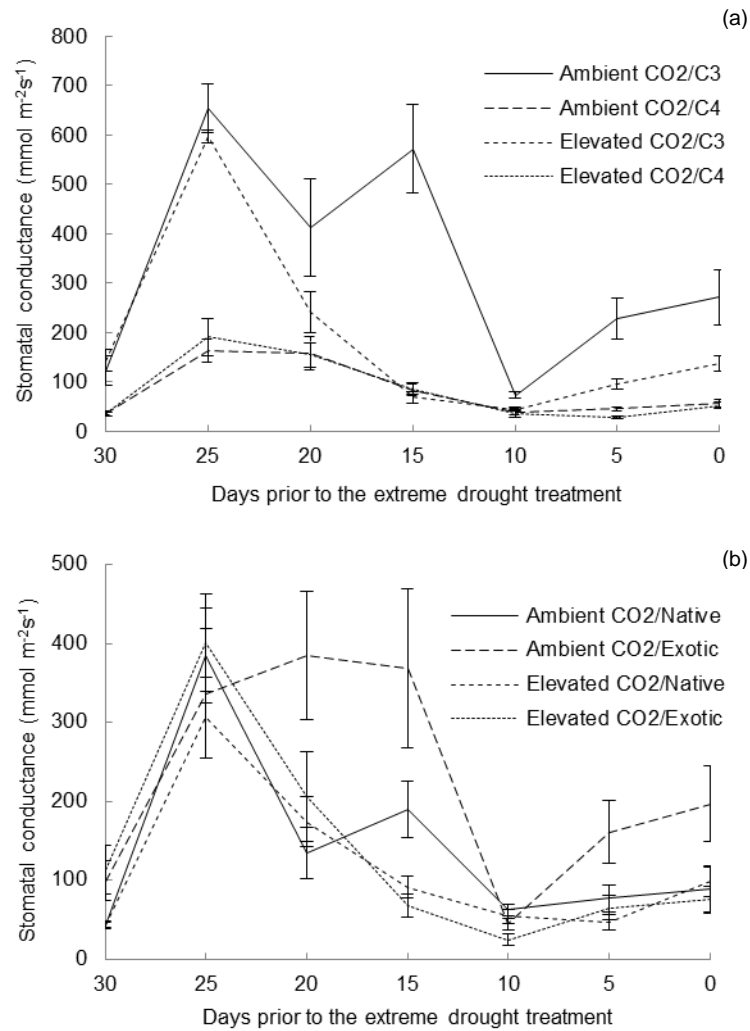


Figure 2: Mean stomatal conductance across all grass species for each (a) CO<sub>2</sub> × physiology and (b) CO<sub>2</sub> × origin combination, during the 30 days prior to the extreme drought treatment. Vertical bars represent one standard error.

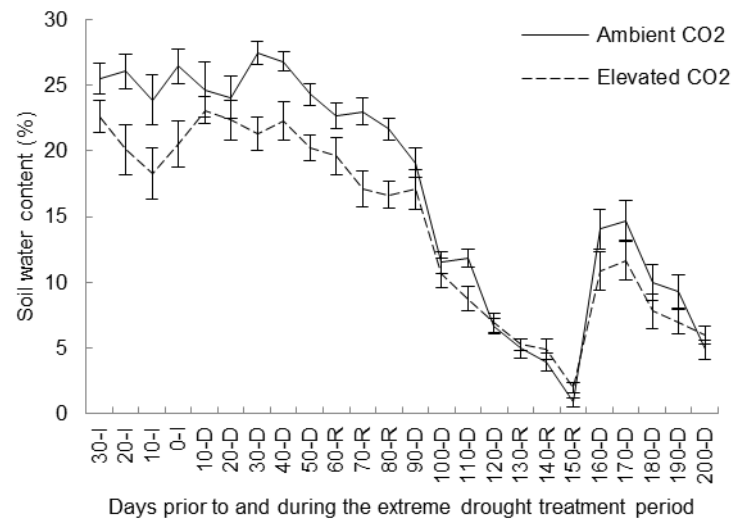


Figure 3: Mean soil water content across all mesocosms under ambient and elevated CO<sub>2</sub> levels over the 30 days prior to the extreme drought treatment and the drought/recovery cycles. The letters on the x-axis represent different phases of the experiment with I=the 30 day period prior to the extreme drought treatment, D=drought period and R=recovery period. Vertical bars represent one standard error.

## Discussion

In this study we tested the vulnerability of an experimental grassland community to repeat extreme drought events under ambient and elevated CO<sub>2</sub> levels. Our results show that grasses grown under elevated CO<sub>2</sub> levels had significantly higher mortality in response to extreme drought than those grown under ambient CO<sub>2</sub> levels. Our original hypothesis was that SWC of the mesocosms and thus vulnerability of the grasses to extreme drought would not differ between the CO<sub>2</sub> treatments because the reductions in stomatal conductance would be offset by increases in LAI. Our results did not support this hypothesis: SWC was significantly lower in the elevated CO<sub>2</sub> mesocosms thus increasing extreme

drought-related mortality of grasses in those mesocosms. The 32% reduction in stomatal conductance under elevated CO<sub>2</sub> levels was offset by a 30% increase in LAI. This offset is shown by the non-significant difference in canopy transpiration rate of the grasses between the CO<sub>2</sub> treatments. These results contrast the results of previous studies that have found that the SWC in semi-arid grasslands increased under elevated CO<sub>2</sub> levels because the increases in total leaf area (related to LAI) were insufficient to offset the reductions in stomatal conductance (LeCain et al. 2003; Morgan et al. 2011).

As the amount of water lost through canopy transpiration did not differ between the CO<sub>2</sub> treatments, differences in SWC must have been due to the amount of water reaching the soil surface. Rain throughfall decreases with LAI because of increased interception and subsequent evaporation of rainfall from the surfaces of leaves (Woodward 1990). In this experiment the grasses had significantly higher LAI under elevated CO<sub>2</sub> levels. This would have increased rainfall interception consequently causing differences in the SWC between the CO<sub>2</sub> treatments prior to the extreme drought treatments. As the experiment progressed through each drought/recovery cycle, the initial difference in SWC between the CO<sub>2</sub> treatments converged. This convergence of SWC between the CO<sub>2</sub> treatments coincided with the mortality of grasses in the elevated CO<sub>2</sub> mesocosms. We suggest that this is because there were fewer individuals (due to the extreme drought-related mortality) in the elevated CO<sub>2</sub> mesocosms which would have reduced the canopy transpiration and rainfall interception in those mesocosms.

We hypothesised that decreases in stomatal conductance under elevated CO<sub>2</sub> levels would be greater for the C<sub>4</sub> and native grasses. Surprisingly we found that the exotics had the greatest reduction in stomatal conductance among the grasses under elevated CO<sub>2</sub> levels. It is often assumed that native plants are less influenced by water limited conditions (e.g. extreme drought) in comparison to exotic plants (Funk and Zachary 2010; Diez et al. 2012). Our findings suggest that the exotic grasses would be less influenced by extreme drought than C<sub>4</sub> and native grasses in a high CO<sub>2</sub> world. However it is difficult to suggest if this could significantly alter the species composition of grasslands. This is because changes in SWC can alter the structure and function of grasslands in aspects other than productivity that may influence species composition. SWC can contribute to species shifts within grasslands by changing seed production and seedling recruitment among species (Niklaus et al. 2001; Morgan et al. 2004) and altering competitive interactions among established plants (Polley et al. 2003; Dijkstra et al. 2010; Polley et al. 2012).

The findings from our study suggest that CO<sub>2</sub> concentration and soil water availability are important in mediating grassland productivity through biomass die-back and mortality. It has been projected that a drastic shift in the annual global precipitation patterns may result in up to a 20% loss in soil water (Schiermeier 2008). This is reinforced by global climate models which predict that large areas on every inhabitable continent will experience intensified droughts and widespread decreases in soil water (IPCC 2011). However, grassland productivity is projected to increase under elevated CO<sub>2</sub> levels. Parton et al. (1995) modelled the effects of elevated CO<sub>2</sub> levels on 31 temperate and tropical grassland sites,



using the CENTURY model. They found that with the exception of cold desert steppe regions grassland productivity increased under elevated CO<sub>2</sub> levels (Parton et al. 1995). From our results we suggest that this projected global increase in the grassland productivity under elevated CO<sub>2</sub> levels may be negated (by biomass die-back and mortality) due to the projected soil water constraints in the future, both as a direct consequence of changed precipitation and changes in plant-level traits such as stomatal conductance and LAI.

This study has shown that vegetation response to climatic extremes is likely to be affected by CO<sub>2</sub> concentration, with extreme drought-related mortality of grasses increasing under elevated CO<sub>2</sub> levels. The suggested mechanism for this increased mortality is an increase in the LAI of the grasses which offset reductions in stomatal conductance and increased rainfall interception prior to extreme drought events. This highlights the importance of considering the interactions between climatic extremes and other aspects of climate change such as CO<sub>2</sub> concentration on plant communities. Our results also highlight the importance of better quantification of soil water in global climate and vegetation models as this may be a key driver affecting global vegetation patterns and responses to climate change. It should be noted that this study only examined the response of a single growth stage (i.e. mature) of grasses to repeated extreme drought events under different CO<sub>2</sub> treatments. However, the vulnerability of plants to climatic extremes may be different in the early stages of their growth and development in comparison to mature plants. Therefore it is important to also consider the effect of climatic extremes on seed germination and seedling establishment as these stages are likely to strongly influence vegetation dynamics (Allen et al. 2010).

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## CHAPTER THREE

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**Extreme drought associated reductions in native grass biomass facilitates the invasion of an exotic grass into a temperate model grassland system.**

My contribution to the research and paper: Concept – 90%; Data collection – 80%; Analysis – 100%; Writing – 90%.





## Abstract

The invasion success of exotic plant species is often dependent on resource availability. Aspects of climate change such as rising atmospheric CO<sub>2</sub> concentration and extreme climatic events will directly and indirectly alter resource availability in ecological communities. Understanding how these climate change associated changes in resource availability will interact with one another to influence the invasion success of exotic plants is complex. The aim of this study was to identify the mechanism by which the invasion of exotic plants into grasslands occurs when there are climate change associated changes in resource availability (CO<sub>2</sub> levels and soil water availability as a result of extreme drought). We grew grassland mesocosms consisting of four co-occurring grass species common to the Cumberland Plain Woodland of western Sydney, Australia under ambient and elevated CO<sub>2</sub> levels and subjected them to an extreme drought treatment. We then added seeds of a highly invasive C<sub>3</sub> grass, *Ehrharta erecta*, and assessed its invasion success (biomass production and reproductive output). We found that reduced biomass production of the native grasses in response to the extreme drought treatment enhanced the invasion success of *E. erecta* by creating resource pulses in light and space. Surprisingly CO<sub>2</sub> level did not affect the invasion success of *E. erecta*. Our results suggest that changes to the invasion risk of grasslands in the future will be context-dependent, with invasion risk being strongly coupled to soil water availability and the subsequent response of resident native vegetation.

## Introduction

Biological invasions of exotic species have been identified as a major threat to biodiversity (Mack et al. 2000; van der Wal et al. 2008; Lambertini et al. 2011). Over the past two decades, the potential for exotic species to invade and alter ecosystem structure and function has been increasingly recognized (Levine et al. 2003; Blackburn et al. 2011). Biological invasions may negatively impact plant and animal community structure, nutrient cycling and hydrology (Levine et al. 2003; Simberloff et al. 2013) and are considered a significant management and economic concern (Pimentel et al. 2005; Vila et al. 2009).

A number of hypotheses have been proposed to explain the successful establishment and spread of exotic plants in novel environments (Dietz and Edwards 2006). Perhaps the best supported hypothesis is that invasive exotic plants are most successful in environments where there is high resource availability (Davis et al. 2000; Daehler et al. 2003; Leishman and Thomson 2005; Blumenthal et al. 2006). For example, it has been shown that N-addition to Californian serpentine grasslands resulted in a 2-fold and 9-fold increase in biomass production and reproductive output respectively of three invasive exotic grass species (Going et al. 2009). Climate change may cause profound changes in resource availability within communities, creating opportunities for exotic plants to invade (Bradley et al. 2010). Rising atmospheric CO<sub>2</sub> concentration and extreme climatic events are two aspects of climate change that will directly and indirectly (through resident native species responses) affect resource availability in ecological communities (Dukes and Mooney 1999; Diez et al. 2012; Sorte et al. 2013).

Atmospheric CO<sub>2</sub> concentration has been rising steadily since the Industrial Revolution from a concentration of ~270 ppm to current levels of ~400 ppm (IPCC 2013). This increase is predicted to continue under a range of emission scenarios (IPCC 2013). It has been shown that plants with fast growth rates tend to be favoured by elevated CO<sub>2</sub> levels (Tangley 2001; Poorter and Navas 2003). Invasive exotic plants often have leaf-level traits, such as high specific leaf area, high leaf N content and high photosynthetic capacity, that are associated with rapid carbon capture strategies resulting in fast growth when resources are not limited (Grotkopp and Rejmanek 2007; Leishman et al. 2007; Leishman et al. 2010). Several studies have shown that elevated CO<sub>2</sub> levels enhance biomass production and reproductive output of invasive exotic plants relative to their native counterparts in a range of grassland systems (Smith et al. 2000; Baruch and Jackson 2005; Dukes et al. 2011; Manea and Leishman 2011; Blumenthal et al. 2013). Therefore it is likely that rising atmospheric CO<sub>2</sub> concentration may be an important driver in enhancing the invasion success of exotic plants in grasslands, particularly when resources are not limited.

Conversely, aspects of climate change that reduce resource availability could inhibit the growth of invasive exotic plants (Bradley et al. 2010). It is widely acknowledged that the magnitude and frequency of climatic extremes, such as extreme drought, are likely to increase under future climate change conditions. Low soil water availability caused by extreme drought is one of the major limitations to the growth and reproductive output of plants (De Boeck et al. 2010). This suggests that the invasion risk of ecological communities under extreme

drought conditions will be reduced particularly as fast-growing invasive exotic plants tend to have a larger water demand than their native counterparts (Cavaleri and Sack 2010; Funk and Zachary 2010; Diez et al. 2012; see exception Funk and Vitousek 2007). For example, Baruch and Jackson (2005) found that a widespread native grass species (*Trachypogon plumosus*) was more tolerant of vapour-pressure deficits and soil water-limited conditions than two common invasive African grass species (*Hyparrhenia rufa* and *Melinis minutifolia*) in Venezuela. However this less conservative water use of invasive exotic plants may be negated to some extent by water savings of plants in general under elevated CO<sub>2</sub> levels as a result of reductions in the stomatal conductance (Blumenthal et al. 2013).

An additional complication is that resource availability may also affect the resident native vegetation within ecological communities (Davis et al. 2000). This may lead to changes in the competitive interactions between the resident native and invasive exotic species (Davis et al. 2000). Similarly to invasive exotic grassland species, native grassland species have been shown to have increased biomass production and reproductive output under elevated CO<sub>2</sub> levels (Owensby et al. 1999; Morgan et al. 2001; Shaw et al. 2002; Dijkstra et al. 2010). This in turn would increase the competitive effect of the resident native species on the invasive exotic species thus increasing the resilience of the grassland to invasion. Elevated CO<sub>2</sub> levels may also facilitate exotic plant invasion into grasslands by increasing the amount of soil water available to the invasive exotic species through reductions in the stomatal conductance of the native vegetation. This was the case in mixed-grass Wyoming Prairie, United States where a reduction in stomatal conductance of the dominant C<sub>3</sub> native grass species, *Pascopyrum smithii*,

contributed to an increase in soil water content (SWC) (Blumenthal et al. 2013). This in part contributed to a 13-fold increase in biomass production and 32-fold increase in reproductive output of an introduced forb, *Linaria dalmatica* (Blumenthal et al. 2013). In contrast, reduced soil water availability as a result of extreme drought may cause biomass die-back in resident native grassland species (Kreyling et al. 2008; Beierkuhnlein et al. 2011). If the native resident species do not immediately recover when the extreme drought ends, then a resource pulse of light and space will be created resulting in colonisation opportunities for potential invaders (Jimenez et al. 2011; Diez et al. 2012). It is not clear how these complex interactions between different climate change-driven changes in resource availability will affect invasion success of exotic species (Sorte et al. 2013).

Grasslands are dynamic systems that are particularly vulnerable to exotic plant invasion due to rising atmospheric CO<sub>2</sub> concentration, soil nutrient enrichment, changes in disturbance regimes, increased introduction of exotic plant species and other human associated impacts (Seastedt and Pysek 2011). In this study we asked if climate change-associated changes in resource availability (CO<sub>2</sub> levels and soil water availability as a result of extreme drought) alter the invasion success of exotic plants into grasslands, and if so, what is the mechanism? Experimental mesocosms containing native grass species from the grassy open woodland community known as Cumberland Plain Woodland (CPW) that occurs in western Sydney, Australia were grown under ambient and elevated CO<sub>2</sub> levels and exposed to a one in 20 year extreme drought event. Seeds from the invasive exotic grass species, *Ehrharta erecta*, were then added to the experimental grassland system and invasion success was assessed based on biomass production and reproductive

output of the invader. We selected CPW as our study system because its highly fragmented nature coupled with heavy grazing by cattle in the past has meant exotic plants have been able to invade most CPW remnants and pose a major problem for management (Benson and Howell 2002).

## **Materials and methods**

### **Species selection**

We selected four native and one invasive exotic grass species which commonly co-occur in CPW. All of the selected grass species are perennial. Within the native group we included two  $C_3$  (*Austrodanthonia racemosa* and *Microlaena stipoides*) and two  $C_4$  (*Bothriochloa macra* and *Themeda australis*) species. We selected *Ehrharta erecta* Lam. (Panic Veldtgrass) as our invasive exotic grass species because it is considered a high risk invader in Cumberland Plain plant communities (Little 2003). It is a tufted  $C_3$  grass that grows up to 0.6 m and is native to South Africa (The Royal Botanic Gardens and Domain Trust, Sydney, NSW, Australia, <http://www.plantnet.com>). Seeds for the four native grass species were obtained from a commercial supplier (Nindethana Seed Service, Albany, WA, Australia). *E. erecta* seeds were collected from a number (>50) of individual plants from Macquarie University campus (33.78°S, 151.11°E). The seeds for each of the four native grass species were germinated on moist paper towels within covered aluminium trays. To spread the risk of germination failure, each species was germinated in a number of different aluminium trays.

### Experimental design and treatments

We established 24 mesocosms containing a mix of the native grass species. Within each mesocosm two individuals from each of the four native grass species (eight grasses per mesocosm) were planted at the stage of second true leaf emergence in random positions along two rows. Each mesocosm consisted of a 65 L tub (60 cm long × 40 cm wide × 28 cm deep), containing 60 L of field-collected clay soil from the Cumberland Plain. The soil was collected from Mt Annan (34.07°S, 150.76°E) and Luddenham (33.88°S, 150.69°E) in western Sydney and was homogenised into one batch using a cement mixer.

The mesocosms were divided evenly between the ambient and elevated CO<sub>2</sub> levels (each CO<sub>2</sub> level contained 12 mesocosms), across four glasshouses (two ambient and two elevated). All mesocosms were mist watered for one minute twice daily (~2.25mm daily) which is representative of the average daily amount of rainfall (828 mm annually) on the Cumberland Plain. This daily rainfall average was based on the Australian Bureau of Meteorology historical records from Camden airport (1943-2004).

CO<sub>2</sub> treatments were set to two levels: ambient (380-420 ppm) and elevated (530-570 ppm). These CO<sub>2</sub> concentration ranges were maintained and monitored continuously by a CO<sub>2</sub> dosing and monitoring system (Canary Company Pty Ltd, Lane Cove, NSW, Australia). The elevated CO<sub>2</sub> treatment represents the predicted atmospheric CO<sub>2</sub> concentration by 2060 under the RCP 4.5 emissions scenario (IPCC 2013). The temperature of the glasshouses was set for a minimum of 16°C and a maximum of 24°C. Relative humidity of the glasshouses was

monitored daily at 9am and 3pm using a HOBO temperature/RH/2 external channel data logger (OneTemp, Parramatta, NSW, Australia). Using a paired t-test with a Bonferroni adjustment we found no significant difference in the relative humidity between the ambient and elevated CO<sub>2</sub> glasshouses ( $t_{1,38}=1.00$ ,  $p=0.326$ ).

