Growth and photosynthetic responses of Acacia (Vachellia) seedlings to atmospheric CO_2 increased from glacial to current concentrations: Underlying mechanisms and ecological implications

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By

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Abstract

The African Acacia species *Vachellia karroo, V. robusta, V. nilotica,* and *V. tortilis* are some of the most invasive species implicated in bush encroachment and woody thickening of historically open savannas in southern Africa. This is partially explained by historic increases in atmospheric CO_2 concentrations, which are proposed to have promoted the growth and survivorship of C_3 tree seedlings relative to C_4 grasses. However, the uniformity of CO_2 responsiveness and differences among *Vachellia* species remain largely undetermined. Here we investigate the growth and photosynthetic responses of four *Vachellia* species, all implicated in woody encroachment, but originating from distinct climatic niches.

Exposing these species to a range of sub-ambient CO_2 concentrations (12 – 40 Pa) showed that *V. karroo*, *V. robusta, V. nilotica* and *V. tortilis* all responded strongly and fairly consistently to increasing CO_2 concentrations, acting as a 'functional type' despite being selected from different geographic regions and having different climatic niches. Combined average net CO_2 assimilation rates increased by 130% despite significant, but low levels of down-regulation and decreased stomatal conductance. The increased photosynthetic rates stimulated growth and biomass production in all compartments, with no significant differences in interspecific above and below ground allocation. Growth rates and dry biomass increased by 50% and 186%, respectively, while leaf level water use efficiency (ratio of net CO_2 assimilation rate to transpiration rate) increased by an average of 218%. When this was scaled to the whole plant level, this stimulation was decreased to 80%. The decrease was the result of the CO_2 stimulated increase in canopy areas, which increased leaf area for water loss. The seedlings' total number of spinescent physical defenses, as well as the average mass and spine mass fraction also increased with rising CO_2. These thicker spines could act as better deterrents against vertebrate browsers. Spine density was unchanged, however, showing that the increased spine numbers were associated with larger seedlings at higher CO_2 rather than an increase in the number of spines per stem length.

The stimulatory effects of increasing CO_2 concentrations since the last glacial maximum and resultant increases in seedling growth and biomass are likely to have had important consequences for the survival and establishment of Acacia seedlings. Tolerance of drought and disturbance has been related to seedling size, hence

stimulating the growth rate could confer disturbance tolerance and this tolerance would develop more rapidly with increasing CO_2 concentrations. Furthermore, increased nitrogen and water use efficiency have the potential to support seedling establishment in environments where these resources would otherwise be limited at lower atmospheric CO_2 concentrations. Resulting in a larger proportion of CO_2 fertilization responsive woody seedlings surviving the seedling size classes, and persisting within historically open savannas.

Where interspecific differences occurred they are likely to have arisen from adaptation to specific climates where these species are native and selection would have been driven by factors such climate, resource availability, levels of disturbance and competitive interactions. *V. karroo* had the highest growth rates and strong CO_2 driven increases in biomass accumulation, despite having the lowest inherent photosynthetic rates. *V. karroo* also had the lowest increase in water use efficiency and high transpiration rates could potentially increase access to soil nutrients through mass flow. This species had the highest mean spine mass and showed significant increases in spine mass fraction at elevated CO_2 concentrations, which may be important for deterring herbivores.

V. robusta's distribution to the mesic east coast of Africa suggests that water is an important limitation to its distribution. Hence, the CO_2 stimulated increase in water use efficiency at both leaf and whole canopy level allows speculation that this may be an important driver of this species' range expansion, which might continue if increasing levels of CO_2 continue to promote water use efficiency.

V. nilotica occupies a broad range of habitats, inhabiting large areas of the sub tropics both north and south of the equator, with the strongest climatic correlates being the precipitation of the wettest quarter followed by high temperature seasonality. In response to increasing CO_2, *V. nilotica* showed overall strong increases in growth, water use efficiency, and physical defenses. These responses may explain why *V. nilotica* has been such a successful encroacher in a broad range of habitats where limitations are likely to include multiple climatic factors and disturbances.

V. tortilis has the widest distribution of all the species studied, covering broad ranges of Africa and only being excluded from the wettest parts of the equator and driest parts of the deserts. In these experiments this species showed the lowest biomass responsiveness to CO_2, but had especially large increases in water use efficiency at both the leaf and canopy level. This may have been an important driver for this species’ encroachment into the more arid parts of its distribution, however this link will need to be verified with further experimentation.

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A	Net CO_2 assimilation rate
A/Ci	Photosynthetic rate/ intra-stomatal CO_2 concentration
Amax	Maximum Photosynthetic rate
$_{growth}$*Amax*	Maximum Photosynthetic rate at seedlings growth CO_2
$_{ambient}$*Amax*	Maximum Photosynthetic rate at CO_2 (40 Pa)
ANOVA	Analysis of variation
Ca	Extra stomatal CO_2 concentration
CO_2	Carbon Dioxide
$[CO_2]$	Carbon Dioxide Concentration
Ci	Intra-stomatal CO_2 concentration
iCO_2	Increased carbon dioxide concentration
eCO_2	Elevated carbon dioxide concentration
^0C	Degrees Celsius
Jmax	Maximum rate of electron transport
LGM	Last glacial maximum
mm	Millimeters
ml min^{-1}	Millimetres per minute
O_2	Oxygen
OTC	Open top chamber
Pa	Pascal partial pressure
PPFD	Photosynthetic Photon Flux Density
REML	Restricted Maximum Likelihood
RGR	Relative growth rate

R_{SL}	Relative stomatal limitation
RuBP	Ribulose 1,5-bisphosphate
miniOTP	Mini open top chamber
NUE	Nitrogen use efficiency
SLA	Specific leaf area
SMATR	Standardised Major Axis Estimation and Testing Routines
SMF	Spine mass fraction
VPD	Vapour pressure deficit
Vcmax	Maximum rate of carboxylation
WUE	Water use efficiency

Chapter 1 –The effects of increasing CO_2 concentrations on African savanna ecosystems.