The native grasses were grown in the mesocosms for 12 weeks until they were mature (i.e. had several tillers). At this stage each mesocosm received 30 g of Osmocote slow release low (16N:1.6P:8K) P fertiliser (Scotts Australia Pty Ltd, Bella Vista, NSW, Australia). After the 12 week growth period an extreme drought treatment was applied to six of the mesocosms under each CO<sub>2</sub> level. We defined a drought as the number of consecutive days with <1mm of rainfall. This definition is a part of the ETCCDI/CRD climate change indices (CLIVAR, Laoshan, QIN, China, <http://www.etccdi.pacificclimate.org>). We used the IPCC (2011) definition of climatic extreme which is an extreme that occurs once every 20 years, on average. Gumbel I distributions were fitted to the annual drought extremes of the Cumberland Plain for each year from 1867-2010. Rainfall on the Cumberland Plain is not seasonal so the time of year that the annual drought extremes occurred differed from year to year. The data used were obtained from the Australian Bureau of Meteorology historical records of Brownlow Hill (34.03°S, 150.65°E, 1867-1969), Kentlyn (34.05°S, 155.88°E; 1970-1971), Camden Airport (34.04°S, 150.69°E; 1972-1992, 1998-2001), Ruse (34.06°S, 150.85°E; 1993-1997) and Mt Annan Botanical Gardens (34.07°S, 150.76°E; 2002-2010). A one in 20 year extreme drought event for the Cumberland Plain was calculated to last for a period of 53 days. The extreme drought was simulated by turning off the watering system for the treatment period.



After the extreme drought treatment, 100 *E. erecta* seeds were then added to each of the mesocosms. Seeds were added by digging 15mm deep trenches between the established native grasses, sprinkling the seeds into the trenches and then backfilling the trenches with soil. Once the *E. erecta* seeds were added, every mesocosm received the control watering treatment (~2.25mm daily). After 16 weeks all native and *E. erecta* grasses in each mesocosm were harvested, washed free of soil and separated into their following components: leaf biomass, stem biomass and root biomass. The total leaf area of each individual native grass was measured using a LI-3100C Area Meter (Li-Cor, Lincoln, NE, United States). The leaf area index (LAI) for each individual native grass was then calculated as total leaf area divided by ground area in the mesocosm (0.24 m<sup>2</sup>). The plant components were then oven-dried at 60°C for 72 hours and weighed using an analytical electronic balance (Mettler Toledo, Port Melbourne, VIC, Australia).

At the beginning of the extreme drought treatment the stomatal conductance of two randomly selected individuals from each native grass species was measured for each CO<sub>2</sub> level × watering treatment combination every 10 days for the remainder of the experiment using a Porometer AP4 (Delta-T Devices, Burwell, CB, United Kingdom). Measurements began at 10 am and took approximately two hours to complete. Stomatal conductance was measured at the widest part of the newest fully developed leaf. Also at the beginning of the extreme drought treatment, the SWC of each mesocosm was measured every 10 days for the remainder of the experiment. It was measured at a depth of 15 cm using a Hydrosense II Portable Soil Moisture System (Campbell Scientific Australia Pty Ltd,

Garbutt, QLD, Australia). Seeds produced by *E. erecta* individuals were collected, counted and then oven-dried at 60°C for 48 hours and weighed. The reproductive output of *E. erecta* individuals was calculated by multiplying mean seed mass by seed number.

### Data analysis

We used General Estimating Equation (GEE) models with a Bonferroni adjustment to determine if there was a difference in mesocosm SWC between CO<sub>2</sub> treatment (ambient vs elevated) and watering treatment (control vs drought) from the beginning of the extreme drought treatment through to the end of the experiment. For all GEE models we specified a gamma distribution with log-link model using an exchangeable correlation matrix for the continuous variable (SWC).

We used three-way mixed model nested ANOVAs to test if biomass, root to shoot ratio (R:S) and canopy transpiration rate across all the native grass species differed between CO<sub>2</sub> level (ambient vs elevated), watering treatment (control vs drought) and plant type (C<sub>3</sub> vs C<sub>4</sub>). The fixed factors were CO<sub>2</sub> level, watering treatment and plant type and the random factor was species which was nested within plant type. The canopy transpiration rate for each individual native grass was calculated as the mean species stomatal conductance multiplied by their LAI.

Ordinary least-squares regression models were generated to determine relationships between total mesocosm native grass biomass and (1) mean mesocosm SWC, (2) total mesocosm *E. erecta* biomass and (3) total mesocosm *E. erecta* reproductive output.

We used two-way ANOVAs to test for a CO<sub>2</sub> level (ambient vs elevated) and watering treatment (control vs drought) effect on *E. erecta* biomass and *E. erecta* reproductive output.

All statistical analyses were performed using IBM SPSS statistical software, Version 21.0.0 (SPSS Inc., 2012, IBM, Illinois, United States, <http://www.spss.com>) with the significance level set at 0.05. Data were log<sub>10</sub> transformed when necessary to satisfy requirement for normality and fulfil the assumptions of ANOVA.

## Results

There was no significant interaction between CO<sub>2</sub> level and watering treatment for SWC (Wald  $\chi^2=1.07$ , df=1, p=0.302). SWC of the extreme drought treatment mesocosms was significantly lower (43% on average) than the control watering treatment mesocosms (Wald  $\chi^2=86.27$ , df=1, p<0.001; Fig. 1). SWC did not differ between the ambient and elevated CO<sub>2</sub> mesocosms (Wald  $\chi^2=0.428$ , df=1, p=0.513; Fig. 1).

Native grasses that were exposed to the extreme drought treatment had significantly less biomass ( $F_{1,156}=19.20$ , p<0.001; 35% on average; Fig. 2a) and lower canopy transpiration rates ( $F_{1,156}=18.68$ , p<0.001; 34% on average; Fig. 2b) than those grown under the control watering treatment but did not differ in their R:S ( $F_{1,156}=2.95$ , p=0.088). R:S was significantly greater ( $F_{1,156}=5.57$ , p=0.020; 25% on average) under elevated CO<sub>2</sub> levels while biomass ( $F_{1,156}=0.24$ , p=0.625) and canopy transpiration rates ( $F_{1,156}=2.83$ , p=0.094) did not differ between the two CO<sub>2</sub> levels. The C<sub>3</sub> and C<sub>4</sub> native

grasses did not have significantly different biomass ( $F_{1,156}=0.77$ ,  $p=0.472$ ), R:S ( $F_{1,156}=0.81$ ,  $p=0.461$ ) and canopy transpiration rates ( $F_{1,156}=0.27$ ,  $p=0.655$ ). There were significant differences between native grass species for biomass ( $F_{2,156}=153.17$ ,  $p<0.001$ ), R:S ( $F_{2,156}=5.11$ ,  $p=0.007$ ) and canopy transpiration rates ( $F_{2,156}=184.11$ ,  $p<0.001$ ) (Table S2). There were no significant higher order interactions among factors (Table S3).

Total mesocosm native grass biomass was strongly positively related with mean mesocosm SWC ( $F_{1,22}=24.13$ ,  $p<0.001$ , slope=0.42,  $r^2=0.52$ ; Fig. 3a). In contrast, total mesocosm native grass biomass was negatively related with total mesocosm *E. erecta* biomass ( $F_{1,18}=10.54$ ,  $p=0.004$ , slope=-3.24,  $r^2=0.37$ ; Fig. 3b) and reproductive output ( $F_{1,18}=12.17$ ,  $p=0.003$ , slope=-4.26,  $r^2=0.40$ ; Fig. 3c). These relationships suggest that SWC drives native grass biomass production which in turn drives the invasion success (biomass production and reproductive output) of *E. erecta*.

There was no significant interaction between CO<sub>2</sub> level and watering treatment for the biomass production ( $F_{1,99}=0.59$ ,  $p=0.442$ ) and reproductive output ( $F_{1,99}=0.02$ ,  $p=0.893$ ) of *E. erecta*. *E. erecta* biomass production ( $F_{1,99}=4.51$ ,  $p=0.036$ ; Fig. 4a) and reproductive output ( $F_{1,99}=5.01$ ,  $p=0.027$ ; Fig. 4b) were significantly greater (190% and 307% on average respectively) in the extreme drought mesocosms. In contrast CO<sub>2</sub> level did not significantly affect biomass production (Fig. 4a) and reproductive output (Fig. 4b) of *E. erecta*.

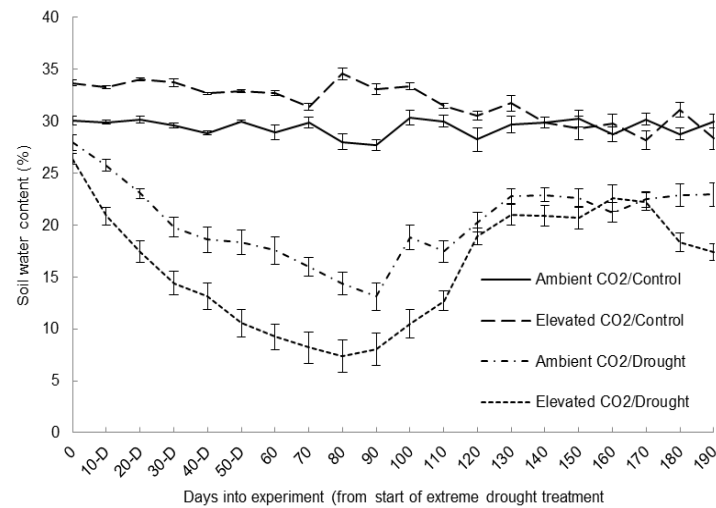


Figure 1: Mean soil water content across all mesocosms for each CO<sub>2</sub> level (ambient vs elevated) × watering treatment (control vs drought) combination over the extreme drought treatment period and remainder of the experiment. The letter ‘D’ on the x-axis represent the extreme drought treatment period of the experiment. Vertical bars represent one standard error.

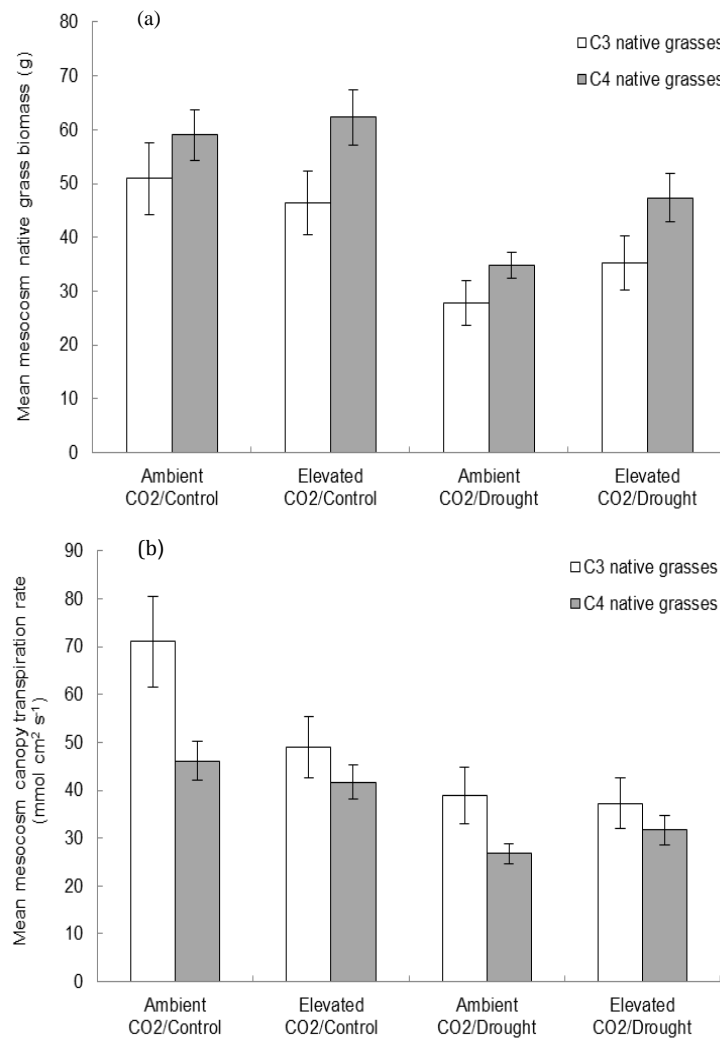


Figure 2: Mean mesocosm C<sub>3</sub> and C<sub>4</sub> native grass (a) biomass and (b) canopy transpiration rate for each CO<sub>2</sub> level (ambient vs elevated) × watering treatment (control vs drought) combination. Vertical bars represent one standard error.

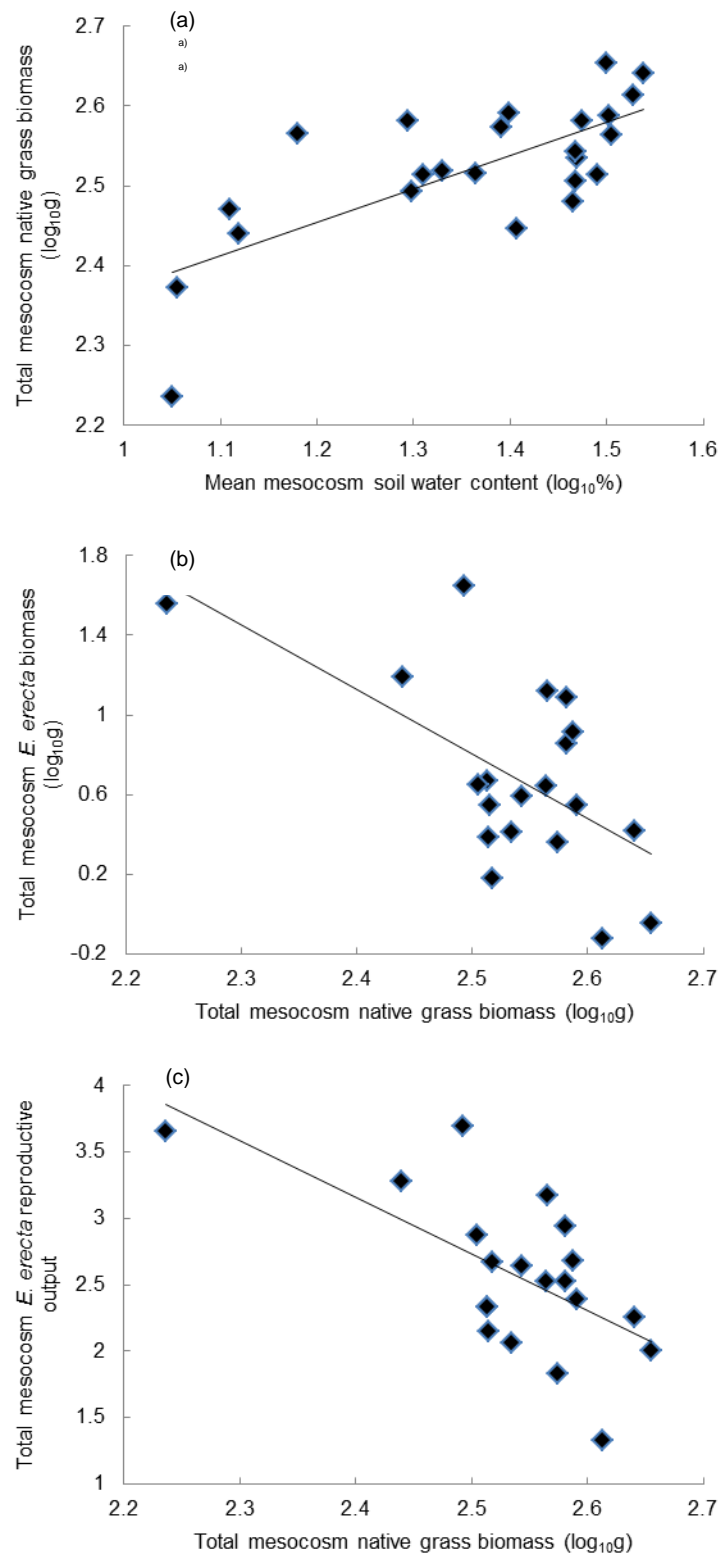


Figure 3: Relationships between total mesocosm native biomass and (a) mean mesocosm SWC, (b) total mesocosm *E. erecta* biomass and (c) total mesocosm *E. erecta* reproductive output. Points represent variable values at the mesocosm-level.

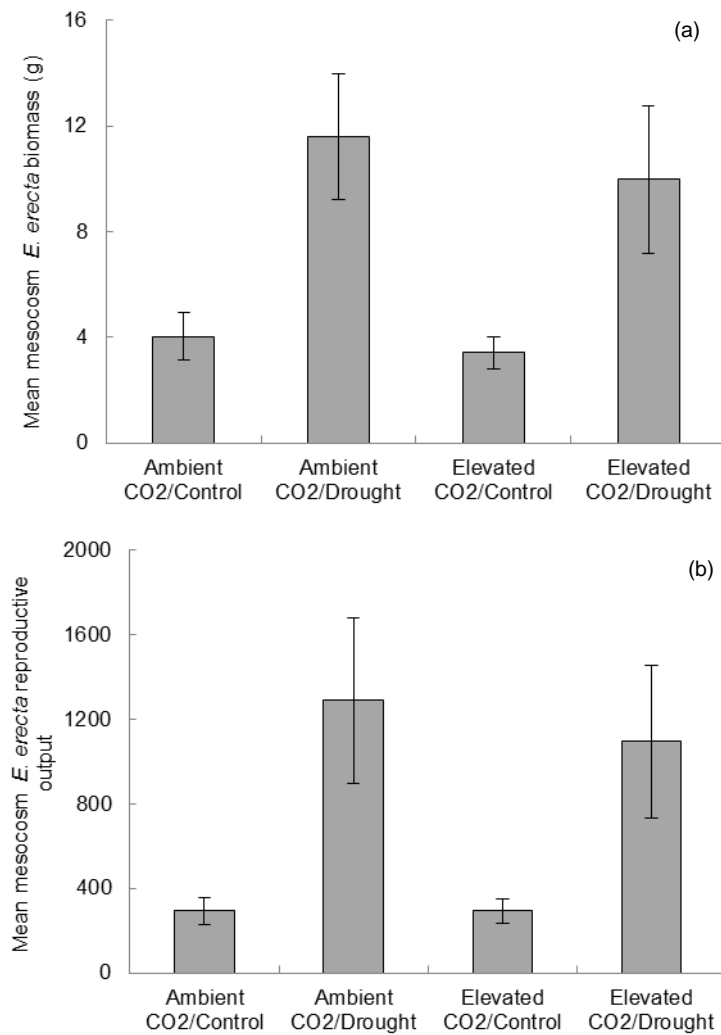


Figure 4: Mean mesocosm *E. erecta* (a) biomass and (b) reproductive output for each CO<sub>2</sub> level (ambient vs elevated) × watering treatment (control vs drought) combination. Vertical bars represent one standard error.



## Discussion

The aim of this study was to identify the mechanism by which the invasion of exotic plants into grasslands may occur when there are climate change-associated changes in resource availability. We found that mesocosm SWC was significantly reduced (43% on average) by the extreme drought treatment which resulted in significant less native grass biomass (35% on average). It should be noted that this reduction in native grass biomass was a result of both reduced production and individuals dying. Furthermore native grass biomass production was strongly positively related to SWC at the mesocosm-level. These findings suggest, not surprisingly, that SWC was an important driver of native grass biomass production in the mesocosms. This is consistent with previous studies that have shown biomass production in grasslands is strongly mediated by soil water availability (Asner et al. 2004; Morgan et al. 2004; Fay et al. 2008; Morgan et al. 2011). Surprisingly there was no difference in relative biomass response between the C<sub>3</sub> and C<sub>4</sub> native grasses within the extreme drought treatment. This was unexpected because at a given CO<sub>2</sub> concentration C<sub>4</sub> grasses should be able to retain more water compared to C<sub>3</sub> grasses (Osborne and Sack 2012; see Ghannoum 2009 for exception) thus making them less vulnerable to extreme drought. There were strong negative relationships between native grass biomass production and the biomass production and reproductive output of *E. erecta*. The reduction in native grass biomass as a result of the extreme drought treatment in our experiment translated into an increase in mesocosm biomass (190% on average) and reproductive output (307% on average) of *E. erecta*. Our results suggest that reduced biomass production of resident native species in grasslands in response to extreme drought may facilitate exotic plant invasion by creating resource pulses in

light and space. Similar findings have been reported for mixed-grasslands in New Zealand and United States where native biomass die-back resulting from extreme temperature and drought respectively has facilitated invasion into the system (White et al. 2001; Scott et al. 2010).

It is evident from our results that soil water availability is an important driver of native biomass production in grasslands which in turn strongly mediates their susceptibility to exotic plant invasion. In addition to changes in precipitation patterns (e.g. extreme drought), soil water availability can also be driven in part by complex interactions between factors such as temperature, soil properties and CO<sub>2</sub> level (Morgan et al. 2011). Temperature and soil properties were standardised in our study while CO<sub>2</sub> level was not. Elevated CO<sub>2</sub> levels induce stomatal closure, which reduces canopy transpiration thus increasing soil water availability (Morgan et al. 2001; Leakey et al. 2009). This was the case in native mixed-grass Wyoming Prairie in the United States where annual SWC increased on average by 17.3% over a three year period under elevated CO<sub>2</sub> levels due partly to reductions in stomatal conductance (Morgan et al. 2011). In our study, stomatal conductance was reduced on average by 26% in the native grasses under elevated CO<sub>2</sub> levels. However the amount of water that is lost through canopy transpiration not only depends on the stomatal conductance of vegetation but also on its LAI (Woodward 1990). We found that the LAI of the native grasses increased (17% on average) under elevated CO<sub>2</sub> levels which negated to some extent the reductions in stomatal conductance. This resulted in non-significant differences in native grass canopy transpiration rates and mesocosm SWC between the two CO<sub>2</sub> levels. Although similar findings have been reported for CPW grassland (Manea and Leishman

2014), contrary findings have been reported for other grassland systems (LeCain et al. 2003; Morgan et al. 2011). From these results we suggest that CO<sub>2</sub> driven changes in soil water availability (and subsequently invasion risk) are highly dependent on changes in LAI as stomatal conductance responses to elevated CO<sub>2</sub> levels are often predictable (Ainsworth and Long 2005; Ainsworth and Rogers 2007; Leakey et al. 2009).

The effect of climate change-associated changes in resource availability on the invasion risk of ecological communities is complex and context-dependent. For example, although invasive exotic species may initially be better able to take advantage of resource pulses resulting from extreme drought and colonise an area, native species in drought-prone ecosystems may be better adapted to survive prolonged drought conditions (Diez et al. 2012). Addressing this complex issue will require a range of approaches including manipulative experiments, observational studies and modelling (Diez et al. 2012). Controlled manipulative experiments such as this study provide a powerful approach for isolating mechanisms that are driving plant responses to these changes in resource availability (Smith 2011). Although our study only provides an insight into the short-term effects of climate change-associated changes in resource availability on the invasion risk of grasslands it has shown that we need to consider the indirect (through resident native vegetation responses) as well as the direct responses of these resource changes on invasive exotic species. It is vital that we continue to gain a better understanding of how climate change-associated changes in resource availability will alter the invasion success of exotic species as we develop effective policies and programs to manage these species in the future (Diez et al. 2012).

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## CHAPTER FOUR

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**Competitive interactions between established grasses and woody plant seedlings under elevated CO<sub>2</sub> levels are mediated by soil water availability.**

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**Abstract**

The expansion of woody plants into grasslands has been observed worldwide and is likely to have widespread ecological consequences. It has been proposed that woody plant expansion into grasslands is driven in part by the rise in atmospheric CO<sub>2</sub> concentration. We examined the effect of CO<sub>2</sub> concentration on the competitive interactions between established C<sub>4</sub> grasses and woody plant seedlings in a model grassland system. We grew woody plant seedlings in mesocosms together with established C<sub>4</sub> grasses in three competition treatments (root competition, shoot competition and root plus shoot competition) under ambient and elevated CO<sub>2</sub> levels. We found that woody plant seedling growth was suppressed by competition from grasses, with root and shoot competition having the similar competitive effects on growth. In contrast to expectations, woody plant seedling growth was reduced under elevated CO<sub>2</sub> levels across all competition treatments, with the most plausible explanation being reduced light and soil water availability in the elevated CO<sub>2</sub> mesocosms. Reduced light and soil water availability in the elevated CO<sub>2</sub> mesocosms was associated with increased leaf area index of the grasses which offset the reductions in stomatal conductance and increased rainfall interception. The woody plant seedlings also had reduced 'escapability' (stem biomass and stem height) under elevated CO<sub>2</sub> levels. Our results suggest that the expansion of woody plants into grasslands in the future will likely be context-dependent, with establishment success of woody plant seedlings being strongly coupled to the CO<sub>2</sub> response of competing grasses and to soil water availability.

## Introduction

Grass-dominated ecosystems (hereon referred to as grasslands) range from open grassland to densely canopied savanna and woodland with up to 80% woody plant cover (Sankaran et al. 2005). They occupy more than 30% of the global terrestrial landscape (Asner et al. 2004) and act as a sink for large amounts of soil carbon, subsequently playing an important role in the global carbon cycle (Briggs et al. 2005; Grace et al. 2006). Historically, the major threat to grasslands was their conversion to an agricultural landscape (Briggs et al. 2005), however it is being increasingly recognised that global grasslands face a new threat from the increased cover and abundance of woody plants (Briggs et al. 2005; Ward 2005). The expansion of woody plants into grasslands has been observed worldwide, with examples from Africa (Roques et al. 2001; Buitenwerf et al. 2012), North America (Briggs et al. 2002; Knapp et al. 2008), South America (Cabral et al. 2003) and Australia (Bowman et al. 2010; Lunt et al. 2010). Woody plant encroachment of grasslands can significantly impact on ecosystem processes such as carbon and water fluxes (Scott et al. 2006), which is likely to have widespread ecological consequences (Briggs et al. 2005).

Woody plant encroachment of grasslands is generally attributed to changes in climate, grazing intensity and fire regimes (Archer et al. 1995; Scholes and Archer 1997). Recently, it has been proposed that increases in woody plant cover are linked to the rising atmospheric CO<sub>2</sub> concentration that has occurred over the last 200 years (Polley et al. 1997; Bond and Midgley 2000; Buitenwerf et al. 2012). This trend is predicted to continue, with dynamic global vegetation models suggesting a continued worldwide increase in woody plant biomass in a high CO<sub>2</sub> world (Prentice et al. 2011). For example, a five-year CO<sub>2</sub> open-top chamber experiment in a semi-arid Colorado

grassland found that doubling of present-day CO<sub>2</sub> concentration resulted in a 20-fold increase in cover of the common shrub *Artemisia frigida* (Morgan et al. 2007). The cover of adult woody plants in grasslands is strongly determined by seedling establishment and sapling escape from the flame zone (Bond 2008; Werner and Prior 2013). Bond and Midgley (2012) suggest that rising atmospheric CO<sub>2</sub> concentration may be enhancing the establishment success of woody plant seedlings in grasslands, resulting in increased woody plant cover. It has been suggested that the enhancement in woody plant seedling establishment success may be due to reductions in the canopy transpiration rates of grasses under elevated CO<sub>2</sub> levels resulting in an increase in soil water availability (Polley et al. 1997). For example, Morgan et al. (2011) found that in the Wyoming mixed-grass prairies in the United States, the annual soil water content (SWC) increased on average by 17.3% over a three year period under elevated CO<sub>2</sub> levels due partly to reductions in canopy transpiration rates. This increase in soil water availability coupled with elevated CO<sub>2</sub> levels may particularly favour invasive exotic and N<sub>2</sub>-fixing woody plants which are most successful in environments where there is high resource availability (Davis et al. 2000; Blumenthal et al. 2006; De Graaff et al. 2006).

The amount of water that is lost through canopy transpiration depends on the stomatal conductance and leaf area index (LAI) of the vegetation (Woodward 1990). Reductions in stomatal conductance of grasses under elevated CO<sub>2</sub> levels have been well-documented (Anderson et al. 2001; Morgan et al. 2001; Morgan et al. 2004). In contrast to reductions in stomatal conductance, grasses often have higher LAI under elevated CO<sub>2</sub> levels (Ward et al. 1999; Newingham et al. 2013; Manea and Leishman 2014), resulting in increased canopy transpiration. Therefore reductions in stomatal

conductance of grasses under elevated CO<sub>2</sub> levels may be offset by increases in their LAI (Piao et al. 2007).

Atmospheric CO<sub>2</sub> concentration may also affect woody plant seedling establishment by influencing 'escapability.' 'Escapability' is defined as the capacity for the woody plant seedlings to gain sufficient height to escape the shaded conditions of the grass-layer canopy. Woody plant seedling establishment, particularly under elevated CO<sub>2</sub> levels, is enhanced in open, well-lit conditions but not under closed canopy conditions (Bond and Midgley 2000). In order to achieve this benefit woody plant seedlings need to gain height and overtop the grass canopy. The large carbon demand required to produce woody structures can be met more efficiently under elevated CO<sub>2</sub> levels (Drake et al. 1997), allowing woody plant seedlings to grow taller more rapidly and escape the shade imposed by the grass canopy. This was the case in the Kgope et al. (2010) study where the stem height of both *A. karroo* and *A. nilotica* seedlings grown individually in pots significantly increased along a CO<sub>2</sub> gradient.

In this study we used a model grassland system representative of the Cumberland Plain Woodland (CPW) of western Sydney, Australia. The CPW is an open woodland community with a diverse grassy under-storey (Little 2003). We established mesocosms of common C<sub>4</sub> grass species of the CPW under ambient and elevated CO<sub>2</sub> levels. We then planted seedlings of common woody plant species into the mesocosms and imposed different competition treatments to assess the relative importance of root competition, shoot competition and root plus shoot competition from the grasses on woody plant seedling growth. We asked (1) is woody plant seedling growth suppressed by competition from grasses?, (2) is the competitive effect of grasses due mainly to root



or shoot competition or to the combination of both?, (3) are the competitive interactions between grasses and woody plant seedlings modified under elevated CO<sub>2</sub> levels, and if so what is the mechanism?, and (4) do some woody plant types (invasive exotics vs natives, N<sub>2</sub>-fixers vs non N<sub>2</sub>-fixers) when grown in competition with grasses have relatively better growth under elevated CO<sub>2</sub> levels?

## **Material and methods**

### Species selection

We selected three C<sub>4</sub> native grass species, four native woody plant species (C<sub>3</sub>) and four invasive exotic woody plant species (C<sub>3</sub>) that commonly co-occur in CPW. All species are perennial. Seeds for each species were collected from a number of individual plants from sites on the Cumberland Plain or were obtained from a commercial supplier (Nindethana Seed Service, Albany, WA, Australia). Information on the biology of each species is provided in Table 1.

### Experimental design and treatments

We established 30 mesocosms containing a mix of the native grass species. Each mesocosm consisted of a 65 L tub (60 cm long × 40 cm wide × 28 cm deep), containing 55 L of field-collected clay soil from the Cumberland Plain. The soil was collected from Mt Annan (34.07°S, 150.76°E) and Luddenham (33.88°S, 150.69°E) in western Sydney and was homogenised into one batch using a concrete mixer. Within each mesocosm four individuals from each grass species (12 grasses per mesocosm) were planted at the stage of second true leaf emergence in random positions along three rows.

Species	Family	Growth form	Photosynthetic pathway	Origin	N <sub>2</sub> -fixing ability
<i>Bothriochloa macra</i> (Steud.) S.T.Blake	Poaceae	Grass	C <sub>4</sub>	Native	No
<i>Chloris truncata</i> R.Br.	Poaceae	Grass	C <sub>4</sub>	Native	No
<i>Themeda australis</i> (R.Br.) Stapf	Poaceae	Grass	C <sub>4</sub>	Native	No
<i>Acacia implexa</i> Benth.	Fabaceae-Mimosoideae	Woody	C <sub>3</sub>	Native	Yes
<i>Bursaria spinosa</i> Cav.	Pittosporaceae	Woody	C <sub>3</sub>	Native	No
<i>Dodonaea viscosa</i> <u>subsp.</u> <i>cuneata</i> Jacq.	Sapindaceae	Woody	C <sub>3</sub>	Native	No
<i>Indigofera australis</i> Willd.	Fabaceae-Faboideae	Woody	C <sub>3</sub>	Native	Yes
<i>Acacia saligna</i> (Labill.) H.L.Wendl.	Fabaceae-Mimosoideae	Woody	C <sub>3</sub>	Exotic	Yes
<i>Cotoneaster glaucophyllus</i> Franch.	Malaceae	Woody	C <sub>3</sub>	Exotic	No
<i>Ligustrum sinense</i> Lour.	Oleaceae	Woody	C <sub>3</sub>	Exotic	No
<i>Senna pendula</i> Willd.	Fabaceae-Caesalpinioideae	Woody	C <sub>3</sub>	Exotic	Yes

Table 1: Selected characteristics of species used in this study. Data obtained from PlantNET (The Royal Botanic Gardens and Domain Trust, Sydney, NSW, Australia, <http://www.plantnet.com>).

The mesocosms were divided evenly between the ambient and elevated CO<sub>2</sub> levels (each CO<sub>2</sub> level contained 15 mesocosms), across four glasshouses (two ambient and two elevated). All mesocosms were mist watered for one minute twice daily (~2.25 mm daily) which is representative of the mean daily amount of rainfall (828 mm annually) on the Cumberland Plain. This daily rainfall mean was based on the Australian Bureau of Meteorology historical records from Camden airport (1943-2004). Each mesocosm received 30 g of Osmocote slow release low P fertiliser (16N:1.6P:8K; Scotts Australia Pty Ltd, Bella Vista, NSW, Australia) at the start of the experiment and again 12 weeks into the experiment.

The grasses were grown in the mesocosms for 12 weeks until they were mature (i.e. had several tillers or were seeding). After the 12 week growth period one individual from each of the eight woody plant species was planted at the stage of cotyledon emergence into random locations within each mesocosm. The mesocosms provided three competition treatments to the woody plants: root competition, shoot competition and root plus shoot competition, with competition treatment randomly allocated among the mesocosms. Each competition treatment had five replicates under each CO<sub>2</sub> level (i.e. 3 competition treatments × 2 CO<sub>2</sub> levels × 5 replicates = 30 mesocosms). Root competition was controlled by planting woody plant seedlings in PVC pipes with a height of 27cm and diameter of 9 cm. The top of these pipes were flush with the soil surface. Shoot competition was controlled by clipping all of the grasses within the mesocosm to a height of 5 mm. We selected clipping as our method to control shoot competition because we were confident that it would eliminate all shoot competition (as opposed to just pushing back the grass canopy). However we do acknowledge that by using this

method we may have also reduced root biomass as the grasses would have had to utilise root non-structural carbohydrates to resprout.

CO<sub>2</sub> treatments were set to two levels: ambient (380-420 ppm) and elevated (530-570 ppm). These CO<sub>2</sub> concentration ranges were maintained and monitored continuously by a CO<sub>2</sub> dosing and monitoring system (Canary Company Pty Ltd, Lane Cove, NSW, Australia). The elevated CO<sub>2</sub> treatment represents the predicted atmospheric CO<sub>2</sub> concentration by 2060 under the RCP 4.5 emissions scenario (IPCC 2013). The temperature of the glasshouses was set for a minimum of 16°C and a maximum of 24°C. Relative humidity of the glasshouses was monitored daily at 9am and 3pm using a HOBO temperature/RH/2 external channel data logger (OneTemp, Parramatta, NSW, Australia). Using a paired t-test with a Bonferroni adjustment we found no significant difference in the relative humidity between the ambient and elevated CO<sub>2</sub> glasshouses ( $t_{1,38}=1.00$ ,  $p=0.326$ ).

The woody plant seedlings were grown in the three competition treatments for 20 weeks. It should be noted that although the woody plant seedlings did not become pot-bound during this growth period the grass species *C. truncata* and *T. australis* did. This may have increased the root density of the grasses and thus their competitive effect on the woody plant seedlings compared to field conditions making it more difficult for the woody plant seedlings to escape root competition. During the 20 week growth period the stomatal conductance of a randomly selected individual of each grass species was measured in each of the root plus shoot competition mesocosms every 10 days using a Porometer AP4 (Delta-T Devices, Burwell, CB, United Kingdom). Measurements began at 10 am and took

approximately two hours to complete. Stomatal conductance was measured at the widest part of the newest fully developed leaf. Also during the 20 week growth period the SWC of the root plus shoot competition mesocosms was measured at a random location within each mesocosm at a depth of 15 cm every five days using a Hydrosense II Portable Soil Moisture System (Campbell Scientific Australia Pty Ltd, Garbutt, QLD, Australia).

After the growth period, the woody plant seedlings from all mesocosms and the grasses from the root plus shoot competition mesocosms were harvested. The woody plant seedlings and grasses were separated into the following components: leaf biomass, stem biomass and root biomass. The total leaf area of each individual grass was measured using a LI-3100C Area Meter (Li-Cor, Lincoln, NE, United States). The LAI for each individual grass was calculated as their total leaf area divided by ground area in the mesocosm ( $0.24 \text{ m}^2$ ). Stem height of the woody plant seedlings was measured as the length from the base of the stem to the apical meristem. The plant components were then washed free of soil before being oven-dried at  $60^\circ\text{C}$  for 72 hours and weighed using an analytical electronic balance (Mettler Toledo, Port Melbourne, VIC, Australia).

### Data analysis

Ordinary least-squares regression models were generated to determine relationships between (1) total mesocosm grass biomass and total mesocosm woody plant seedling biomass; (2) total mesocosm grass biomass and total mesocosm grass LAI; (3) total mesocosm grass LAI and mean mesocosm SWC; and (4) mean mesocosm SWC and total mesocosm woody plant seedling biomass.

We used a three-way ANOVA to test if the total biomass across all the woody plant species differed between the two CO<sub>2</sub> levels for the three competition treatments. CO<sub>2</sub> level and competition treatment were fixed factors and species was a random factor. Across competition treatment differences were tested for using Tukey's HSD post-hoc comparisons. Within competition treatment differences in CO<sub>2</sub> effect were tested for using two-way ANOVAs with CO<sub>2</sub> level and species as the factors.

We analysed total woody plant seedling biomass of the root plus shoot competition mesocosms at a plant type-level using two analyses. The first analysis used origin (native *vs* invasive exotic) and the second analysis used N<sub>2</sub>-fixing ability (N<sub>2</sub>-fixing *vs* non N<sub>2</sub>-fixing) as the plant-type factor. Both analyses used a two-way mixed model nested ANOVA with CO<sub>2</sub> level and plant type as fixed factors and species which was nested within plant type as a random factor. Within plant-type differences in CO<sub>2</sub> effect were tested for using one-way ANOVAs with CO<sub>2</sub> level as the factor.

We used a two-way MANOVA to test for a CO<sub>2</sub> effect on the stem biomass and stem height of the woody plant seedlings in the root plus shoot competition mesocosms across all woody plant species. We then used a two-way MANOVA to test for a CO<sub>2</sub> effect on the total biomass, root biomass, LAI and canopy transpiration rate of the grasses in the root plus shoot competition mesocosms across all grass species. The canopy transpiration rate of each grass species was calculated as their mean stomatal conductance multiplied by their mean LAI. The

factors for the MANOVA analyses were CO<sub>2</sub> level and species. Univariate ANOVAs were only conducted when MANOVA significance was granted.