Since the last glacial maximum (22 thousand years ago) atmospheric carbon dioxide (CO_2) concentrations have increased from ~15 partial pressure (Pa) to the current values of around 40 Pa. Increases from the glacial concentrations are associated with planetary warming and variation in the amount of incoming solar radiation and associated Milankovitch forcing (Steffen et al., 2007). Since the industrial revolution, dramatic increases in atmospheric CO_2 (28 – 40 Pa) have been associated with anthropogenic activity, including deforestation and the burning of fossil fuels. CO_2 concentrations have strong impacts on plant photosynthesis and function as well as secondary impacts on the earth's thermodynamics (Beerling and Osborne, 2006; Steffen et al., 2007).

CO_2 fertilization

Plants respond to increasing CO_2 concentrations by becoming more efficient and productive; this response is known as the CO_2 fertilization effect (Sage, 1994). The effect has been extensively studied and reveals a wide range and magnitude of responses, C_3 photosynthetic plants show strong CO_2 responsiveness while C_4 grasses, such as those that inhabit African savannas, are weak responders (Temme et al., 2013). Increased productivity in response to CO_2 fertilization results from an increase in rates of carboxylation, a reduction in photorespiration and increase in water and nutrient use efficiency (Drake et al., 1997; Körner, 2006; Sage, 1994). In C_4 plants these responses are reduced as a result of a CO_2-concentrating mechanism which saturates the Ribulose- 1, 5- bisphosphate carboxylase/ oxygenase (Rubisco) carboxylation reactions with $CO_{2,}$ reducing photorespiration reactions (Sharkey, 1988).

Photorespiration occurs when Rubisco binds to an O_2 molecule causing the oxygenation of Ribulose 1,5-bisphosphate (RuBP) in the presence of light; this process can result in net photosynthetic inefficiencies of between 20 - 50% in C_3 plants (Drake et al., 1997; Long and Bernacchi, 2003). The amount of photorespiration that the plant experiences depends on the ambient air temperature and the ratio of O_2 to CO_2 present in the atmosphere (Long and Bernacchi, 2003).

eCO_2 (elevated CO_2) reduces photorespiration by favouring the carboxylation reaction of Rubisco, causing the plant to become more efficient until Rubisco is CO_2 saturated. A doubling of CO_2 (20 – 40 Pa) can lead to as much as a 50% reduction in photorespiration in C_3 plants, causing significant improvements to plant efficiency (Drake et al., 1997; Sage, 1994).

The primary nitrogen sinks for plants are photosynthetic enzymes within the leaves. As CO_2 levels increase, photosynthetic efficiency is increased requiring fewer enzymes to sequester the same amount of carbon (Drake et al., 1997). The increased productivity allows plants to reduce the enzyme concentrations within their leaves with no reduction in photosynthetic productivity and this increases their nitrogen use efficiency (NUE). Under elevated CO_2 the ratio of nitrogen to carbon in plant leaves increases by 15- 20% irrespective of the nitrogen concentration in the soil, having a significant effect on the total nitrogen requirement of C_3 plants (Cramer et al., 2009; Drake et al., 1997)

CO_2 concentration has a direct effect on the plant's leaf level WUE (water use efficiency), which is independent of the stimulation of photosynthesis. A doubling of atmospheric CO_2 (20 – 40 Pa) can lead to an increase in leaf level WUE of between 40% and 100% in C_3 plants (Drake et al., 1997; Morgan et al., 2001). As atmospheric CO_2 concentrations increase, the diffusion of CO_2 into the leaf increases intercellular [CO_2], allowing for a reduction in stomatal conductance. This decreases plant water loss per molecule of CO_2 sequestered thus, increasing plant WUE (Drake et al., 1997). Studies have shown that over longer time periods, seasons to years, stomatal density and size can also be affected, forming a mechanism for long-term water use optimization (Franks et al., 2012). These increases in WUE can cause a significant change in the water availability within ecosystems (Drake et al., 1997; Polley et al., 1992).

Acclimation to elevated CO_2

Most plants respond to *e*CO_2 with strong initial increases in productivity, particularly when space, nutrients and water are not limiting (Körner, 2006). The initially strong CO_2 fertilization responses are not linear and often not sustained over longer periods of time and productivity slows by, on average, as much as 18%

(Drake et al., 1997; Moore et al., 1999; Temme et al., 2013). This reduction in productivity is termed ‘down-regulation’ (Sage, 1994) and is a regulatory response involving several mechanisms, the most important of which are adjustments of leaf concentrations of the photosynthetic enzymes in response to leaf sugar levels (Drake et al., 1997). When leaf sugars exceed demand then hexokinase functions as a hexose flux sensor, ultimately affecting transcription of the genes for photosynthetic enzymes, decreasing their concentration and slowing photo assimilate production (Moore et al., 1999). The levels of sugars in leaves depend both on rates of production (source) and consumption via use and storage (sinks). Hence, down regulation is closely correlated to sink strength. Sink strength depends both on the phenology and growth habit of a species, including its developmental stage, reproductive stage, and its ability to develop storage organs.

The *Vachellia (*Fabaceae Mimosoideae Acacieae *Vachellia*) species selected for this study all have below ground storage organs known as lignotubers, a starch storage organ located in the stem of the plant below the soil surface. The evolutionary purpose for the lignotuber is to store resources which can be remobilised to help the plant survive and regrow after disturbance and top-kill events such as fire or herbivory (Kgope et al., 2010; Wigley et al., 2009). As growth [CO_2] increases the lignotuber provides the plant with a large carbohydrate sink where starch can be stored, keeping starch from building up in photosynthetic areas where it would trigger starch-mediated down regulation (Moore et al., 1999). Provided the plant has enough resources to sustain growth, the lignotuber becomes an important sink structure, potentially reducing down regulation while also improving the plant’s potential to re-sprout and regrow after disturbance.

To sustain the high growth rates with CO_2 fertilization, plants need to be nutrient-, water- and light-saturated. This resource saturated state is known as an uncoupled system and will almost never occur naturally except in highly disturbed systems (Körner, 2006). The vast majority of natural ecosystems are limited by either one or a combination of these resources, making them coupled systems. In coupled systems CO_2 fertilization is capped by the availability of the most limiting resource (Körner, 2006). Down regulation is strongly coupled to resource limitation, reducing the potential for plants to benefit from CO_2 fertilization in many arid or nutrient-poor areas (Drake et al., 1997; Körner, 2006). CO_2 fertilization does, however, increase

WUE and NUE, potentially reducing the resource limiting on species in marginal habitats (Drake et al., 1997; Kriticos, 2003).