We used General Estimating Equation models with a Bonferroni adjustment to determine if there was a difference in stomatal conductance of the grass species and the SWC of the mesocosms between the ambient and elevated CO<sub>2</sub> levels. We specified a gamma distribution with log-link model using an exchangeable correlation matrix for the continuous variables (stomatal conductance and SWC).

All statistical analyses were performed using IBM SPSS statistical software, Version 21.0.0 (SPSS Inc., IL, United States, <http://www.spss.com>) with the significance level set at 0.05. Data were log<sub>10</sub> transformed when necessary to satisfy requirement for normality and fulfil the assumptions of ANOVA.

## Results

There were clear relationships between grass biomass, grass LAI, SWC and woody plant seedling biomass. Total woody plant seedling biomass linearly decreased with total grass biomass ( $F_{1,8}=16.71$ ,  $p=0.003$ , slope=-0.62,  $r^2=0.68$ ; Fig. 1a). Grass LAI was strongly positively correlated with total grass biomass ( $F_{1,8}=31.61$ ,  $p<0.001$ , slope=0.04,  $r^2=0.80$ ; Fig 1b) and negatively correlated with SWC at the mesocosm level ( $F_{1,8}=12.83$ ,  $p=0.007$ , slope=-1.52,  $r^2=0.62$ ; Fig. 1c). SWC was positively correlated with woody plant seedling biomass ( $F_{1,8}=5.25$ ,  $p=0.051$ , slope=5.10,  $r^2=0.40$ ; Fig. 1d). These relationships suggest that grass biomass and LAI drive SWC which in turn drives woody plant biomass.

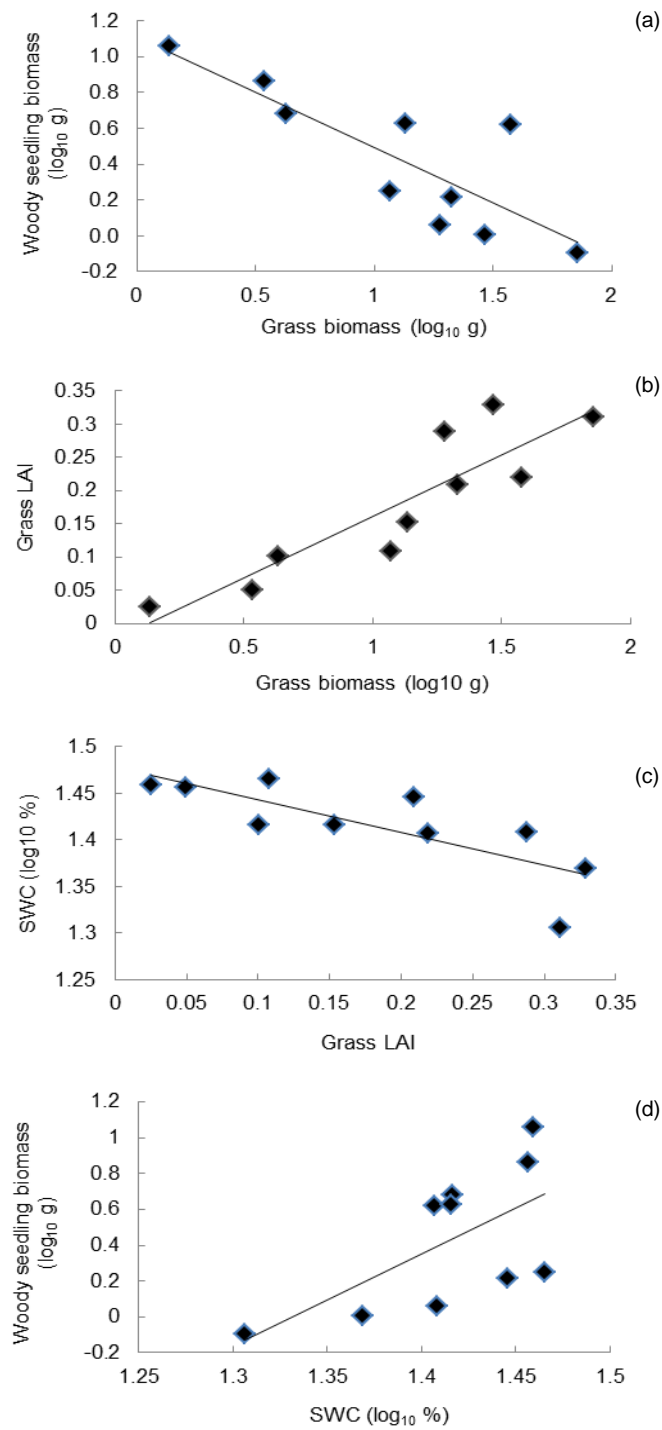


Figure 1: Relationships between (a) total grass biomass and total woody plant seedling biomass, (b) total grass biomass and grass leaf area index (LAI), (c) grass LAI and soil water content (SWC) and (d) SWC and total woody plant seedling biomass. Points represent mean mesocosm values of the root and shoot competition mesocosms.



There was no significant interaction between competition, CO<sub>2</sub> and species for total woody plant seedling biomass ( $F_{14,192}=1.36$ ,  $p=0.175$ ). Furthermore CO<sub>2</sub> did not have a significant interaction with either competition ( $F_{2,192}=3.00$ ,  $p=0.052$ ) or species ( $F_{7,192}=1.55$ ,  $p=0.152$ ). However there was a significant interaction between competition and species ( $F_{14,192}=5.14$ ,  $p<0.001$ ). Post-hoc comparisons showed that woody plant seedlings grown in root plus shoot competition produced 86% and 81% less total biomass than seedlings grown in root competition ( $t=-20.05$ ,  $p<0.001$ ) or shoot competition ( $t=-18.06$ ,  $p<0.001$ ), respectively. The total biomass of woody plant seedlings grown in root competition did not significantly differ from those grown in shoot competition ( $t=1.19$ ,  $p=0.631$ ). Total woody plant seedling biomass was reduced by 46% under elevated CO<sub>2</sub> levels ( $F_{1,192}=26.46$ ,  $p<0.001$ ) (Fig. 2). This was consistent across all competition treatments (root competition  $F_{1,64}=5.17$ ,  $p=0.026$ ; shoot competition  $F_{1,64}=10.00$ ,  $p=0.002$ ; root plus shoot competition  $F_{1,64}=34.92$ ,  $p<0.001$ ) (Fig. 2).

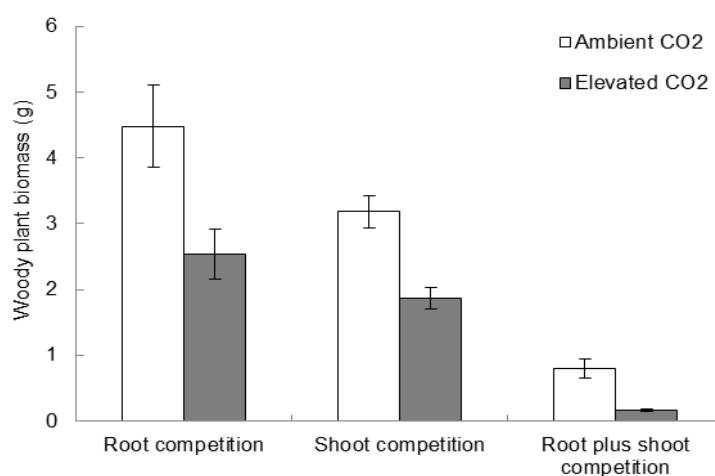


Figure 2: Total woody plant seedling biomass under ambient and elevated CO<sub>2</sub> levels for each competition treatment. Vertical bars represent one standard error.

At the plant type-level for the root plus shoot competition treatment, there was a significant interaction between CO<sub>2</sub> level and N<sub>2</sub>-fixing ability ( $F_{1,70}=5.41$ ,  $p=0.023$ ) but not origin ( $F_{1,70}=0.30$ ,  $p=0.589$ ) for total biomass. Post-hoc comparisons showed that N-fixing woody plant seedling biomass ( $F_{1,38}=7.88$ ,  $p=0.008$ ) was reduced by 82% on average under elevated CO<sub>2</sub> levels while non N-fixing woody plant seedling biomass ( $F_{1,38}=2.14$ ,  $p=0.151$ ) was not affected by CO<sub>2</sub> level. There was no significant difference in total biomass between the native and invasive exotic woody plant seedlings ( $F_{1,70}=2.32$ ,  $p=0.179$ ). However there were significant biomass differences between woody species ( $F_{6,70}=31.90$ ,  $p<0.001$ ).

For all woody plant traits we found significant differences between CO<sub>2</sub> levels, as well as between species when all traits were included in the MANOVA model. There were significant differences between woody species in their response to the CO<sub>2</sub> treatments for stem biomass ( $F_{7,64}=3.61$ ,  $p=0.002$ ; Table S4). The stem biomass for all woody species except *B. spinosa* was significantly greater under ambient compared to elevated CO<sub>2</sub>. Species did not differ significantly in their response to the CO<sub>2</sub> treatments for stem height ( $F_{7,64}=1.21$ ,  $p=0.308$ ; Table S4). The woody plant seedlings had 78% greater stem height ( $F_{7,64}=17.57$ ,  $p<0.001$ ) under ambient compared to elevated CO<sub>2</sub>. There was also a significant species effect for stem height ( $F_{7,64}=33.36$ ,  $p<0.001$ ).

For all grass traits we found significant differences between CO<sub>2</sub> levels, as well as between species when all traits were included in the MANOVA model. Analysis of the grasses found no significant differences between species in their

response to the CO<sub>2</sub> treatments for total biomass ( $F_{2,24}=0.01$ ,  $p=0.991$ ), root biomass ( $F_{2,24}=0.05$ ,  $p=0.955$ ), LAI ( $F_{2,24}=0.30$ ,  $p=0.746$ ) and canopy transpiration rate ( $F_{2,24}=0.42$ ,  $p=0.661$ ) of the grasses. The grasses had significantly 154% greater total biomass ( $F_{1,24}=13.77$ ,  $p=0.001$ ), 266% greater root biomass ( $F_{1,24}=10.97$ ,  $p=0.003$ ), 127% greater LAI ( $F_{1,24}=8.62$ ,  $p=0.007$ ) and 71% increase in canopy transpiration rate ( $F_{1,24}=5.17$ ,  $p=0.032$ ) under elevated CO<sub>2</sub> levels. There were significant total biomass ( $F_{2,24}=11.54$ ,  $p<0.001$ ), root biomass ( $F_{2,24}=7.84$ ,  $p=0.002$ ) and LAI ( $F_{2,24}=4.15$ ,  $p=0.028$ ) differences between grass species (Table S5).

Stomatal conductance was on average 31% lower under elevated compared to ambient CO<sub>2</sub> levels, but this difference was not statistically significant (Wald  $\chi^2=3.67$ ,  $df=1,96$ ,  $p=0.055$ ). The SWC was significantly lower in the elevated CO<sub>2</sub> mesocosms compared to ambient CO<sub>2</sub> mesocosms (Wald  $\chi^2=6.44$ ,  $df=1,280$ ,  $p=0.011$ ).

## Discussion

In this study we first asked whether woody plant seedling growth is suppressed by competition from grasses. As expected this was the case with total woody plant seedling biomass being negatively related to total grass biomass at the mesocosm-level. It has previously been suggested that the competitive effect of grasses on woody plant seedling growth is primarily due to root competition in C<sub>4</sub> grasslands (Bond 2008; Cramer et al. 2010; see Kiær et al. 2013). Our experimental design enabled us to disentangle the effects of root and shoot competition from the grasses on woody plant seedlings. This showed that root and shoot competition

from the grasses had a similar suppressive effect on the growth of the woody plant seedlings, with no significant difference between root and shoot competition.

We then asked if the competitive interactions between woody plant seedlings and grasses are modified by elevated CO<sub>2</sub> levels. When grown in root plus shoot competition with grasses, the total biomass of the woody plant seedlings was significantly greater (400% on average) under ambient CO<sub>2</sub> levels. This is contrary to much of the literature which has shown that when resources are not limited, elevated CO<sub>2</sub> levels enhances C<sub>3</sub> woody plant growth (Ainsworth and Long 2005; Morgan et al. 2007; Kgope et al. 2010). In contrast to the woody plant seedlings, the grasses had significantly greater total biomass (154% on average) under elevated CO<sub>2</sub> levels. The increase in the total biomass of the grasses was associated with greater LAI which reduced light and soil water availability at the mesocosm-level. Thus the reduction in woody plant seedling growth under elevated CO<sub>2</sub> levels in our experiment can be attributed at least in part to increased grass growth in response to elevated CO<sub>2</sub> levels that resulted in reduced light and soil water availability. Previous studies (Polley et al. 1997; Morgan et al. 2011) have suggested that soil water availability should increase under elevated CO<sub>2</sub> levels due to decreased canopy transpiration rates. In our study the non-significant decrease in stomatal conductance (31% on average) of the grasses under elevated CO<sub>2</sub> levels was more than offset by an increase in LAI (127% on average), resulting in a significant increase in canopy transpiration rate (71% on average) of the grasses under elevated CO<sub>2</sub> levels. Furthermore we suggest that the reduction in soil water availability under elevated CO<sub>2</sub> levels was due to (1) increased rainfall interception due to increased grass LAI, and (2) increased grass root biomass

(266% on average) resulting in increased competition for soil water. However elevated CO<sub>2</sub> levels may not enhance growth of grass species in all grasslands. For example, a five-year CO<sub>2</sub> growth chamber experiment in semi-arid Colorado grassland found that the total biomass of the C<sub>4</sub> grass *Bouteloua gracilis* was not significantly altered by elevated CO<sub>2</sub> levels (Morgan et al. 2007). Thus variability in responsiveness of the grass species within grasslands may be important in determining the outcome of elevated CO<sub>2</sub> levels on other species within the system.

The growth response of plants to elevated CO<sub>2</sub> levels in grasslands is not only limited by light and soil water but also by the availability of soil nutrients such as N (Reich et al. 2006; Reich and Hobbie 2012) and P (Stocklin et al. 1998; Grunweig and Korner 2003). Cumberland Plain soils are more fertile than the other main vegetation communities of the Sydney region, but relatively unfertile by world standards (Hill et al. 2005). Although our results suggest that soil water availability was an important driver of woody plant seedling growth, competition for nutrients may have also influenced their growth. Similarly to soil water, the greater total grass biomass under elevated CO<sub>2</sub> levels may have increased competition for soil nutrients, limiting nutrient availability and thereby reducing woody plant seedling growth. Because N<sub>2</sub>-fixing woody plant seedlings did not grow better relative to the non N<sub>2</sub>-fixing woody plant seedlings under elevated CO<sub>2</sub> levels, we suggest that N availability was unlikely to be more limited under elevated compared to ambient CO<sub>2</sub> levels. Furthermore, it is unlikely that N was limiting because (1) fertiliser that contained N was added to all mesocosms and (2) by mixing soil from two different locations could have increased N mineralisation. However the fertiliser contained no P so it is possible that increased competition

from the grasses under elevated CO<sub>2</sub> levels reduced P availability which may have influenced woody plant seedling growth.

Woody plant seedlings in grasslands need to manage the trade-off between stem growth to maximise height ('escapability') and leaf and root growth (Clarke and Knox 2009). A major component of 'escapability' in woody plant seedlings in grasslands is rapid stem growth that allows the seedlings to penetrate the grass canopy and escape aboveground competition from grasses (Bond 2008). We hypothesised that the large carbon demand required to make woody structures would be met more efficiently under elevated CO<sub>2</sub> levels (Drake et al. 1997), allowing the woody plant seedlings to grow taller more rapidly and escape the shade imposed by the grass canopy. However, in our experiment 'escapability' of woody plant seedlings was not enhanced under elevated CO<sub>2</sub> levels: stem biomass and stem height were larger under ambient CO<sub>2</sub> levels by an average of 31% and 78% respectively. Kgope et al. (2010) showed for two common woody South African savanna species (*A. karroo* and *A. nilotica*) that stem biomass and stem height increased along an increasing CO<sub>2</sub> gradient. However in their experiment plants were grown individually in pots without competition. We suggest that any increase in 'escapability' of woody plant seedlings due to elevated CO<sub>2</sub> levels will depend on resource availability, particularly soil water. Interestingly we found no significant differences in CO<sub>2</sub> responsiveness between native and invasive exotic woody plant species (see Weltzin et al. 2003; Manea and Leishman 2011).

Rising atmospheric CO<sub>2</sub> concentration has been suggested to be a major driver of the expansion of woody plants into grasslands under past, current and

future conditions (Polley et al. 1997; Bond and Midgley 2000, Buitenwerf et al. 2012). As the cover of adult woody plants in grasslands is most limited by seedling establishment (Bond 2008), rising atmospheric CO<sub>2</sub> concentration that enhance the establishment success of woody plant seedlings in grasslands should have a major influence on the structure and woody plant cover of these systems (Bond and Midgley 2012). Our results were not consistent with this hypothesis with the woody plant seedlings having reduced growth under elevated CO<sub>2</sub> levels. However we do not disagree with the premise that CO<sub>2</sub> may be a major driver of changes in woody plant cover globally. Our results suggest that the establishment success of woody plant seedlings and the subsequent changes in woody plant cover in grasslands in the future will be strongly coupled to the CO<sub>2</sub> response of grass species and resource availability within those systems (see Staver et al. 2011; February et al. 2013). C<sub>4</sub> photosynthesis requires high-light environments and high temperatures for maximum photosynthetic performance (Sage 2004). These were the growth conditions in our experiment to which the C<sub>4</sub> grasses were exposed. In grasslands where an over-storey does exist (savannas and woodlands), conditions will be less favourable for C<sub>4</sub> photosynthesis, thus limiting the CO<sub>2</sub> fertilisation effect on the C<sub>4</sub> grasses (Bond and Midgley 2012). This could lessen competition from grasses for light and soil water, resulting in the potential for enhanced growth of woody plant seedlings. This study has shown that the vulnerability of grasslands, such as the CPW, to woody plant encroachment in the future will be context dependent with grasslands that are open and well-lit or have low soil water availability likely to be most resilient.

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## CHAPTER FIVE

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**Leaf flammability and fuel load accumulation increase under elevated CO<sub>2</sub> levels in a model grassland system.**

In press at *International Journal of Wildland Fire*

My contribution to the research and paper: Concept – 90%; Data collection – 100%; Analysis – 100%; Writing – 90%.





## Abstract

Fire is a common process that shapes the structure of grasslands globally. Rising atmospheric CO<sub>2</sub> concentration may have a profound influence on grassland fire regimes. In this study we asked (1) does CO<sub>2</sub> and/or soil P availability alter leaf flammability (ignitibility and fire sustainability), (2) are leaf tissue chemistry traits drivers of leaf flammability, and are they modified by CO<sub>2</sub> and/or soil P availability?; (3) does CO<sub>2</sub> and/or soil P availability alter fuel load accumulation in grasslands?; and (4) does CO<sub>2</sub> and/or soil P availability alter the resprouting ability of grassland species? We found that leaf flammability increased under elevated CO<sub>2</sub> levels due to decreased leaf moisture content and foliar N, while fuel load accumulation increased due to decreased foliar N (slower litter decomposition rates) and increased aboveground biomass production. These plant responses to elevated CO<sub>2</sub> levels were not modified by soil P availability. The increase in leaf flammability and fuel load accumulation under elevated CO<sub>2</sub> levels may alter grassland fire regimes by facilitating fire ignition as well as shorter fire intervals. However, the increased root biomass of grasses under elevated CO<sub>2</sub> levels may enhance their resprouting capacity relative to woody plants, resulting in a shift in the vegetation structure of grasslands.

## Introduction

Wildland fires influence the structure of many of the world's tropical, sub-tropical and temperate Mediterranean grass-dominated ecosystems (hereon referred to as grasslands) (Bond *et al.* 2005; Bond and Midgley 2012). Empirical and physical based modelling studies have demonstrated that the structure of grasslands can deviate strongly from their climate potential due to fire (Bond *et al.* 2005; Lenihan *et al.* 2008; Higgins *et al.* 2010). Grassland fire regimes are likely to be altered by projected increases in atmospheric CO<sub>2</sub> concentration, extreme drought and temperatures associated with climate change (IPCC 2011). Increased atmospheric CO<sub>2</sub> concentration may affect grassland fire regimes by altering leaf flammability and fuel load accumulation (via changes in litter decomposition rates and aboveground biomass production). However, rising atmospheric CO<sub>2</sub> concentration may also enhance the resprouting ability (via increased storage capacity) of grassland species after fire.