The response to CO_2 fertilization and the subsequent down regulating responses vary widely between plant functional groups and species, with a pronounced difference between C_3 woody species and C_4 grasses, underpinned by differences in photosynthetic physiology (Anderson et al., 2001; Leakey, 2009). As CO_2 levels are pushed higher through natural and anthropogenic drivers, these differences in responses can cause shifts in competitive ecosystems, affecting the way in which responsive and non-responsive species interact within ecosystems (Polley et al., 1992). In order to understand how increasing CO_2 concentrations can impact grassy ecosystems and savanna, it is necessary to understand how these systems came into being and how they are maintained, particularly through the dynamic interaction of trees and grasses.

The origin and maintenance of open savannas

Savannas are one of the earth's major ecosystems covering an area of around 33 million km^2 globally (Beerling and Osborne, 2006). Savannas are defined as having a discontinuous C_3 woody canopy with a continuous C_4 grass layer. They are highly productive ecosystems that support high biodiversity as well as a significant proportion of the human population (Archibald et al., 2005). The spread of savannas occurred fairly rapidly during the late Miocene and was preceded by the evolution of the C_4 photosynthetic grasses. The timing of the expansion of savannas coincides with a period of low atmospheric CO_2, which would have given the C_4 grasses a competitive advantage over the woody species with the ancestral C_3 photosynthetic system (Beerling and Osborne, 2006; Higgins et al., 2000). Facilitated by the low CO_2 concentrations the C_4 grasses spread through areas that were historically woodland and forest, encouraging the frequency and intensity of fires. Once the areas were open the grasses could form a continuous layer, suppressing the recruitment of woody species (Beerling and Osborne, 2006; Higgins et al., 2000). Interestingly, savannas occur in climates that can support closed forests or woodlands. Open savannas are maintained through a combination of biological interactions and disturbance events (Staver et al., 2011).

Disturbances and stress events in savannas include fire and herbivory, the frequency and impact of which depend on having climates with high seasonality, dry seasons, and periods of drought (Bond, 2008; Ratnam et al., 2011). In isolation these events often cannot maintain open savannas, so a combination of fire, herbivory and seasonality is often required (Bond, 2008). Because savannas occupy broad climatic ranges and species compositions vary, the importance of the different disturbances and stress events varies depending on the specific savanna. Mesic savannas tend to have more intense fire and more intense grazing and browsing intensities whereas in arid savannas desiccation contributes to a large percentage of seedling and sapling mortality (Staver et al., 2009; Pillay and Ward, 2014).

Savannas have evolved to interact with fire. The C_4 grasses actively promote it by building up dry flammable biomass and going dormant during the dry season, whereas the C_3 woody species have evolved to tolerate fire (Archibald et al., 2005; Archibald and Bond, 2003). Young C_3 woody plants store resources below ground and resprout after damage; mature plants grow tall enough to protect their canopies and have fire-resistant bark (Staver et al., 2009). Fire is crucial in the maintenance of healthy open savannas, as it removes old biomass that smothers new growth and hinders seedling development, and it regulates woody species densities by killing or damaging any woody species trapped within the grass layer (Bond, 2008).

Herbivory is historically intense within savannas, with a range of grazers and browsers removing large quantities of biomass from the vegetation (Staver et al., 2009). The composition of the herbivores present and their feeding strategies have the potential to strongly alter species compositions. Herds of grazing species such as cattle or buffalo graze largely on the grass layer, reducing its ability to compete with woody C_3 species, and creating open areas for seedlings to germinate (Augustine and McNaughton, 2006; Kutt and Woinarski, 2007). When browsing species or mega herbivores are present, however, they concentrate on foraging on woody species, often causing significant damage to mature plants and selectively browsing on young more nutritious and less defended saplings (Augustine and McNaughton, 2006; Kutt and Woinarski, 2007). Herbivory is also influenced by both the physical and chemical defences, which plants invest in to reduce damage and alter herbivore preferences. These defences and preferences add an interesting biological interaction as each plant community has unique herbivore defences and levels of tolerance (Gowda and Palo, 2003; Hean and Ward, 2012).

The frequent disturbance events and seasonal stresses act as bottlenecks to the recruitment of C_3 species. To compensate for this woody C_3 savanna species produce vast quantities of seeds, but only a small fraction of the seedlings will reach mature size classes (Higgins et al., 2000; O'Connor et al., 2014; Ward, 2005). The majority of the seedlings suffer mortality events from herbivory, fire, or desiccation before reaching mature size classes (Pillay and Ward, 2014). The effect of recruitment bottlenecks is variable; if the frequency or intensity of the disturbance events are reduced such as through fire suppression or the removal of herbivores, it can lead to all of the trapped individuals rapidly reaching mature size classes, known as a mass escape event, giving the impression of rapid encroachment (Bond, 2008).

Bush encroachment and increasing atmospheric CO_2 concentrations

Woody thickening, or bush encroachment is the increase in density of woody species within areas that were historically open grasslands or savannas. It was first noticed and described in South Africa in the early 1900s and has become one of the world's largest ecological phenomena to occur within living memory (O'Connor et al., 2014; Ward et al., 2014). Between 10 – 20 million hectares of historically open savanna have been encroached in South Africa alone, with the rate of encroachment increasing (O'Connor et al., 2014; Ward, 2005). Once an area becomes encroached to the point where the canopies interlock (45 – 50%), the woody C_3 species shade out the shade intolerant C_4 grass layer, removing the flammable understorey and creating an irreversible state shift (Staver et al., 2009; Charles-Dominique et al., 2015). This state shift changes a biodiverse and highly productive savanna into a monoculture woodland with decreased ecological or economical value (Ward, 2005).

This process of bush encroachment is thought to have been, at least partially, facilitated by $eCO_{2.}$ This is underpinned by C_3 versus C_4 differences in response to eCO_2, giving C_3 woody species a competitive advantage over the C_4 grasses, as well as increasing the probability of the C_3 woody species surviving disturbance events and outgrowing disturbance traps (Bond and Midgley, 2000; O'Connor et al., 2014). Elevated CO_2 is not the sole factor responsible for increasing encroachment and is thought to interact with factors such as altered land use, fire and climate which, in combination, drive encroachment. In fact the extent of role of eCO_2 has recently been questioned (Venter et al., 2018), although factors correlated to bush encroachment in this paper are not themselves unaffected by eCO_2 and an interaction remains a likely explanation.

CO_2 fertilization has been shown to increase the growth rates and biomass accumulation of certain woody savanna species, as well as increase the rate at which they can resprout after disturbances (Kgope et al., 2010). These responses improve the probability of woody savanna species surviving disturbance events, and increasing the rate at which they can outgrow vulnerable size classes. The improved survival and reduced time that the seedlings are trapped within disturbance zones means disturbance events become less effective than they would have been under low CO_2 atmospheres, requiring either more frequent or more intense events to achieve the same suppressing effect on woody species (Bond and Midgley, 2000).

Woody savanna species, especially those from the *Vachellia* genus, can resprout after disturbance events, re-growing their above ground biomass and surviving events that would kill other species (Bond, 2008; Higgins et al., 2000; Wigley et al., 2009). This is achieved by protecting meristems below ground and storing carbohydrates in a dedicated lignotuber, the stored carbohydrates are mobilized following a disturbance event and used to regrow the damaged canopy (Wigley et al., 2009). Once this lignotuber is established plants become relatively impervious to disturbances; however, briefly, as young seedlings, they are completely vulnerable and die if disturbed. Between germination and reaching disturbance tolerant size classes, a large percentage of woody seedlings are killed by desiccation and disturbance events (O’Connor, 1995; Perumal, 2016). As CO_2 fertilization increases the growth rates of responsive species, the rate at which they can reach disturbance tolerant size classes will be reduced, lowering the probability of experiencing a fatal disturbance and increasing the percentage of the population reaching the next size class.

A large component of the response to CO_2 fertilization is the effect it has on plant resource use efficiency. As CO_2 increases C_3 species become more nutrient, water and light use efficient (Drake et al., 1997). In ecosystems where the species were already present, this can increase their growth rates and lead to positive feedback loops on growth and resource foraging (Drake et al., 1997). More efficient resource use allows the plants to develop bigger canopies and root systems with the available resources which in turn, gives them access to more resources (Kirschbaum and Lambie, 2015). Increased resource use efficiency could also allow species to expand into ranges which were historically inhospitable for them. The increased climatic tolerances could be especially relevant in arid environments, potentially increasing the range in which encroaching species can survive, allowing them to encroach on previously open areas (Kriticos, 2003).

The woody species implicated in encroachment vary across continents and ecosystems; however, in southern Africa encroachment is dominated by former members of the *Acacia* genus - now split into the *Vachellia* and *Senegalia* genera. Both genera are made up of medium-sized nitrogen-fixing trees with high productivities which thrive in highly competitive and disturbance-prone savannas (Murphy et al., 2010). In the last century they have been aggressively expanding their historic ranges, even becoming invasive in foreign countries (Kriticos, 2003). Motivated by their prevalence for encroaching (Stevens et al., 2016), and known for their strong responses to eCO_2 (Kgope et al., 2010), a range of species from the *Vachellia* genus were selected as the focus species of this study. *V. karroo* was selected for its prominent role in encroachment and its known strong CO_2 responses (Kgope et al., 2010; O'Connor et al., 2014), *V. nilotica* is a strong encroacher, but has shown weaker varied responses to eCO_2 (Kgope et al., 2010; Kriticos, 2003). *V. tortilis* is an encroaching species from an arid environment with slower growth rates, and hasn't been included in as many CO_2 studies as the other encroaching species (Moleele et al., 2002). *V. robusta* is mentioned the least with regards to encroachment, but its fast growth rates make it a likely responder to [CO_2] and its affinity for mesic forest margins is a characteristic that separates it from the other species (Atkin et al., 1999; Coates Palgrave, 2005). The results of the seedlings responses to a range of historical [CO_2]s (CO_2 concentrations) can be used to predict how the species will respond to future [CO_2]s and whether these responses will promote woody encroachment at future [CO_2]s.

Seedlings of the selected species were exposed to sub-ambient CO_2 concentrations ranging from pre-glacial (12 Pa) to current ambient (40 Pa) concentrations. The sub-ambient gradient was chosen to describe the historic effects that CO_2 increase would have had on seedling performance and growth. The study aimed to assess all aspects of seedlings' growth at eCO_2, which could affect and promote seedlings' establishment and survival within the savanna, including growth rates, biomass accumulation, photosynthetic productivity and water use. *Vachellia* seedlings were grown under the selected range of CO_2 concentrations. After eight weeks they were subjected to a range of gas exchange measurements, before being destructively sampled. The aim was to determine if all four of these *Vachellia* species showed equally strong responses to increasing $CO_{2,}$ and whether responses are underpinned by common mechanisms. Alternatively, species might respond differently, or by different mechanisms, and these may originate from species level adaptations to the habitats from which they originate. If differences exist then they may have important ecological implications for bush encroachment; common responses, however, may identify mechanisms that enable these species to respond to *e*CO_2 thereby promoting their ability to encroach savanna.

Having set the context for this research, the following is a guide to the subsequent chapters of the thesis.

Chapter 2 details the equipment used to achieve the desired CO_2 concentrations as well as provide species descriptions for the selected *Vachellias* as well as modelled geographic habitats.

Chapter 3 focuses on the seedlings' growth and morphological responses to increasing [CO_2]'s with the aims to test;

i. Whether the four species of *Vachellia* seedlings (*V. karroo*, *V. nilotica*, *V. robusta*, and *V. tortilis*) investigated in this study all respond in a similar way to *i*CO_2. We expected that the response would include increased RGR and dry biomass production, altered biomass allocation, and an increased investment in physical defences.

ii. Whether interspecific differences in the response to *i*CO_2 occurred, and whether these would confer advantages specific to the climatic conditions prevalent in the natural ranges where these different species occur.