Plant flammability has been defined in terms of three components: ignitibility, combustibility and sustainability (Anderson 1970). Ignitibility is defined as how well the fuel ignites, combustibility as how well the fuel burns, and sustainability as the duration of time the fuel burns (Gill and Zylstra 2005). Leaves are considered to be the most important plant structure determining plant flammability as they are often the first structures to ignite during fires, thus facilitating the spread of the fire (Pickett *et al.* 2009). The flammability of a leaf is strongly associated with its tissue chemistry (leaf moisture content, cellulose content, concentration of fire-retarding salts and nutrients, concentration of secondary compounds) and structural traits (leaf area, surface area to volume ratio and orientation) (Keith 2012). CO<sub>2</sub> level can alter the tissue chemistry and structural traits of leaves (Ward and Strain 1999) thereby changing their flammability. In the first part of this

study we examined if leaf tissue chemistry (leaf moisture content and foliar C, N and P) influences leaf flammability (ignitibility and fire sustainability) and how these leaf tissue chemistry traits are modified by elevated CO<sub>2</sub> levels.

Leaf moisture content is an important correlate of leaf flammability, as leaves with high leaf moisture content take longer to ignite, resulting in lower flammability (De Lillis *et al.* 2009; Schwilk and Caprio 2011; Murray *et al.* 2013). It is well documented that photosynthesis is often stimulated by elevated CO<sub>2</sub> levels (Ainsworth and Long 2005; Leakey *et al.* 2009). Leaf moisture content increases with photosynthesis (Lawlor *et al.* 2002) suggesting leaves will have higher moisture content under elevated CO<sub>2</sub> levels and hence lower ignition potential. The concentration of structural carbohydrates (foliar C), such as cellulose, in leaves often promotes leaf flammability (Keith 2012). However, previous studies have shown that the concentration of these structural carbohydrates in foliar tissues is not altered by elevated CO<sub>2</sub> levels (Billings *et al.* 2003; Hall *et al.* 2005). Under elevated CO<sub>2</sub> levels photosynthesis requires less N to meet the requirements for Rubisco (Drake *et al.* 1997), which leads to a reduction in foliar N (Cotrufo *et al.* 1998; Curtis and Wang 1998; Ainsworth and Long 2005). In contrast to foliar N, foliar P increases under elevated CO<sub>2</sub> levels as the stimulation of photosynthesis increases the P requirement of the phosphorylated photosynthetic intermediates (Gifford *et al.* 2000; see Duval *et al.* 2012 for exception). Both foliar N and P act as flame retardants by favouring the production of non-flammable char, meaning less flammable tar is supplied to the flame which slows heat release (Knicker *et al.* 2008). Thus leaf flammability is likely to be modified by elevated CO<sub>2</sub> levels due to decreased foliar N and increased foliar P.

Grasslands tend to have a sparse or non-existent tree canopy therefore the spread of fire is dependent on the fuel load and fuel bed structure on the ground (Scarff and Westoby 2006; Engber *et al.* 2011). Fuel loads are made up of standing live biomass and curing dead biomass whose decomposition is primarily controlled by photo-degradation (Austin and Vivanco 2006) which is independent of CO<sub>2</sub> level. However elevated CO<sub>2</sub> levels may affect fuel loads by altering litter decomposition rates through changes in leaf tissue chemistry traits (foliar C, N and P) and aboveground biomass production. This was examined in the second part of our study. We can assume that predicted reductions in foliar N under elevated CO<sub>2</sub> levels will result in slower litter decomposition rates (Taylor *et al.* 1989; Cornwell *et al.* 2008) thus increasing fuel loads. Although fire regulates biomass, biomass is also likely to regulate fire regime in grasslands (Lunt *et al.* 2012). That is, increases in grassland aboveground biomass will lead to a more rapid accumulation of fuel (live and dead) which may facilitate fire earlier (Bond and Midgley 2012). Several studies have shown that elevated CO<sub>2</sub> levels stimulate aboveground biomass production in grasslands when resources are not limited (Morgan *et al.* 2001; Shaw *et al.* 2002; Dijkstra *et al.* 2010).

Although increased aboveground biomass production in grasslands under elevated CO<sub>2</sub> levels may facilitate fire earlier, increased root non-structural carbohydrate storage may enhance the capacity of plants to resprout, thereby increasing their persistence after fire (Clarke *et al.* 2013). The amount of root non-structural carbohydrate in plants is related to the amount of root biomass they have accumulated (Knox and Clarke 2005; Clarke *et al.* 2013), thereby making root biomass a good proxy for resprouting ability. In the fourth part of our study we examined how root biomass production will be affected by elevated CO<sub>2</sub> levels. A recent review by Lawes and Clarke (2011) identified a critical knowledge gap on the interactive effects of fire and CO<sub>2</sub> on plant resprouting ability (see

Hoffmann *et al.* 2000; Tooth and Leishman 2013, Tooth and Leishman 2014). Findings from previous studies suggest that the root biomass of grassland species will respond strongly to elevated CO<sub>2</sub> levels (LeCain *et al.* 2006; Anderson *et al.* 2010). We suggest that this strong response of root biomass to elevated CO<sub>2</sub> levels will enhance the resprouting ability of grassland species.

In addition to CO<sub>2</sub> level, we also incorporated soil P availability as a factor in our study as it can directly affect leaf flammability and fuel load accumulation as well as mediate the CO<sub>2</sub> response of plants. Soil P availability may directly influence leaf flammability by allowing plants to increase the uptake and retention of P into their foliar tissues (Tissue and Lewis 2010). Foliar P acts as a flame retardant to reduce flammability (Scarff and Westoby 2008; Scarff *et al.* 2012) and increases litter decomposition rates (Vitousek 1998; Wardle *et al.* 2002). In grasslands throughout the world, it is common for soil P availability to constrain aboveground biomass production (Elser *et al.* 2007; Craine and Jackson 2010), therefore influencing fuel load accumulation. The biomass response of plants to elevated CO<sub>2</sub> levels is also likely to be strongly constrained by soil P availability (Stocklin *et al.* 1998; Grunzweig and Korner 2003).

It is important to understand these complex interactions between CO<sub>2</sub> concentration, soil P availability, leaf flammability, fuel load accumulation and plant persistence as it will enable us to better predict vegetation dynamics, ecosystem processes and plant persistence in a high CO<sub>2</sub> world. In this study we attempted to understand these complex interactions by asking (1a) is leaf flammability (ignition time, flame duration and smouldering duration) modified by elevated CO<sub>2</sub> levels and/or soil P availability?; (1b) are leaf tissue chemistry traits (leaf moisture content and foliar C, N and P) drivers of leaf

flammability, and are they modified by elevated CO<sub>2</sub> levels and/or soil P availability?; (2) does elevated CO<sub>2</sub> levels and/or soil P availability alter litter decomposition rates (foliar C, N and P) and fuel production (leaf and stem biomass) in grasslands?; and (3) does elevated CO<sub>2</sub> levels and/or soil P availability alter the resprouting ability (root biomass) of grassland plant species? To address these questions we grew a range of grass and woody plant species from an open woodland community in a fully factorial glasshouse experiment with two factors: CO<sub>2</sub> level and soil P availability, and measured leaf flammability, foliar C, N and P and biomass production.

## **Materials and methods**

### Species selection

We selected two C<sub>3</sub> grasses, two C<sub>4</sub> grasses, two non N<sub>2</sub>-fixing woody plants and two N<sub>2</sub>-fixing woody plants which are all native perennial species from Cumberland Plain Woodland (CPW) which is an open woodland community with a grassy understorey that occurs in western Sydney, Australia. By selecting species that differed in their photosynthetic pathway and N<sub>2</sub>-fixing ability, we aimed to capture the range of variability of potential responses to elevated CO<sub>2</sub> (Leahey et al. 2009). Seeds for each species were obtained from a commercial supplier (Nindethana Seed Service, Albany, WA, Australia). Information on the biology of each species is provided in Table 1.

Species	Family	Growth form	Photosynthetic pathway	Resprouting type
<i>Austrodanthonia racemosa</i> (R.Br.) H.P.Linder	Poaceae	Grass	C <sub>3</sub>	Basal
<i>Microlaena stipoides</i> (Labill.) R.Br.	Poaceae	Grass	C <sub>3</sub>	Basal
<i>Bothriochloa macra</i> (Steud.) S.T.Blake	Poaceae	Grass	C <sub>4</sub>	Basal
<i>Themeda australis</i> (R.Br.) Stapf	Poaceae	Grass	C <sub>4</sub>	Basal
<i>Acacia implexa</i> Benth.	Fabaceae-Mimosoideae	Woody	C <sub>3</sub>	Basal
<i>Acacia parramattensis</i> Tindale	Fabaceae-Mimosoideae	Woody	C <sub>3</sub>	Epicormic
<i>Eucalyptus creba</i> F.Muell.	Myrtaceae	Woody	C <sub>3</sub>	Epicormic
<i>Eucalyptus moluccana</i> Roxb.	Myrtaceae	Woody	C <sub>3</sub>	Basal or epicormic

Table 1: Grass and woody plant species used in this study, with information on the growth form, photosynthetic pathway and resprouting type of each species. Information on taxonomy, photosynthetic pathway and resprouting type were obtained from PlantNET (The Royal Botanic Gardens and Domain Trust, Sydney, NSW, Australia, <http://www.plantnet.com>).

### Experimental design and treatments

The experiment was a full factorial design with two factors: CO<sub>2</sub> level and soil P availability. We grew 24 replicates of each species that were divided evenly between the ambient and elevated CO<sub>2</sub> levels (i.e. each CO<sub>2</sub> level contained 12 replicates of each species), across four glasshouses (two ambient and two elevated). CO<sub>2</sub> treatments were set to two levels: ambient (380-420 ppm) and elevated (530-570 ppm). These CO<sub>2</sub> concentration ranges were maintained and monitored continuously by a CO<sub>2</sub> dosing and monitoring system (Canary Company Pty Ltd, Lane Cove, NSW, Australia). The elevated CO<sub>2</sub> treatment represents the predicted atmospheric CO<sub>2</sub> concentration by 2060 under the RCP 4.5 emissions scenario (IPCC 2013). Half of the 12 replicates of each species under each CO<sub>2</sub> level were given either a low or high P treatment (i.e. each CO<sub>2</sub> level × soil P availability combination contained six replicates of each species). Soil P treatments were provided by applying 10 g of Osmocote slow release low (16N:1.6P:8K) or high (16N:4.4P:8K) P fertiliser (Scotts Australia Pty Ltd, Bella Vista, NSW, Australia) at the start of the experiment and again 10 weeks into the experiment.

The temperature of the glasshouses was set for a minimum of 16°C and a maximum of 24°C. Relative humidity of the glasshouses was monitored daily at 9am and 3pm using a HOBO temperature/RH/2 external channel data logger (OneTemp, Parramatta, NSW, Australia). Using a paired t-test with a Bonferroni adjustment we found no significant difference in the relative humidity between the CO<sub>2</sub> glasshouses ( $t_{1,38}=1.00$ ,  $p=0.326$ ).

The plants were grown in 12 L pots (21 cm long × 21 cm wide × 28 cm deep), containing 11.5 L of field-collected clay soil from the Cumberland Plain. The soil was collected from Mt Annan (34.07°S, 150.76°E) and Luddenham (33.88°S, 150.69°E) in



western Sydney and was homogenised into one batch using a concrete mixer. The plants were grown for 20 weeks. During this growth period all pots were mist watered for one minute twice daily which is representative of the average daily amount of rainfall (828 mm annually) on the Cumberland Plain. This daily rainfall average was based on the Australian Bureau of Meteorology historical records from Camden airport (1943-2004).

### Measurement of leaf flammability

We set up our experiment in a way that would allow us to measure ignitibility and fire sustainability (flame and smouldering durations). After the growth period, a species was randomly selected daily and a fully expanded outer-canopy leaf was removed from each plant within that species. From each leaf, a 3 cm<sup>2</sup> (0.5 cm × 6 cm) rectangular section of leaf was cut. The section was cut lengthwise as far toward the tip as possible and included the leaf vein. Sections were cut between 10 am and 12 pm and refrigerated until their flammability was tested later on the same day. The leftover leaf material was weighed using an analytical electronic balance (Mettler Toledo, Port Melbourne, VIC, Australia) then oven-dried at 60°C for 72 hours and then weighed again so leaf moisture content (% of oven-dried weight) could be calculated. The individual cut leaf sections were exposed to a temperature of 400°C in a controlled muffle furnace (15 cm long × 10 cm wide × 23 cm deep), following procedures outlined by Gill and Moore (1996). The furnace door was kept open during the experiments to ensure an adequate oxygen supply and to allow the experiments to be digitally recorded. The furnace was located in a well-ventilated room with a constant air-flow. As a result of the open door there was a temperature gradient within the furnace. This temperature gradient was monitored using two type-k (chromel/alumel) thermocouples that were secured to a steel gauze perpendicular to the open furnace door. The thermocouples were connected to a computer via a CR10WP-data

logger (Campbell Scientific, Garbutt, QLD, Australia). When the average temperature value of the two thermocouples was c. 400°C (+/- 10°C) leaf sections were inserted into the furnace.

Leaf sections were placed horizontally in the middle of the furnace, in a direction parallel to the furnace door. They were held just above the steel gauze, 3.5 cm above the furnace floor. Leaf sections were held on their edge by tongs. The source of ignition was a high frequency electrical spark gun held approximately 5 mm above the centre of each leaf section. This spark gun was inserted simultaneously with the leaf section and removed as soon as the section ignited. Filter paper was used as a control, to ensure that the method used was repeatable. The experiments were digitally recorded so that ignition time and flame and smouldering durations could be measured (in seconds) using VideoPad video editor software (NCH Software, Greenwood Village, CO, United States).

#### Measurement of leaf chemistry traits and biomass production

After the 20 week growth period, the plants were harvested, washed free of soil and separated into the following components: leaf biomass, stem biomass and root biomass. The plant components were then oven-dried at 60°C for 72 hours and weighed using an analytical electronic balance (Mettler Toledo, Port Melbourne, VIC, Australia).

After weighing the oven-dried leaf biomass, 0.5 g of leaf biomass from each plant was ground using a cross beater mill (Glen Creston, Stanmore, MIDDX, England). The ground leaf material was then used to obtain the foliar C, N and P of each sample. Foliar C and N were obtained by combustion using a TruSpec CHN analyser (LECO, St Joseph, MI, United States). Foliar P was obtained by digestion using an Ethos-1 microwave digester

(Milestone, Shelton, CT, United States) followed by analysis on a Vista Pro ICPOES (Varian, Palo Alto, CA, United States).

### Data analysis

We used three-way mixed model nested ANOVAs to test if leaf flammability (ignition time, flame duration and smouldering duration), leaf tissue chemistry traits (leaf moisture content, foliar C, N and P) and biomass production (leaf biomass, stem biomass and root biomass), across all species within each growth form (grasses and woody plants), differed between CO<sub>2</sub> level, soil P availability and plant type (grasses-C<sub>3</sub> vs C<sub>4</sub>, woody plants-non N<sub>2</sub>-fixers vs N<sub>2</sub>-fixers). The fixed factors were CO<sub>2</sub> level, soil P availability and plant type and the random factor was species. Across factor combination differences were tested for using Tukey's HSD post-hoc comparisons.

To determine the relationships between leaf tissue chemistry traits and flammability, we used ordinary least-square regression models. Leaf tissue chemistry traits (leaf moisture content and foliar C, N, and P) were the predictor variables and the measures of leaf flammability (ignition time, flame and smouldering durations) were the response variables.

All data analyses were performed using IBM SPSS statistical software, Version 21.0.0 (SPSS Inc., 2012, IBM, Illinois, United States, <http://www.spss.com>) with the significance level set at 0.05. Data were log<sub>10</sub> transformed when necessary to satisfy requirement for normality and fulfil the assumptions of ANOVA.

## Results

### CO<sub>2</sub> and soil P availability effects on leaf flammability

Both the grasses ( $F_{1,86}=10.37$ ,  $p=0.002$ ) and woody plants ( $F_{1,86}=8.01$ ,  $p=0.006$ ) had shorter ignition times (16% and 19% on average respectively; Tables S8 and S11) under elevated CO<sub>2</sub> levels. In addition the grasses ( $F_{1,86}=6.77$ ,  $p=0.011$ ) and woody plants ( $F_{1,86}=5.09$ ,  $p=0.027$ ) had longer flame and smouldering durations (47% and 16% on average respectively; Tables S8 and S11) under elevated CO<sub>2</sub> levels. A similar trend was evident for when they were grown in soils with high P availability (G:  $F_{1,86}=6.21$ ,  $p=0.015$ ; WP:  $F_{1,86}=7.94$ ,  $p=0.006$ ; 33% and 16% on average respectively; Tables S8 and S11). There were interactions between soil P availability and CO<sub>2</sub> level ( $F_{1,86}=6.39$ ,  $p=0.013$ ) and plant type ( $F_{1,86}=12.85$ ,  $p=0.001$ ) for smouldering duration of the grasses. The C<sub>4</sub> grasses (Fig. 1a) and grasses in general grown under ambient CO<sub>2</sub> levels (Fig. 1b) had longer smouldering durations when grown in low P soils.

### Leaf tissue chemistry traits-leaf flammability regressions

Across the eight woodland species, ignition time was weakly positively related to leaf moisture content but was not related to foliar C, N and P (Table 2). Flame and smouldering durations were strongly positively related to foliar C and negatively related to leaf moisture content and foliar N and P (Table 2).

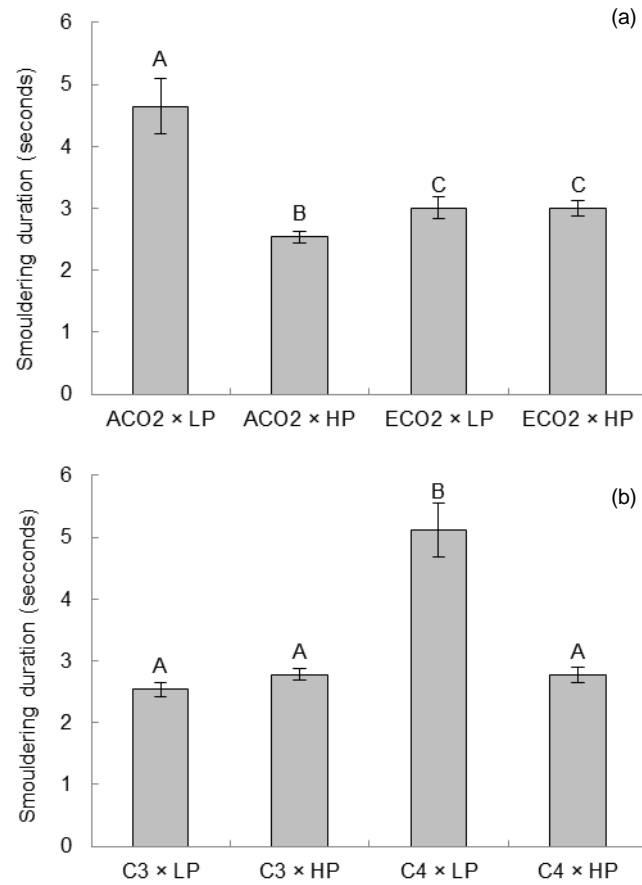


Figure 1: Mean smouldering duration of the grasses for CO<sub>2</sub> level (ambient CO<sub>2</sub> [ACO<sub>2</sub>] vs elevated CO<sub>2</sub> [ECO<sub>2</sub>]), plant type (C<sub>3</sub> vs C<sub>4</sub>) and soil P availability (low P [LP] vs high P [HP]) treatment combinations where a significant interaction was found. Different capital letters indicate significant differences at p<0.05. Vertical bars represent one standard error.

### Leaf tissue chemistry traits

Both the grasses ( $F_{1,86}=10.86$ ,  $p<0.001$ ) and woody plants ( $F_{1,86}=8.34$ ,  $p=0.005$ ) had lower leaf moisture content (14% and 10% on average respectively; Tables S9 and S12) under elevated  $\text{CO}_2$  levels while high soil P availability resulted in lower leaf moisture content (16% on average; Table S12) in woody plants ( $F_{1,86}=12.49$ ,  $p=0.001$ ) but not grasses ( $F_{1,86}=1.91$ ,  $p=0.170$ ).

There was an interaction between  $\text{CO}_2$  level and plant type for foliar C of the grasses ( $F_{1,86}=12.10$ ,  $p=0.001$ ; Fig. 2a) and foliar N of the grasses ( $F_{1,86}=5.18$ ,  $p=0.025$ ; Fig. 2b) and woody plants ( $F_{1,86}=5.26$ ,  $p=0.024$ ; Fig. 3a). In general, the  $\text{C}_3$  grasses had lower foliar C than the  $\text{C}_4$  grasses. The  $\text{C}_4$  grasses and non  $\text{N}_2$ -fixing woody plants had lower foliar N than the  $\text{C}_3$  grasses and  $\text{N}_2$ -fixing woody plants respectively. Foliar C of the  $\text{C}_3$  grasses and foliar N of the  $\text{C}_4$  grasses and non  $\text{N}_2$ -fixing woody plants were further reduced (2%, 14% and 15% on average respectively) under elevated  $\text{CO}_2$  levels. Foliar N of the grasses ( $F_{1,86}=8.16$ ,  $p=0.005$ ) and woody plants ( $F_{1,86}=7.56$ ,  $p=0.007$ ) was higher (12% and 7% on average respectively; Tables S9 and S12) when they were grown in soil with low P availability. There was no  $\text{CO}_2$  or soil P availability effect on foliar P for the grasses or woody plants (Tables S6 and S7).

Leaf tissue chemistry traits	Ignition time			Flame duration			Smouldering duration		
	Slope	r <sup>2</sup>	P	Slope	r <sup>2</sup>	P	Slope	r <sup>2</sup>	P
Leaf moisture content	0.49	0.12	0.050*	-5.89	0.58	<0.001*	-1.41	0.45	<0.001*
Foliar C	-2.15	0.01	0.593	0.37	0.63	<0.001*	-0.09	0.47	<0.001*
Foliar N	0.45	0.02	0.460	-4.58	0.31	0.001*	-1.54	0.46	<0.001*
Foliar P	-0.01	0.01	0.527	-8.12	0.18	0.016*	-2.90	0.30	0.001*

Table 2: Relationships between leaf flammability (ignition time and flame and smouldering durations; measured in seconds) and leaf tissue chemistry traits (leaf moisture content, foliar C, N and P) across all CO<sub>2</sub> level × soil P availability × plant type treatment combinations. Asterisks represent significant relationships (p<0.05).

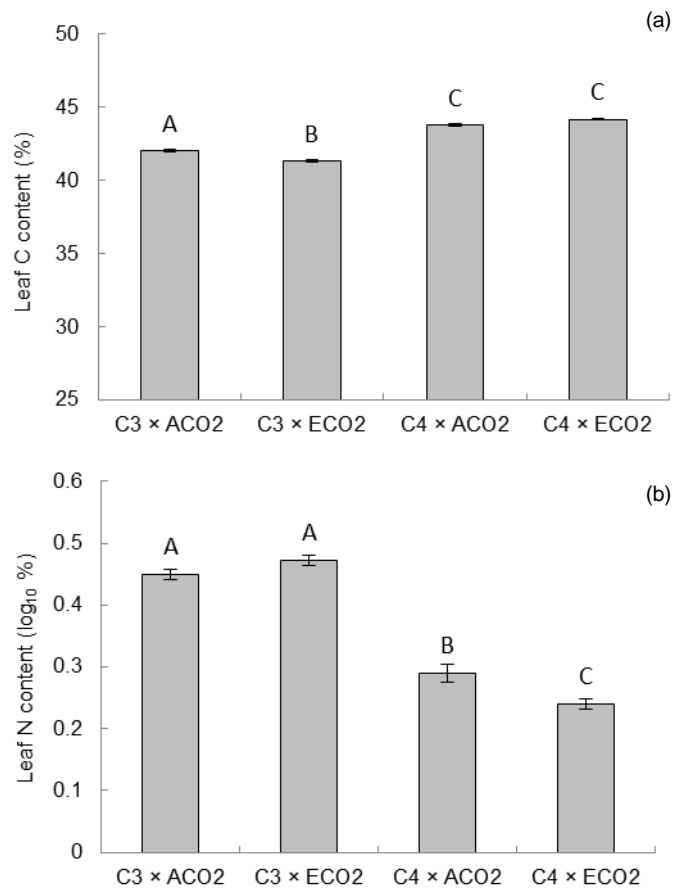


Figure 2: Mean (a) foliar C and (b) foliar N of the grasses for plant type (C<sub>3</sub> vs C<sub>4</sub>) and CO<sub>2</sub> level (ambient CO<sub>2</sub> [ACO<sub>2</sub>] vs elevated CO<sub>2</sub> [ECO<sub>2</sub>]) treatment combinations where a significant interaction was found. Different capital letters indicate significant differences at p < 0.05. Vertical bars represent one standard error.



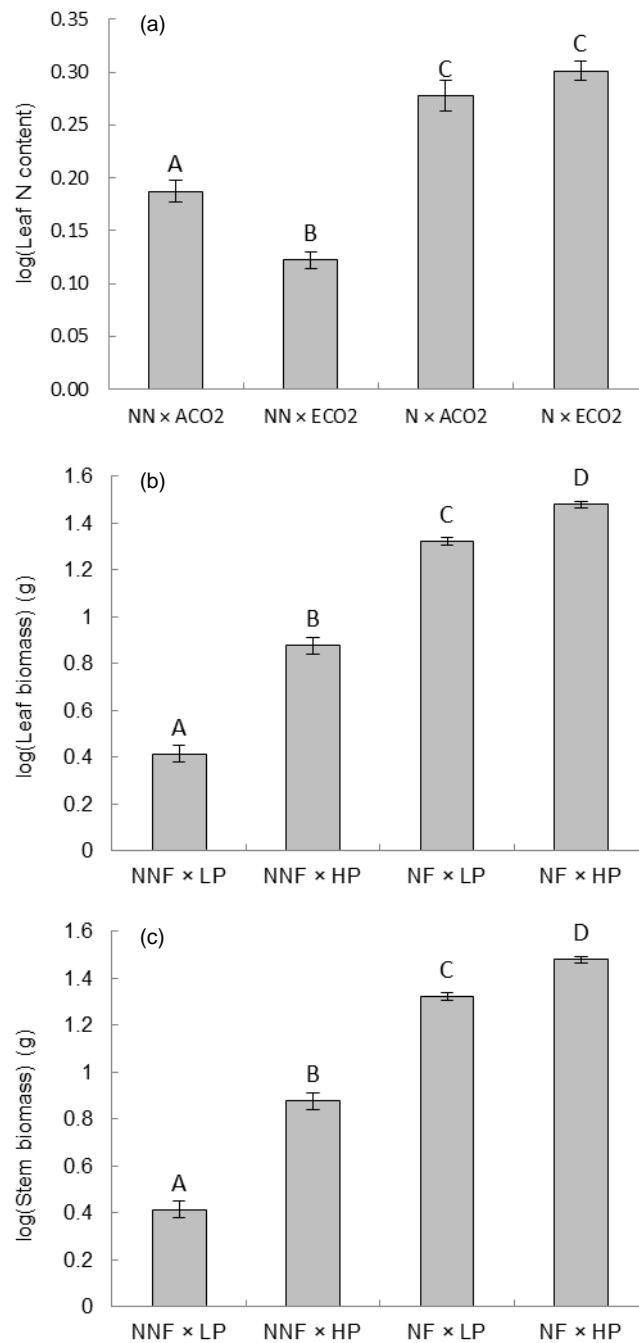


Figure 3: Mean (a) foliar N, (b) leaf biomass and (c) stem biomass of the woody plants for plant type (non N<sub>2</sub>-fixers [NNF] vs N<sub>2</sub>-fixers [NF]), CO<sub>2</sub> level (ambient CO<sub>2</sub> [ACO<sub>2</sub>] vs elevated CO<sub>2</sub> [ECO<sub>2</sub>]) and soil P availability (low P [LP] vs high P [HP]) treatment combinations where a significant interaction was found. Different capital letters indicate significant differences at  $p < 0.05$ . Vertical bars represent one standard error.

### Biomass production

Leaf ( $F_{1,86}=5.49$ ,  $p=0.021$ ) and root biomass ( $F_{1,86}=17.75$ ,  $p<0.001$ ) were greater (27% and 61% on average respectively; Table S10) for the grasses grown under elevated  $\text{CO}_2$  levels. Although non-significant, stem biomass of the grasses ( $F_{1,86}=3.11$ ,  $p=0.082$ ) and leaf ( $F_{1,86}=3.84$ ,  $p=0.053$ ) and root biomass ( $F_{1,86}=3.70$ ,  $p=0.058$ ) of the woody plants were notably greater (14%, 12% and 20% on average respectively; Table S13) under elevated  $\text{CO}_2$  levels. There was no  $\text{CO}_2$  effect on stem biomass ( $F_{1,86}=0.46$ ,  $p=0.499$ ) of the woody plants.

There was an interaction between soil P availability and plant type for leaf ( $F_{1,86}=10.43$ ,  $p=0.002$ ; Fig. 3b) and stem biomass ( $F_{1,86}=5.26$ ,  $p=0.024$ ; Fig. 3c) of the woody plants. The non  $\text{N}_2$ -fixers had greater leaf and stem biomass when grown in high soil P availability but had less leaf and stem biomass than the  $\text{N}_2$ -fixers in general. The grasses had greater leaf ( $F_{1,86}=37.88$ ,  $p<0.001$ ), stem ( $F_{1,86}=51.82$ ,  $p<0.001$ ) and root biomass ( $F_{1,86}=42.73$ ,  $p<0.001$ ) (73%, 97% and 99% on average respectively; Table S10) when grown in soil with high P availability while the woody plants ( $F_{1,86}=31.35$ ,  $p<0.001$ ) had greater (134% on average; Table S13) root biomass.

### **Discussion**

Understanding the complex interactions between  $\text{CO}_2$ , soil P availability, leaf flammability, fuel load accumulation and plant persistence is an important first step in predicting how grassland fire regimes may change in the future. In this study we addressed these complex interactions by measuring the effect of  $\text{CO}_2$  concentration and soil P availability on a range of leaf tissue chemistry traits and

biomass production of grassland plant species which we then used as proxies for leaf flammability (leaf moisture content, foliar C, N and P), fuel load accumulation (foliar C, N and P and aboveground biomass) and plant persistence (root biomass).

We first asked if leaf flammability is modified by elevated CO<sub>2</sub> levels, and if so, is it driven by changes in leaf tissue chemistry traits. Consistent with previous studies (De Lillis *et al.* 2009; Schwilk and Caprio 2011; Murray *et al.* 2013), we found that ignition time was positively related to leaf moisture content although this relationship was weak. Contrary to expectations leaf moisture content in both the grasses and woody plants was reduced under elevated CO<sub>2</sub> levels. However, this reduction in leaf moisture content was consistent with our finding of shorter ignition times of the grasses and woody plants under elevated CO<sub>2</sub> levels providing evidence to suggest grassland species will ignite more quickly in future CO<sub>2</sub> conditions.

As expected, we found that foliar C was positively related to fire sustainability while foliar N and foliar P were negatively related. In general, the C<sub>4</sub> grasses had higher foliar C and lower foliar N than the C<sub>3</sub> grasses suggesting that C<sub>4</sub> grasses should burn for longer (fire sustainability). The grasses had longer flame duration and the woody plants had longer smouldering duration under elevated CO<sub>2</sub> levels. This increase in fire sustainability under elevated CO<sub>2</sub> levels coincided with reductions in foliar N in the C<sub>4</sub> grasses and non N<sub>2</sub>-fixing woody plants which is consistent with the findings of previous studies for these plant types (Curtis and Wang 1998; Ross *et al.* 2002; Billings *et al.* 2003). As foliar N was negatively related to fire sustainability it can be suggested that it was important in driving the

increase in fire sustainability under elevated CO<sub>2</sub> levels. However, the reduction in foliar C in the C<sub>3</sub> grasses under elevated CO<sub>2</sub> levels is counterintuitive to the increase in fire sustainability of the grasses. Therefore it is evident that other leaf chemistry traits (e.g. concentration of fire-retarding salts and nutrients and concentration of secondary compounds) that we did not measure were influenced by elevated CO<sub>2</sub> levels to promote fire sustainability in the grasses. In contrast to foliar C and N, foliar P was not affected by elevated CO<sub>2</sub> levels. Surprisingly, we found leaf moisture content was strongly negatively related to both flame and smouldering durations. As a leaf will generally not ignite until most of its moisture is lost through evaporation (Murray *et al.* 2013) it can be expected that leaf moisture content should not really influence fire sustainability. Our results suggest that C<sub>4</sub> grasses and non N<sub>2</sub>-fixing woody plants should burn for longer (fire sustainability) than C<sub>3</sub> grasses and N<sub>2</sub>-fixing woody plants respectively due to higher foliar C (C<sub>4</sub> grasses) and lower foliar N (both). Furthermore, fire sustainability may be exacerbated by rising atmospheric CO<sub>2</sub> concentration because of further reductions in foliar N in the C<sub>4</sub> grasses and non N<sub>2</sub>-fixing woody plants.

The second part of our study was to determine how fuel load accumulation may change under elevated CO<sub>2</sub> levels through changes in litter decomposition rates (foliar C, N and P) and fuel production (aboveground biomass). We used foliar C, N and P as proxies for litter decomposition with decomposition rates predicted to decrease with foliar C and increase with foliar N and P (Vitousek 1998; Cornwell *et al.* 2008). As described above, foliar C (C<sub>3</sub> grasses) and foliar N (C<sub>4</sub> grasses and non N<sub>2</sub>-fixing woody plants) were reduced under elevated CO<sub>2</sub>

levels while foliar P was not affected by CO<sub>2</sub> level. These results suggest that litter produced by C<sub>4</sub> grasses and non N<sub>2</sub>-fixing woody plants under elevated CO<sub>2</sub> levels may have slower decomposition rates leading to greater fuel load accumulation. This increase in fuel load accumulation under elevated CO<sub>2</sub> levels may be exacerbated by increases in aboveground biomass production particularly when resources (e.g. soil P availability) are not limited (Cary *et al.* 2012). Similarly to previous studies (Morgan *et al.* 2001; Shaw *et al.* 2002; Dijkstra *et al.* 2010) we found that the leaf and stem biomass (non-significant) of the grasses increased under elevated CO<sub>2</sub> levels. In contrast, the aboveground biomass production of the woody plants was not affected by CO<sub>2</sub> level. This lack of a CO<sub>2</sub> effect on woody plant aboveground biomass is surprising as the large carbon demand required to produce woody structures can be met more efficiently under elevated CO<sub>2</sub> levels (Drake *et al.* 1997). Our results suggest that grasslands, particularly C<sub>4</sub>-dominated ones, may accumulate greater fuel loads under elevated CO<sub>2</sub> levels due to slower litter decomposition rates (C<sub>4</sub> grasses) and increased aboveground biomass production (grasses). As fuel load accumulation is related to fire frequency this increase in fuel loads may facilitate shorter fire intervals in grasslands, particularly in drought conditions (Bowman *et al.* 2009; Pausas and Ribeiro 2013).

The third part of our study looked at resprouting ability of grassland species under elevated CO<sub>2</sub> levels using root biomass production as a proxy (see Knox and Clarke 2005; Clarke *et al.* 2013). Resprouting ability will be vitally important in the future because it is projected that increases in extreme drought and temperatures associated with climate change may increase the frequency and intensity of fire (IPCC 2011). We found that root biomass increased under elevated

CO<sub>2</sub> levels for the grasses (LeCain *et al.* 2006; Anderson *et al.* 2010) but not the woody plants. This increase in root biomass suggests that grasses will be able to recover more quickly after fire under elevated CO<sub>2</sub> levels giving them a better chance of out-competing the slower recovering woody plants. This change in grass/woody plant interactions could inhibit woody plant seedling establishment which in turn will reduce the cover of adult woody plants (Bond 2008), thereby potentially shifting the vegetation structure of grasslands under elevated CO<sub>2</sub> levels to a more grass-dominated form (Bond and Midgley 2012).

Surprisingly, the leaf tissue chemistry traits and biomass production responses of the plants to elevated CO<sub>2</sub> levels were not influenced by soil P availability which suggests that the CO<sub>2</sub> response of plants in Cumberland Plain Woodland is not constrained by soil P availability. This may be because the plants already have an ample soil P supply due to the shale derived clayey Cumberland Plain soils being quite fertile (Hill *et al.* 2005). However, we found that high soil P availability may influence leaf flammability and fuel load accumulation directly. Flame duration of the grasses and smouldering duration of the woody plants were prolonged when grown in high soil P availability. Similarly to the elevated CO<sub>2</sub> levels, this increase in fire sustainability of the grasses and woody plants when grown in high soil P availability was associated with decreases in foliar N. Therefore it can be suggested that in fertile grasslands the leaves from both the grasses and woody plants will burn for longer. We also found high soil P availability stimulated leaf and stem biomass production in the grasses and non N<sub>2</sub>-fixing woody plants. These increases in biomass production suggest that grasslands with an ample soil P supply will have slower litter decomposition rates

(foliar N) and produce more aboveground biomass thereby increasing fuel load accumulation and potentially facilitating fire earlier. However, high soil P availability also stimulated root biomass production in the grasses and woody plants which may enhance their resprouting ability thereby increasing their chance of persisting after fire.

Fire plays an important role in shaping the structure of many of the world's grasslands (Bond et al. 2005; Bond and Midgley 2012). Rising atmospheric CO<sub>2</sub> concentration may have a profound effect on the fire regimes in grasslands by altering leaf flammability and fuel load accumulation. We have provided evidence to suggest that rising atmospheric CO<sub>2</sub> concentration may increase the ignitability and fuel load accumulation of grassland species in general due to lower leaf moisture content and increased aboveground biomass production respectively. More specifically, grasslands dominated by C<sub>4</sub> species, such as the Cumberland Plain Woodland, may be particularly vulnerable under elevated CO<sub>2</sub> levels as litter from C<sub>4</sub> grasses will likely burn for longer and decompose slower due to reductions in foliar N. It should be noted that grassland flammability may also be driven by leaf tissue chemistry and structural traits that were not considered in this study. For example, leaf size (leaf area and surface area to volume ratio) is an important determinant of leaf flammability and fuel bed structure as larger leaves have thicker boundary layers making it harder for them to lose heat and therefore making ignition easier (Leigh et al. 2012; Murray et al. 2013), as well as creating a more open flammable fuel bed (Scarff and Westoby 2006; Engber and Varner 2012). Empirical studies such as this are important in providing the data necessary for modelling fire behaviour and vegetation dynamics under future climatic

conditions. Our results suggest that rising atmospheric CO<sub>2</sub> concentration may have a profound effect on global fire regimes resulting in substantial changes to the vegetation structure of grasslands with flow-on effects to ecosystem processes and biodiversity.