Chapter 4 highlights the seedlings photosynthetic and leaf level responses to changes in growth $CO_{2,}$ with the aims to test;

i. How the *Vachellias* seedlings photosynthetic efficiency and performance is affected by changes in growth CO_2 as well as what levels of photosynthetic down regulation are experienced.
ii. Whether and to what extent water use efficiency is affected by growth CO_2, both on a leaf level as well as whole plant canopy scale.
iii. How the seedlings' photosynthetic performance and water use responses correlate to the growth and morphological responses discussed in Chapter 3.

Chapter 5 will use the observations from Chapters 3 and 4 to discuss the ways in which the *Vachellias* have responded to the increases in atmospheric [CO_2], and how these responses could act to trigger or facilitate the rapid increase in woody encroachment within historically open savanna ecosystems.

Chapter 2 – General Methods: CO_2 control system and species description

Sub-ambient CO_2 system design

My experimental design used a sub-ambient CO_2 system to continually provide the seedlings with a predetermined CO_2 concentration **(Figure 2-1)**. The sub-ambient concentrations were achieved by passing atmospheric air through a column of soda lime. This process removed all of the CO_2 from the air which was then mixed with atmospheric air via a system of manually operated aquarium valves (J.W. Pet Company, US) to attain the desired CO_2 concentration.

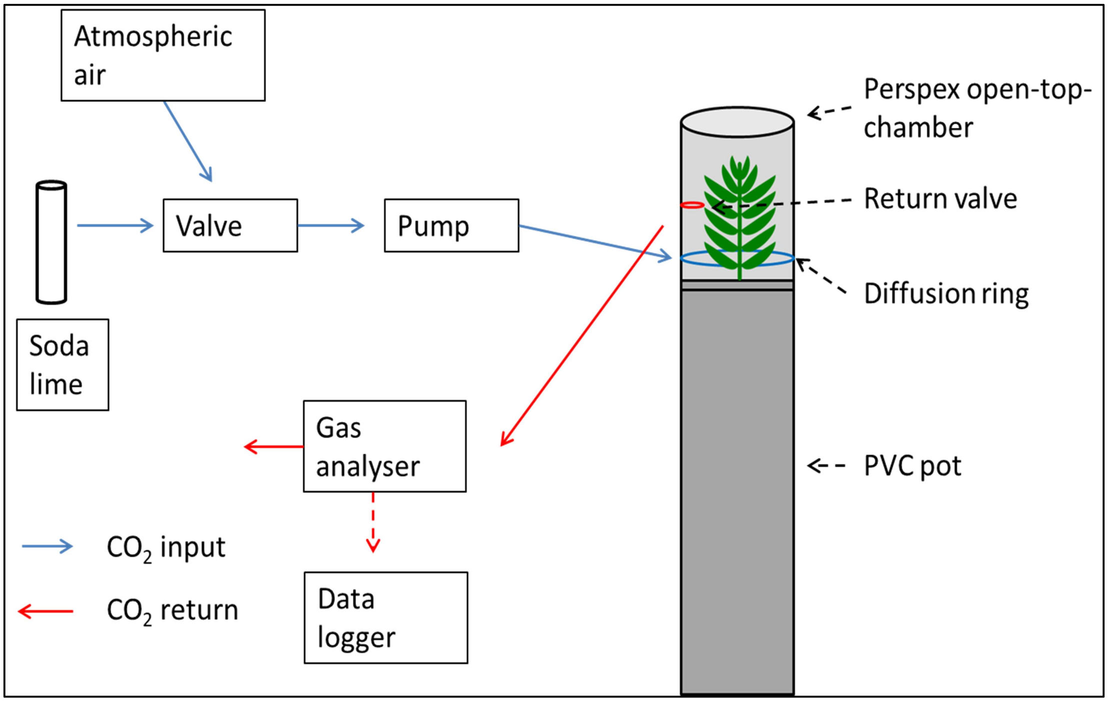

Figure 2-1. Experimental setup for the CO_2 concentration control and analysis

The CO_2 mixture was then pumped to mini open top chambers (OTCs) which were constructed out of 1.12 mm thick transparent polycarbonate sheeting. Each OTC had a volume of 1L. Each OTC consisted of a cylinder 105 mm wide, and 110 mm high which was extended a further 30 mm by a frustum with a 75 mm opening at the upper end. Diffuser rings 25 mm above the soil surface injected the air evenly into the chambers at a rate of 900 ml min^{-1}. The CO_2 concentration in each chamber was monitored for the entire experimental period and was measured once an hour, as an average value integrated over a 6-minute period. A solenoid-valve system controlled via a CR-1000 logger and 16-Channel AC/DC Relay Controller (Campbell Scientific, Logan, Utah, USA) sequentially diverted air (100 ml min^{-1}) to a LI-820 infra-red gas analyser (LI-COR Inc., Lincoln, NE, USA). After every hourly cycle the analyser was zeroed using scrubbed air, and the span calibrated using a premixed atmospheric gas cylinder with a known concentration.

Experimental design

Three experiments were performed in the sub-ambient system (**Table 2-1**) using two different experimental approaches. The first used a gradient approach where each of the ten chambers were set at an individual [CO_2]. Single *V. karoo* plants in each chamber were then exposed to a particular [CO_2] and the collective responses from all plants were analysed via regression analysis. The second experiment used a similar approach but investigated three species (*V. nilotica, V. robusta and V. tortilis*) simultaneously using a gradient approach and seven individual [CO_2]s. The third experiment investigated only *V. karroo* and used a classical design where treatments were replicated, with seven chambers set to each of the three distinct [CO_2]s (**Table 2-1**). Responses could thus be analysed using an analysis of variance (ANOVA) approach. Experiments 1 and 3 allowed *V. karroo* responses to be compared between replicated and regression approaches. The CO_2 concentrations were selected to provide a growth gradient from pre glacial to ambient concentrations.

Table 2-1. Summary of the experimental setups run in the sub-ambient CO_2 system

Experiment	Species	[CO_2] (Pa)	Approach	Replication
1	*V. karroo*	12 – 44	Regression	10 chambers staggered at a range of [CO_2] from 12 - 44
2	*V. nilotica* *V. robusta* *V. tortilis*	13 – 41	Regression	7 chambers per species staggered at a range of [CO_2] from 13 – 41
3	*V. karroo*	16, 31,39	Replicated	7 chambers per each distinct [CO_2]

CO_2 chamber performance and control of [CO_2]

The hourly [CO_2] data, analysed over the duration of each of the experiments, is presented in **Figure 2-2**. Considering the manual control of the concentrations and duration of the studies the desired concentrations were remarkably stable. The chambers at low [CO_2] experienced more variation than those at higher [CO_2]. This is a result of the stronger diffusion gradient between the chamber and atmosphere. In experiment 2 (**Figure 2-2 B**) there was a problem with chamber 14 which persisted even after problem solving, resulting in a higher [CO_2] variability than the other chambers.

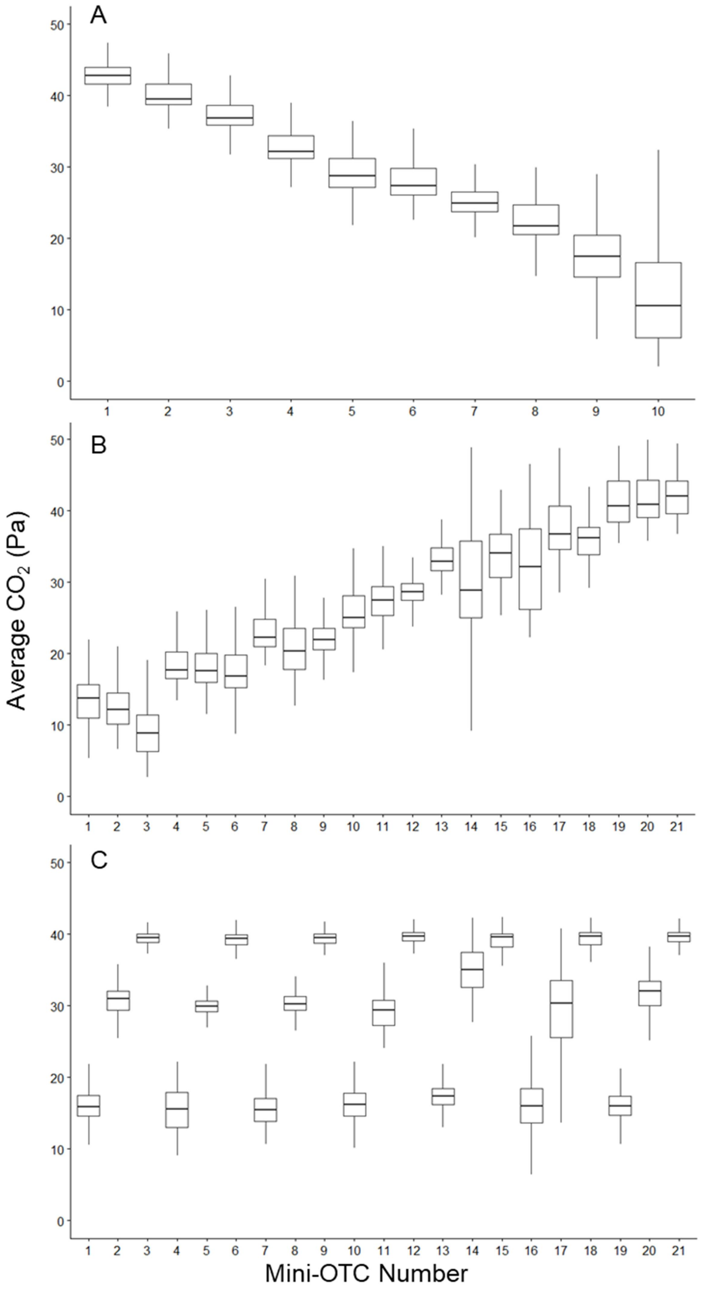

Figure 2-2. CO_2 concentration results for the mini open top chambers measured hourly for the duration of the study

(**A** = Experiment 1 with gradient approach and 10 [CO_2]s **B** = Experiment 2 with gradient approach and three species (*V. niliotica*, *V. robusta*, *V. tortilis)* and seven [CO_2]s, **C** = Experiment 3 with a replicated approach using *V. karroo* and three chambers at each [CO_2]).

Plant growth and maintenance

The study species selected are all closely related *Vachellia* species, which have been noted as encroaching species, but occupy different climatic ranges. The seed was sourced through a commercial seed supplier, and the selected *Vachellia* seeds were scarified before being soaked in warm water for 24 hours. Seeds were then placed in sterile petri dishes lined with moist filter paper and housed within a controlled environment set to 31^0C until they germinated. Once the seeds had produced a substantial apical root the most uniform individuals were selected and planted in naturally sourced topsoil, with typical savanna characteristics. The pots that the seedlings were planted in were purpose-designed deep PVC pots, with the dimensions 105 mm (diameter) x 300 mm (depth) to reduce pot effects on the roots.

The seedlings were observed daily and watered manually when required, water was used sparingly to avoid leaching; no fertilizers were added for the duration of the study. The sub-ambient CO_2 system was housed in a poly-tunnel where average (± S.D.) chamber day/night temperatures within the OTCs were 28.4 ± 3.7 and 15.3 ± 0.9, respectively, measured with a thermocouple mounted in one of the chambers and averaged across the duration of the experiments. The plants received 11 hours of solar daylight at an average daytime Photosynthetic Photon Flux Density (PPFD) of 490 mol m^{-2} s^{-1} and daily maximum values in excess of 1500 mol m^{-2} $s^{-1}$$_,$ measured with photodiode mounted on the side of one of the PVC pots and averaged across the duration of the experiments.

Study species description and ecological ranges

The species' habitats and abiotic selection pressures were modelled using Maxent. Maxent applies the maximum entropy model to determine the probability of a species being present in an area based on known localities (georeferenced herbarium localities) and overlaid with climatic data layers, to model the possible extent of habitat ranges (Philips et al., 2018)

The model was trained with the following climatic factors; annual mean temperature, annual precipitation, mean temperature of the driest quarter, mean temperature of the wettest quarter, precipitation of the wettest quarter, precipitation of the driest quarter, precipitation seasonality and temperature seasonality.