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## **CHAPTER SIX**

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### **Discussion**



In 1958, CD Keeling started measuring atmospheric CO<sub>2</sub> concentration and soon after found that it was rising rapidly (Keeling 1960). Atmospheric CO<sub>2</sub> concentration is now one of the best documented global changes of the past half century (Prentice 2001) with concentrations continuing to rise at a rapid rate (IPCC 2013). As a result, ecosystems globally are currently exposed to CO<sub>2</sub> levels that have not been experienced since the early Miocene (Pearson and Palmer 2000). The consequences of rising atmospheric CO<sub>2</sub> concentration on many ecosystems remain uncertain (Leakey et al. 2009). Plant responses to elevated CO<sub>2</sub> levels were first tested in short-term experiments lasting days to weeks (Leakey et al. 2009). These experiments were important in revealing the underlying mechanisms driving CO<sub>2</sub> responses in plants. However it has become evident that longer term experiments testing multiple interacting factors (e.g. precipitation and temperature) in addition to elevated CO<sub>2</sub> levels in realistic field conditions are essential for determining ecosystem structure and function in the future (Beier 2004; Leakey et al. 2009). Previous studies on grass-dominated ecosystems (hereon referred to as grasslands) have shown this to be the case (Shaw et al. 2002; Mikkelsen et al. 2007). Currently Free Air Carbon dioxide Enrichment (FACE) experiments represent the best technology to address questions relating to ecosystem structure and function in the future (Nowak et al. 2004). However, these experiments are difficult and costly to run (Nowak et al. 2004). For this reason mesocosm studies in many cases provide a more realistic alternative. Utilising the mesocosm approach this thesis explores how elevated CO<sub>2</sub> levels may modify the impact of the processes that threaten the structure and function of grassland ecosystems. As each chapter already has a lengthy discussion, the aim of this chapter is to concisely synthesize the key findings of this thesis, present these key findings in the context of a conceptual model of grassland dynamics and discuss the adaptive potential of

Cumberland Plain Woodland (CPW) to the identified threatening processes under future CO<sub>2</sub> conditions.

It is evident from the results of chapters 2-4 that soil water availability may be an extremely important factor in driving the vulnerability of grasslands to extreme drought, exotic plant invasion and woody plant encroachment. This is hardly surprising considering grassland productivity is strongly mediated by soil water availability (Asner et al. 2004; Morgan et al. 2004; Fay et al. 2008; Morgan et al. 2011). As expected, canopy transpiration rate was an important driver of differences in soil water content between mesocosms grown under ambient and elevated CO<sub>2</sub> levels. The amount of water that is lost through canopy transpiration depends on the stomatal conductance and leaf area index (LAI) of the plant (Woodward 1990). The results from chapters 2-4 showed that stomatal conductance was consistently reduced by ~30% under elevated CO<sub>2</sub> levels. In contrast LAI was quite variable across the different experiments under elevated CO<sub>2</sub> levels. For example, in the exotic plant invasion experiment (chapter 3) LAI of the grasses increased by 17% under elevated CO<sub>2</sub> levels while in the woody plant encroachment experiment (chapter 4) it increased by 127%. This variability in LAI across experiments was a result of different species assemblages used in each experiment. This suggests that plant responses to CO<sub>2</sub> are often species specific which reinforces the idea that predicting plant and ecosystem responses to elevated CO<sub>2</sub> levels using plant type (e.g. C<sub>3</sub> vs C<sub>4</sub>, native vs exotic) classifications may not be accurate (Reich et al. 2001; Zavaleta et al. 2003; Nowak et al. 2004). Therefore the vulnerability of grasslands to extreme drought, exotic plant invasion and woody plant encroachment under future CO<sub>2</sub> conditions will be context dependent with different species

assemblages having different canopy transpiration rates thereby driving variability in soil water availability and hence vulnerability to these threatening processes.

Furthermore the results of chapters 2-4 reinforce the idea that CO<sub>2</sub> responses of individually-grown species are not good predictors of how the same species will respond under intra- and inter-specific competition (Poorter and Navas 2003). This is evident from the exotic grasses (i.e. *E. erecta*) and woody plant seedlings grown as part of chapters 3 and 4 not being favoured by elevated CO<sub>2</sub> levels even though these plant types are often favoured by elevated CO<sub>2</sub> levels when grown in isolation (Curtis and Wang 1998; Kgope et al. 2010; Manea et al. 2011). This highlights the need for future research to integrate competition into experimental designs in order to make valid conclusions about plant and ecosystem responses to elevated CO<sub>2</sub> levels regardless of the process (threatening or otherwise) being studied. However testing the response of every possible species assemblage is unrealistic so it is important that we gain a mechanistic understanding that can be used to predict outcomes rather than examining species on a case by case basis.

Predicting responses at local and regional scales to global change is often dependent on scaling up from plant-level mechanisms (Woodward 1990). Although we attempted to make our glasshouse based mesocosm experiment as realistic a representation of the field conditions as possible, it is almost an impossible exercise to exactly recreate these conditions. The three main examples where this is evident in this thesis include:

- (1) Soil depth in the mesocosms was 28 cm which is shallow compared to soil depth in the field. This may have caused higher mortality of the grasses in

response to extreme drought (chapter 2) as they did not have access to deeper soil water that grasses in the field may have, as well as increasing competition within the mesocosms by reducing available root gaps.

(2) Under the control watering treatment used in the experiments, mesocosms received regular water (i.e. daily). In addition mesocosms were provided with ample nutrients. This resource supply may have yielded different growth responses to elevated CO<sub>2</sub> levels compared to field conditions as increases to grassland productivity under elevated CO<sub>2</sub> levels are often dependent on resource availability that is not limited (Shaw et al. 2002; Dijkstra et al. 2010).

(3) Mist watering was used to apply water to the mesocosms which may have increased canopy interception in comparison to rainfall in field conditions. This may have exaggerated the effect that LAI had on soil water in my experiments. Although this may appear to be counterintuitive to the above point it should be noted that the above point refers to the frequency of watering while this point refers to the magnitude of watering.

Irrespective of these caveats, mesocosm studies such as this are important for understanding mechanisms and stimulating further research, rather than simply assessing outcomes (Benton et al. 2007). It is important to note that I did not try to quantify exact grassland responses to threatening processes, but rather I aimed to test potential mechanisms that may be important in determining the grassland responses to threatening processes which can then be scaled up to a local and regional scale.

Chapter 5 was distinct from the other chapters in this thesis as it tested the response of the threatening process (changes in fire regime) to elevated CO<sub>2</sub> levels at

the plant-level rather than the community-level to identify the underlying mechanism. I found that leaf moisture content and foliar N decreased (Cotrufo et al. 1998; Curtis and Wang 1998; Ainsworth and Long 2005) and aboveground biomass production increased (Morgan et al. 2001; Shaw et al. 2002; Dijkstra et al. 2010) under elevated CO<sub>2</sub> levels. This suggests fire frequency may increase under future CO<sub>2</sub> conditions due to increased leaf flammability (foliar N) and fuel load accumulation (foliar N and aboveground biomass production). This may be further exacerbated by extreme drought and the subsequent invasion of exotic plant species (chapter 3) which has been shown to significantly increase fuel load accumulation in grasslands (Smith et al. 2000; Ziska et al. 2005). However the increased root biomass of grasses under elevated CO<sub>2</sub> levels (LeCain et al. 2006; Anderson et al. 2010) may enhance their resprouting capacity relative to woody plants. When this result is coupled with the reduction in woody plant seedling establishment success under elevated CO<sub>2</sub> levels due to the increased competitive effect of co-occurring native grasses (chapter 4) it can be suggested that the structure of grasslands may shift to a more grass-dominated landscape. This could be detrimental to the structure of grassland communities that have a significant woody plant component such as CPW.

The crux of this thesis was to determine the vulnerability of grasslands to threatening processes (extreme drought, exotic plant invasion, woody plant encroachment, changes to fire regime) under elevated CO<sub>2</sub> levels. The results of this thesis suggest that vulnerability of grasslands to these threatening processes will be context dependent with the composition of species assemblages playing an important role. For this reason it would neither be accurate nor useful to make a blanket statement in regards to grassland integrity as a whole under future CO<sub>2</sub> conditions. Despite this

caveat, in figure 1 I present a conceptual model of how threatening processes may interact with increased atmospheric CO<sub>2</sub> concentration and each other to influence the structure and function of grassland ecosystems. More specifically I would venture to suggest that the results of this thesis indicate CPW may be increasingly vulnerable to these threatening processes as atmospheric CO<sub>2</sub> concentration rises. Although many CPW species are capable of withstanding prolonged drought conditions (Benson and Howell 2002) the results of chapters 2 and 3 suggest extreme drought may induce mortality in grassland species under elevated CO<sub>2</sub> levels as well as facilitate exotic plant invasions regardless of CO<sub>2</sub> level. Furthermore the structure of CPW may be altered to a more grass-dominated landscape under future CO<sub>2</sub> conditions due to reduced woody plant seedling establishment success, increased fire frequency and enhanced resprouting ability of grasses relative to woody plants after disturbance.



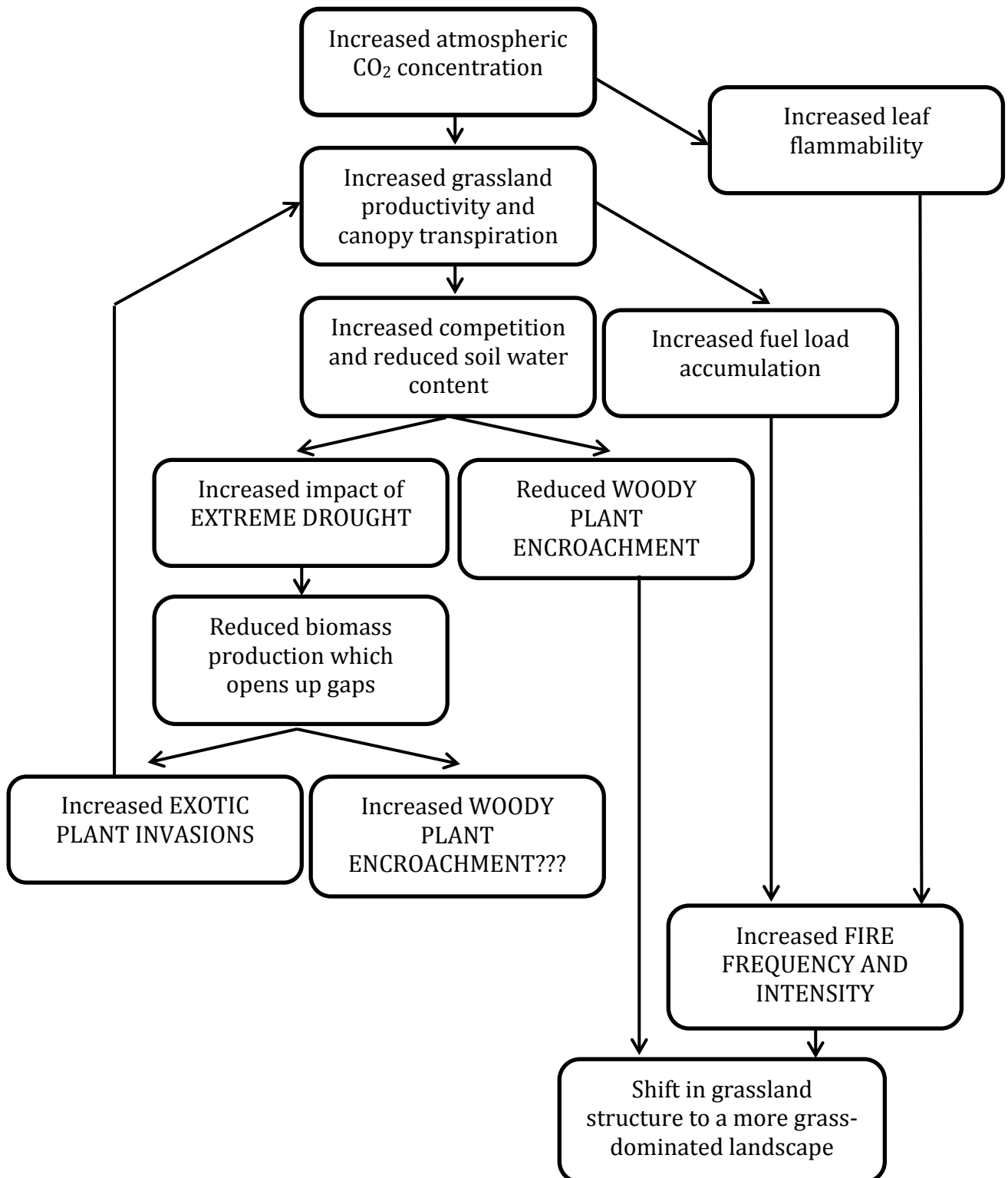


Figure 1: A conceptual model of how grassland threatening processes may interact with increased atmospheric CO<sub>2</sub> concentration and each other.

In the past the structure of CPW has tended to change relatively quickly in response to changes in grazing, fire and disturbance (Benson and Howell 2002). This characteristic may make the management of CPW in terms of adaptation to threatening processes under future CO<sub>2</sub> conditions quite difficult. The ideal solution would be to address the underlying mechanisms that are driving the impacts of the threatening processes. However this may not be realistic as it would mean controlling the competitive interactions between plant species within the community and the environmental factors that drive these interactions such as soil water availability. Thus the best strategy to increase the adaptive capacity of CPW in order to increase its chance of long-term persistence in a high CO<sub>2</sub> world is undertake actions that reduce the impact of threatening processes (i.e. invasive exotic plant management, fire regime management) and use opportunities to increase resilience through re-seeding or re-planting with drought-tolerant provenances of native species.

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## **APPENDICES**

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**Appendix 1: Chapter 2**

Species	Total biomass					Root:shoot					Canopy transpiration rate				
	Ambient CO <sub>2</sub>		Elevated CO <sub>2</sub>		p-value	Ambient CO <sub>2</sub>		Elevated CO <sub>2</sub>		p-value	Ambient CO <sub>2</sub>		Elevated CO <sub>2</sub>		p-value
	Mean	SE	Mean	SE		Mean	SE	Mean	SE		Mean	SE	Mean	SE	
<i>C. gayana</i>	8.206	5.824	28.349	10.833	<b>0.023</b>	0.534	0.043	0.358	0.071	0.076	6.409	4.120	53.704	17.772	<b>0.003</b>
<i>E. curvula</i>	3.247	0.701	7.390	1.212	<b>0.015</b>	0.458	0.109	0.446	0.117	0.923	2.788	0.474	1.526	0.295	<b>0.045</b>
<i>P. clandestinum</i>	35.395	4.533	34.345	4.911	0.815	0.583	0.088	0.530	0.130	0.935	27.072	1.797	30.459	5.255	0.710
<i>B. catharticus</i>	3.995	1.043	6.227	1.720	0.433	0.315	0.052	0.424	0.113	0.386	54.673	13.242	43.345	9.483	0.454
<i>E. erecta</i>	3.753	0.526	5.322	2.630	0.808	0.415	0.097	0.475	0.104	0.688	42.375	9.463	19.141	6.356	0.060
<i>B. macra</i>	0.179	0.092	0.893	0.563	0.107	0.627	0.140	0.471	0.088	0.304	0.356	0.166	1.156	0.659	0.286
<i>C. truncata</i>	2.611	1.728	5.965	2.700	0.104	0.333	0.067	0.303	0.044	0.702	2.466	1.224	1.691	0.491	0.939
<i>T. australis</i>	0.228	0.086	0.818	0.102	<b>0.003</b>	0.578	0.098	0.607	0.259	0.947	0.528	0.260	2.345	0.676	<b>0.008</b>
<i>A. racemosa</i>	1.497	0.781	2.529	0.775	0.307	0.406	0.096	0.603	0.076	0.142	4.175	1.863	8.086	2.307	0.230
<i>M. stipoides</i>	1.175	0.307	1.497	0.614	0.727	0.223	0.023	0.520	0.085	<b>0.014</b>	5.442	1.841	3.370	1.399	0.838

Table S1: Mean (with one standard error) total biomass (g), root:shoot and canopy transpiration rate (mmol m<sup>2</sup> s<sup>-1</sup>) of each grass

species from the 'before drought treatment' mesocosms under ambient and elevated CO<sub>2</sub> levels. One-way ANOVAs were used to test within species differences between the two CO<sub>2</sub> levels. Significant differences (p=0.05) are shown in bold.

**Appendix 2: Chapter 3**

Traits	Species	Ambient CO <sub>2</sub> /Control		Elevated CO <sub>2</sub> /Control		Ambient CO <sub>2</sub> /Drought		Elevated CO <sub>2</sub> /Drought	
		Mean	SE	Mean	SE	Mean	SE	Mean	SE
Total biomass	<i>A. racemosa</i>	67.9840	14.8162	69.3109	13.1564	45.1433	11.1166	57.2283	12.8732
	<i>M. stipoides</i>	8.3935	3.1587	4.5545	1.9397	1.9376	0.2528	2.2853	0.6221
	<i>B. macra</i>	26.9758	3.1327	28.1817	2.1425	21.4150	1.9634	19.4683	3.1534
	<i>T. australis</i>	91.0675	12.7084	99.6200	13.9347	48.2017	7.5063	75.1942	13.2778
Canopy transpiration rate	<i>A. racemosa</i>	95.2467	20.7578	74.0293	14.0520	63.2466	15.5746	61.1242	13.7496
	<i>M. stipoides</i>	10.5187	3.9584	3.1540	1.3433	2.4282	0.3168	1.5826	0.4308
	<i>B. macra</i>	17.7472	2.0610	17.9775	1.3667	14.0888	1.2917	12.4191	2.0116
	<i>T. australis</i>	74.4662	10.3917	67.5292	9.4459	39.4147	6.1379	50.9717	9.0006

Table S2: Mean (with one standard error) biomass (g) and canopy transpiration rate (mmol m<sup>2</sup> s<sup>-1</sup>) of each native grass species for each CO<sub>2</sub> level × watering treatment combination.

Factors	Biomass		Canopy transpiration rate	
	f	p	f	p
CO <sub>2</sub>	0.240	0.625	2.830	0.094
Watering	19.200	<0.001	18.680	<0.001
Plant type	0.770	0.472	0.270	0.655
Species(plant type)	153.170	<0.001	184.110	<0.001
CO <sub>2</sub> × watering	0.370	0.542	0.300	0.588
CO <sub>2</sub> × plant type	0.050	0.821	2.080	0.151
Watering × plant type	0.130	0.723	0.100	0.749
CO <sub>2</sub> × watering × plant type	0.640	0.426	0.540	0.462

Table S3: Results of the three-way mixed model nested ANOVAs (n=166) that tested the effect of CO<sub>2</sub> level (ambient vs elevated), watering treatment (control vs drought) and plant type (C<sub>3</sub> vs C<sub>4</sub>) on native grass biomass and canopy transpiration rate. CO<sub>2</sub> level, watering treatment and plant type were fixed factors and species which was nested within plant type was a random factor.

**Appendix 3: Chapter 4**

Species	Total biomass					Stem weight					Stem height				
	Ambient CO <sub>2</sub>		Elevated CO <sub>2</sub>		p-value	Ambient CO <sub>2</sub>		Elevated CO <sub>2</sub>		p-value	Ambient CO <sub>2</sub>		Elevated CO <sub>2</sub>		p-value
	Mean	SE	Mean	SE		Mean	SE	Mean	SE		Mean	SE	Mean	SE	
<i>A. implexa</i>	0.922	0.137	0.157	0.022	<b>0.003</b>	0.238	0.041	0.032	0.004	<b>0.004</b>	213.800	25.704	91.200	5.088	0.076
<i>A. saligna</i>	4.511	0.794	0.672	0.039	<b>0.001</b>	1.400	0.256	0.118	0.008	<b>0.001</b>	579.400	32.155	238.600	8.603	<b>&lt;0.001</b>
<i>B. spinosa</i>	0.013	0.001	0.013	0.000	0.807	0.002	0.000	0.004	0.000	0.083	45.000	1.102	45.000	3.286	0.860
<i>C. glaucophyllus</i>	0.292	0.078	0.068	0.008	0.058	0.070	0.025	0.011	0.001	0.078	97.850	12.685	47.600	3.341	0.056
<i>D. viscosa</i>	0.037	0.003	0.019	0.001	<b>0.021</b>	0.010	0.001	0.004	0.000	<b>0.019</b>	40.000	4.940	27.600	1.147	0.407
<i>I. australis</i>	0.189	0.037	0.089	0.022	0.356	0.058	0.017	0.033	0.010	0.533	184.800	22.763	148.333	29.510	0.513
<i>L. sinense</i>	0.168	0.012	0.113	0.006	0.065	0.029	0.002	0.026	0.002	0.626	77.000	4.680	62.600	3.697	0.260
<i>S. pendula</i>	0.254	0.058	0.141	0.030	0.304	0.092	0.029	0.042	0.010	0.344	153.600	16.793	121.200	13.767	0.440

Table S4: Mean (with one standard error) total biomass (g), stem biomass (g) and stem height (mm) of each woody plant species from the 'root plus shoot competition' mesocosms under ambient and elevated CO<sub>2</sub> levels. One-way ANOVAs were used to test within species differences between the two CO<sub>2</sub> levels. Significant differences (p=0.05) are shown in bold.