Once modelled the percentage influence of the climatic factor of the species can be extracted (**Figure 2-3**). As intended by our species selection the species showed strong variations in the influence by the climatic variables. The strongest overall habitat determinants were annual precipitation and annual temperature seasonality, while the weakest overall climate influence was precipitation seasonality.

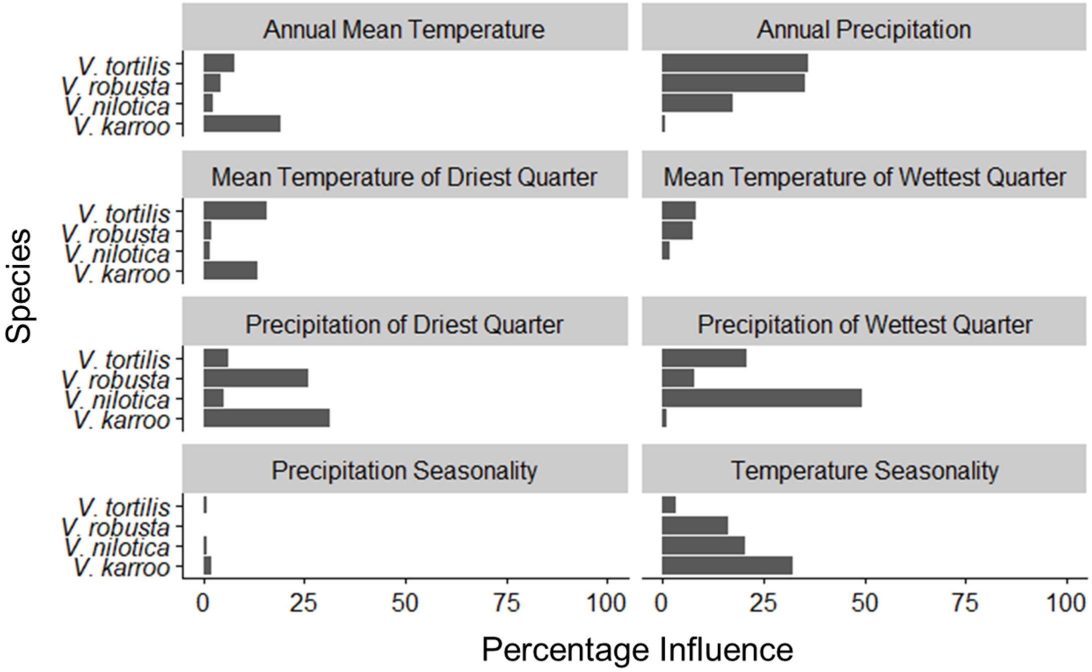

Figure 2-3. Percentage influence of abiotic bioclim data sets on the distribution of the selected *Vachellia* species

Vachellia karroo (Hayne) is a common tree with a dense round canopy, often branching close to the ground. The height is highly variable reaching up to 15 meters in certain areas, but most commonly between 3 to 5 meters. *V. karroo* can be found throughout most of southern Africa, and is tolerant to a wide range of climatic conditions (Coates Palgrave, 2005). *V. karroo's* range was confined to the southern portion of the continent (**Figure 2-3**) favouring areas with moderate rainfall and fairly strong seasonal temperature variability. This particular tree was one of the first indigenous species to be recognised as an encroacher; it has become a problem throughout southern Africa, transforming millions of hectares of profitable farmland into woodland (Kgope et al., 2010; O'Connor, 1995).

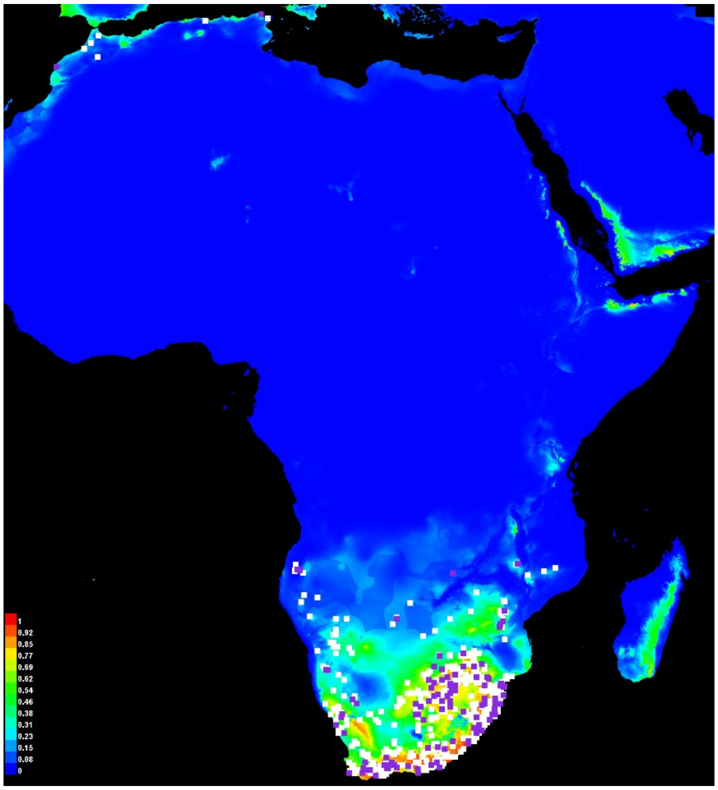

Figure 2-4. Species distribution model for *V. karroo* using biomclim data and herbarium specimen locality data, (n = 442). White squares represent known GPS coordinates of the species, purple squares are plotted when a number of white squares overlap. The scale bar on the left shows the modelled probability of the species occurring in an area with red being 1 and dark blue being 0.