Species	Total biomass					Root biomass					Canopy transpiration rate				
	Ambient CO <sub>2</sub>		Elevated CO <sub>2</sub>		p-value	Ambient CO <sub>2</sub>		Elevated CO <sub>2</sub>		p-value	Ambient CO <sub>2</sub>		Elevated CO <sub>2</sub>		p-value
	Mean	SE	Mean	SE		Mean	SE	Mean	SE		Mean	SE	Mean	SE	
<i>B. macra</i>	0.179	0.092	0.893	0.563	0.107	0.077	0.046	0.273	0.168	0.189	0.043	0.020	0.401	0.261	0.186
<i>C. truncata</i>	2.611	1.728	5.965	2.700	0.104	0.432	0.257	1.579	0.903	0.079	0.200	0.099	0.250	0.073	0.416
<i>T. australis</i>	0.228	0.086	0.818	0.102	<b>0.003</b>	0.068	0.013	0.257	0.085	<b>0.009</b>	0.089	0.044	0.187	0.054	0.089

Table S5: Mean (with one standard error) total biomass (g), root biomass (g) and canopy transpiration rate (mmol m<sup>2</sup> s<sup>-1</sup>) of each native grass species from the 'root plus shoot competition' mesocosms under ambient and elevated CO<sub>2</sub> levels. One-way ANOVAs were used to test within species differences between the two CO<sub>2</sub> levels. Significant differences (p=0.05) are shown in bold.

**Appendix 4: Chapter 5**

Factors	Leaf moisture		Foliar C		Foliar N		Foliar P		Leaf weight		Stem weight		Root weight	
	content													
	f	p	f	p	f	p	f	p	f	p	f	p	f	p
CO <sub>2</sub>	10.86	<b>0.001</b>	1.02	0.315	0.49	0.486	2.24	0.138	5.49	<b>0.021</b>	3.11	0.082	17.75	<0.001
Nutrient	1.91	0.170	0.40	0.530	8.16	<b>0.005</b>	0.88	0.351	37.88	<b>&lt;0.001</b>	51.82	<b>&lt;0.001</b>	42.73	<0.001
Plant type	<0.01	0.997	15.48	0.059	9.74	0.089	3.42	0.206	3.13	0.219	0.54	0.538	0.01	0.946
Species(plant type)	28.61	<b>&lt;0.001</b>	15.72	<b>&lt;0.001</b>	12.04	<b>&lt;0.001</b>	20.94	<b>&lt;0.001</b>	73.68	<b>&lt;0.001</b>	9.16	<b>&lt;0.001</b>	26.84	<b>&lt;0.001</b>
CO <sub>2</sub> × nutrient	0.04	0.833	0.10	0.748	<0.01	0.971	5.61	<b>0.020</b>	2.40	0.125	3.11	0.082	4.66	<b>0.034</b>
CO <sub>2</sub> × plant type	1.23	0.271	12.10	<b>0.001</b>	5.18	<b>0.025</b>	1.53	0.220	2.99	0.087	3.34	0.071	2.91	0.092
Nutrient × plant type	0.40	0.529	1.12	0.293	0.74	0.391	<0.01	0.991	3.36	0.070	1.78	0.186	8.25	<b>0.005</b>
CO <sub>2</sub> × nutrient × plant type	1.74	0.200	0.65	0.423	1.44	0.233	0.98	0.325	0.20	0.879	0.22	0.642	0.01	0.939

Table S6: Results of the three-way mixed model nested ANOVAs (n=96) that tested the effect of CO<sub>2</sub> level (ambient vs elevated), soil P availability (low P vs high P) and plant type (C<sub>3</sub> vs C<sub>4</sub>) on native grass leaf tissue chemistry and biomass production traits. CO<sub>2</sub> level, soil P availability and plant type were fixed factors and species which was nested within plant type was a random factor. Significant differences (p=0.05) are shown in bold.



Factors	Leaf moisture		Foliar C		Foliar N		Foliar P		Leaf weight		Stem weight		Root weight	
	content													
	f	p	f	p	f	p	f	p	f	p	f	p	f	p
Plant type	0.56	0.533	0.85	0.453	6.44	0.126	0.01	0.943	16.60	0.055	4.21	0.177	31.35	<b>0.030</b>
CO <sub>2</sub>	8.43	<b>0.005</b>	0.03	0.857	1.13	0.292	2.24	0.138	3.84	0.053	0.46	0.499	3.70	0.058
Nutrient	12.49	<b>0.001</b>	1.06	0.306	7.56	<b>0.007</b>	0.67	0.414	43.71	<0.001	45.95	<0.001	63.85	<b>&lt;0.001</b>
Species(plant type)	36.37	<b>&lt;0.001</b>	25.71	<b>&lt;0.001</b>	7.45	<b>0.001</b>	9.31	<b>&lt;0.001</b>	15.51	<b>&lt;0.001</b>	33.08	<b>&lt;0.001</b>	16.00	<b>&lt;0.001</b>
CO <sub>2</sub> × plant type	0.03	0.864	1.04	0.311	5.26	<b>0.024</b>	1.95	0.167	2.59	0.111	3.20	0.077	0.48	0.489
Nutrient × plant type	0.01	0.934	3.33	0.072	2.13	0.148	0.20	0.653	10.43	<b>0.002</b>	5.26	<b>0.024</b>	3.88	0.052
CO <sub>2</sub> × nutrient	0.03	0.854	0.78	0.379	0.21	0.645	1.72	0.193	1.92	0.169	3.26	0.074	0.06	0.800
CO <sub>2</sub> × nutrient × plant type	0.84	0.362	1.53	0.220	0.97	0.326	2.12	0.149	0.21	0.650	0.04	0.840	0.55	0.461

Table S7: Results of the three-way mixed model nested ANOVA (n=96) that tested the effect of CO<sub>2</sub> level (ambient vs elevated), soil P availability (low P vs high P) and plant type (non N-fixer vs N-fixer) on native woody plant leaf tissue chemistry and biomass production traits. CO<sub>2</sub> level, soil P availability and plant type were fixed factors and species which was nested within plant type was a random factor. Significant differences (p=0.05) are shown in bold.

Leaf flammability	Species	ACO <sub>2</sub> × LP		ACO <sub>2</sub> × HP		ECO <sub>2</sub> × LP		ECO <sub>2</sub> × HP	
		Mean	SE	Mean	SE	Mean	SE	Mean	SE
Ignition time	<i>A. racemosa</i>	7.8167	0.9293	7.5000	1.0096	7.9667	0.4904	7.5500	0.3490
	<i>M. stipoides</i>	7.0667	0.9142	6.6000	0.9448	5.1000	0.7492	5.1500	0.6566
	<i>B. macra</i>	7.6167	0.6534	6.4000	0.3958	5.5833	0.4238	5.0500	0.5655
	<i>T. australis</i>	14.2000	1.8301	10.8833	0.6590	10.9833	0.2845	10.2167	0.5095
Flame duration	<i>A. racemosa</i>	1.0000	0.2683	0.6000	0.1770	0.8167	0.2548	2.0333	0.1585
	<i>M. stipoides</i>	0.4500	0.1586	0.4833	0.1078	0.5000	0.0816	0.6500	0.1746
	<i>B. macra</i>	0.3667	0.1145	0.6333	0.0882	1.0167	0.3497	0.8167	0.0749
	<i>T. australis</i>	0.4333	0.2092	1.2667	0.1687	1.0833	0.2496	1.1833	0.1447
Smouldering duration	<i>A. racemosa</i>	2.8667	0.5481	2.3500	0.2626	2.2417	0.3351	3.2083	0.4315
	<i>M. stipoides</i>	2.9000	0.5739	2.3667	0.2333	2.0333	0.3106	2.5500	0.3294
	<i>B. macra</i>	2.5000	0.7024	1.6833	0.0749	3.2500	1.0883	1.6333	0.2171
	<i>T. australis</i>	10.4167	2.1225	3.7667	0.2974	4.3000	0.3890	3.9850	0.3277

Table S8: Mean (with one standard error) leaf ignition time, flame duration and smouldering duration (seconds) of each native grass species for CO<sub>2</sub> level (ambient CO<sub>2</sub> [ACO<sub>2</sub>] vs elevated CO<sub>2</sub> [ECO<sub>2</sub>]) and soil P availability (low P [LP] vs high P [HP]) treatment combinations.

Leaf tissue chemistry traits	Species	ACO <sub>2</sub> × LP		ACO <sub>2</sub> × HP		ECO <sub>2</sub> × LP		ECO <sub>2</sub> × HP	
		Mean	SE	Mean	SE	Mean	SE	Mean	SE
Foliar C	<i>A. racemosa</i>	42.7783	0.1422	42.5050	0.2360	41.4120	0.4745	41.2867	0.4604
	<i>M. stipoides</i>	40.8733	0.4076	41.5600	0.2603	40.8217	0.3973	41.7820	0.1159
	<i>B. macra</i>	44.3767	0.1322	44.2633	0.1530	44.7667	0.1709	44.4000	0.2584
	<i>T. australis</i>	43.1900	0.1951	43.3200	0.2162	43.6750	0.1314	43.7717	0.1906
Foliar N	<i>A. racemosa</i>	2.8390	0.2328	2.5987	0.1662	2.9912	0.2026	3.1913	0.2424
	<i>M. stipoides</i>	3.3095	0.1816	2.6598	0.1523	3.2728	0.1738	2.6017	0.1750
	<i>B. macra</i>	2.5883	0.2381	2.5267	0.2726	2.1633	0.1497	1.6773	0.1053
	<i>T. australis</i>	1.5955	0.1480	1.4998	0.0544	1.6767	0.0982	1.5628	0.0626
Foliar P	<i>A. racemosa</i>	0.3051	0.0118	0.2977	0.0202	0.2388	0.0213	0.3535	0.0265
	<i>M. stipoides</i>	0.2394	0.0233	0.1936	0.0173	0.2394	0.0151	0.2089	0.0136
	<i>B. macra</i>	0.1757	0.0236	0.1628	0.0085	0.2093	0.0148	0.2382	0.0273
	<i>T. australis</i>	0.1530	0.0090	0.1574	0.0101	0.1460	0.0065	0.1629	0.0189

Table S9: Mean (with one standard error) foliar C, N and P (%) of each native grass species for CO<sub>2</sub> level (ambient CO<sub>2</sub> [ACO<sub>2</sub>] vs elevated CO<sub>2</sub> [ECO<sub>2</sub>]) and soil P availability (low P [LP] vs high P [HP]) treatment combinations.

Biomass production values	Species	ACO <sub>2</sub> × LP		ACO <sub>2</sub> × HP		ECO <sub>2</sub> × LP		ECO <sub>2</sub> × HP	
		Mean	SE	Mean	SE	Mean	SE	Mean	SE
Leaf biomass	<i>A. racemosa</i>	16.7029	1.8671	29.3752	3.0694	20.6678	5.0259	33.7623	5.2378
	<i>M. stipoides</i>	3.0864	0.1797	3.8446	0.4936	2.7310	0.4069	3.9068	0.3814
	<i>B. macra</i>	24.0606	4.9018	33.8422	3.3611	16.3053	2.6446	44.2404	2.6470
	<i>T. australis</i>	8.6625	1.2518	12.0168	2.1236	16.2311	2.3934	26.6067	1.3568
Stem biomass	<i>A. racemosa</i>	13.2400	1.6609	28.4117	3.6367	12.6783	2.8222	30.6250	4.0525
	<i>M. stipoides</i>	12.3333	1.6909	22.4383	1.8457	12.8483	2.8671	23.7733	2.4610
	<i>B. macra</i>	13.0833	3.0303	18.2283	2.4311	8.2767	1.6886	25.6633	3.1691
	<i>T. australis</i>	6.0383	1.1221	8.6583	1.7597	10.8583	1.8592	20.5583	1.8381
Root biomass	<i>A. racemosa</i>	6.4200	1.1788	13.5917	2.1242	8.0767	1.5896	22.1450	6.5286
	<i>M. stipoides</i>	3.3950	0.4952	5.8400	0.8185	4.6850	0.6962	7.4533	0.4884
	<i>B. macra</i>	5.5033	1.8533	7.9617	0.7444	3.4850	0.2562	15.3150	3.1511
	<i>T. australis</i>	9.3050	1.1480	8.0983	1.9329	13.9567	1.9525	25.4783	1.3872

Table S10: Mean (with one standard error) leaf, stem and root biomass (g) of each native grass species for CO<sub>2</sub> level (ambient CO<sub>2</sub> [ACO<sub>2</sub>] vs elevated CO<sub>2</sub> [ECO<sub>2</sub>]) and soil P availability (low P [LP] vs high P [HP]) treatment combinations.

Leaf flammability	Species	ACO <sub>2</sub> × LP		ACO <sub>2</sub> × HP		ECO <sub>2</sub> × LP		ECO <sub>2</sub> × HP	
		Mean	SE	Mean	SE	Mean	SE	Mean	SE
Ignition time	<i>E. creba</i>	2.8000	0.2683	3.1333	0.3363	2.6333	0.3964	2.6833	0.2212
	<i>E. molluccana</i>	13.3833	2.0969	11.9500	2.3533	7.9333	0.8421	7.9500	0.6667
	<i>A. implexa</i>	7.4000	0.5335	5.7833	0.6140	5.8667	0.8620	5.0333	0.6489
	<i>A. parramattensis</i>	9.9500	1.5558	8.6833	1.0669	10.2333	1.5090	8.3500	1.9028
Flame duration	<i>E. creba</i>	2.3833	0.1515	2.9333	0.4248	2.9500	0.1803	3.5333	0.3461
	<i>E. molluccana</i>	1.3000	0.1549	1.8500	0.4537	1.4167	0.2600	3.4333	1.3552
	<i>A. implexa</i>	2.6500	0.3324	3.0833	0.4020	2.6500	0.3462	2.7500	0.1746
	<i>A. parramattensis</i>	3.1333	0.7566	2.8333	0.5970	2.0000	0.3055	3.3333	0.7003
Smouldering duration	<i>E. creba</i>	5.6500	0.6233	8.2500	0.5110	6.9833	0.6695	8.3000	1.2019
	<i>E. molluccana</i>	12.1833	2.9969	13.7000	1.7506	15.4667	2.2326	16.5667	1.5251
	<i>A. implexa</i>	6.3667	0.5637	5.9667	0.5920	6.6833	0.6321	8.2833	0.7432
	<i>A. parramattensis</i>	7.7333	1.1304	8.5167	0.6426	7.2500	0.9708	9.8500	1.9498

Table S11: Mean (with one standard error) leaf ignition time, flame duration and smouldering duration (seconds) of each native woody plant species for CO<sub>2</sub> level (ambient CO<sub>2</sub> [ACO<sub>2</sub>] vs elevated CO<sub>2</sub> [ECO<sub>2</sub>]) and soil P availability (low P [LP] vs high P [HP]) treatment combinations.

Leaf tissue chemistry traits	Species	ACO <sub>2</sub> × LP		ACO <sub>2</sub> × HP		ECO <sub>2</sub> × LP		ECO <sub>2</sub> × HP	
		Mean	SE	Mean	SE	Mean	SE	Mean	SE
Foliar C	<i>E. creba</i>	46.6000	0.2814	47.1767	0.2496	47.5267	0.1984	46.5967	0.3731
	<i>E. molluccana</i>	46.1150	0.4892	46.0017	0.2703	45.5100	0.4368	45.4217	0.3928
	<i>A. implexa</i>	46.1667	0.3045	46.9350	0.1679	46.3217	0.3302	46.7433	0.3777
	<i>A. parramattensis</i>	47.6250	0.3267	47.7317	0.3463	47.6400	0.4486	48.3383	0.4104
Foliar N	<i>E. creba</i>	1.7002	0.1360	1.3122	0.0999	1.3472	0.0936	1.1790	0.0951
	<i>E. molluccana</i>	1.7837	0.1560	1.5102	0.1194	1.5568	0.0203	1.2922	0.0653
	<i>A. implexa</i>	2.0492	0.1789	2.3747	0.2063	2.3070	0.2195	2.1227	0.1533
	<i>A. parramattensis</i>	1.9061	0.3031	1.6158	0.2046	2.0387	0.1592	1.7132	0.1202
Foliar P	<i>E. creba</i>	0.1582	0.0104	0.1658	0.0244	0.1190	0.0129	0.1553	0.0135
	<i>E. molluccana</i>	0.1796	0.0141	0.1758	0.0227	0.1672	0.0137	0.1396	0.0047
	<i>A. implexa</i>	0.1485	0.0079	0.2248	0.0244	0.1768	0.0280	0.1705	0.0054
	<i>A. parramattensis</i>	0.1289	0.0082	0.1214	0.0119	0.1460	0.0150	0.1268	0.0216

Table S12: Mean (with one standard error) foliar C, N and P (%) of each native woody plant species for CO<sub>2</sub> level (ambient CO<sub>2</sub> [ACO<sub>2</sub>] vs elevated CO<sub>2</sub> [ECO<sub>2</sub>]) and soil P availability (low P [LP] vs high P [HP]) treatment combinations.

Biomass production values	Species	ACO <sub>2</sub> × LP		ACO <sub>2</sub> × HP		ECO <sub>2</sub> × LP		ECO <sub>2</sub> × HP	
		Mean	SE	Mean	SE	Mean	SE	Mean	SE
Leaf biomass	<i>E. creba</i>	2.1393	0.5620	3.0823	0.7481	2.7532	0.5560	7.3594	1.5969
	<i>E. molluccana</i>	4.2099	1.0383	13.5155	3.6755	4.1783	1.0307	15.8167	1.3076
	<i>A. implexa</i>	21.8106	3.3491	24.8006	2.7146	20.5807	3.1419	30.0865	1.8351
	<i>A. parramattensis</i>	23.5174	1.9574	33.8769	3.0984	23.1451	4.1916	38.2447	6.0258
Stem biomass	<i>E. creba</i>	1.7037	0.4931	1.7354	0.4876	2.0564	0.4548	4.7106	1.3182
	<i>E. molluccana</i>	4.5035	1.2141	19.6450	4.6756	4.1509	1.2055	20.5400	2.6936
	<i>A. implexa</i>	17.6283	2.8949	25.0367	1.7902	12.9183	2.5385	26.9983	1.9990
	<i>A. parramattensis</i>	14.7417	1.8575	20.2983	2.2149	10.7650	1.9820	22.7283	4.1487
Root biomass	<i>E. creba</i>	0.2641	0.0800	0.7253	0.2083	0.4645	0.1258	1.0269	0.2594
	<i>E. molluccana</i>	0.5989	0.1246	4.3763	1.8958	0.6194	0.1076	4.8237	1.4594
	<i>A. implexa</i>	17.0317	3.9780	25.5350	2.9430	16.2667	4.5311	32.3217	5.0601
	<i>A. parramattensis</i>	9.7500	1.5683	27.0450	6.0257	11.2250	1.8039	35.4150	4.9737

Table S13: Mean (with one standard error) leaf, stem and root biomass (g) of each native woody plant species for CO<sub>2</sub> level (ambient CO<sub>2</sub> [ACO<sub>2</sub>] vs elevated CO<sub>2</sub> [ECO<sub>2</sub>]) and soil P availability (low P [LP] vs high P [HP]) treatment combinations.