Vachellia nilotica (Rochebr) is a smallish tree with a dense mushroom-shaped canopy, common throughout a range of ecosystems and a wide range of climates throughout Africa (Palgrave and Coates, 2005). *V. nilotica* is an extensively distributed encroaching species within southern Africa, and has even become an alien invasive species in non-African countries such as Australia (Kgope et al., 2010; Kriticos, 2003). *V. nilotica* has an extensive range inhabiting the bulk of the sub-tropic region, both north and south of the equator (**Figure 2-5**). The species distribution was most dependent on the precipitation in the wettest season, annual precipitation, and temperature seasonality, but is largely unaffected by annual mean temperatures (**Figure 2-3**).

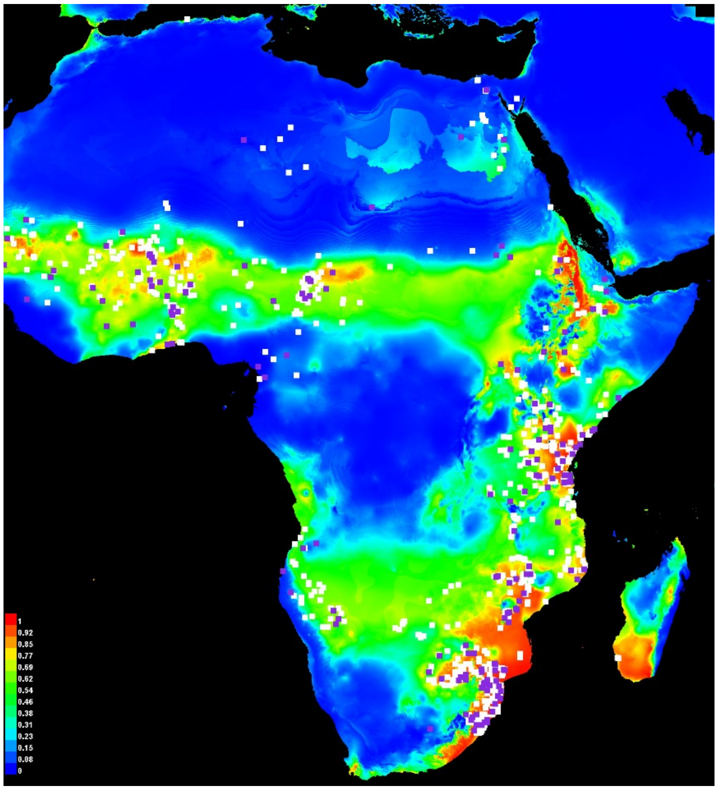

Figure 2-5. Species distribution model for *V. nilotica* using biomclim data and herbarium specimen locality data, (n = 684). White squares represent known GPS coordinates of the species, purple squares are plotted when a number of white squares overlap. The scale bar on the left shows the modelled probability of the species occurring in an area with red being 1 and dark blue being 0.

Vachellia robusta (E. Meyer) is a fast growing medium to large tree, often single-stemmed and reaching heights of around 12 meters, where the canopy spreads into a round crown. *V. robusta* favours wet and shaded areas, often being found in valleys or forest margins. It is found along the east coast of southern Africa, between the Eastern Cape and south of the Zambezi. This species is seldom cited as an encroacher. *V. robusta* has the most climatically restricted range of the species **(Figure 2-6**), being largely confined to the mesic east coast of Africa, the species distribution was strongly influenced by the annual precipitation as well as precipitation of the driest quarter (**Figure 2-3**).

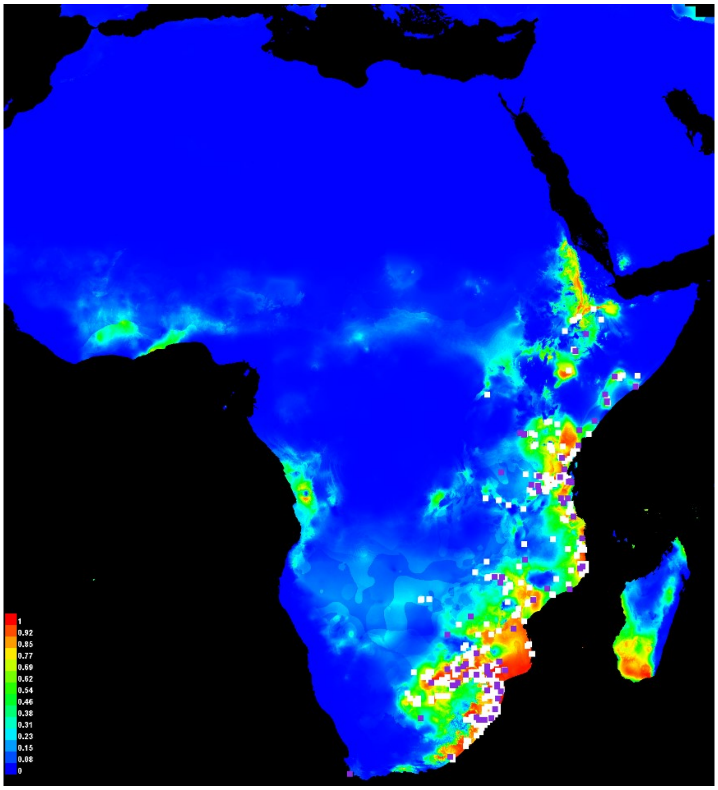

Figure 2-6. Species distribution model for *V. robusta* using biomclim data and herbarium specimen locality data, (n =301) White squares represent known GPS coordinates of the species, purple squares are plotted when a number of white squares overlap. The scale bar on the left shows the modelled probability of the species occurring in an area with red being 1 and dark blue being 0.

Vachellia tortilis (Forsk) is a drought tolerant, slow growing, medium to large tree reaching between 5 and 20 meters in height (**Figure 2-10**). Young trees often have rounded canopies but mature trees form characteristic umbrella-shaped canopies. This particular species is found extensively through low altitude dry areas throughout southern Africa. Although it has a slow growth rate *V. tortilis* has been noted as a prolific encroaching species. *V. tortilis* has an extensive range across the entire African continent (**Figure 2-7**) with modelled ranges extending far into the Sahara desert, only being completely excluded from the tropics and most arid portions of the continent. The species was most strongly influenced by annual precipitation, precipitation of the wettest quarter and mean temperature of the wettest quarter (**Figure 2-3**).

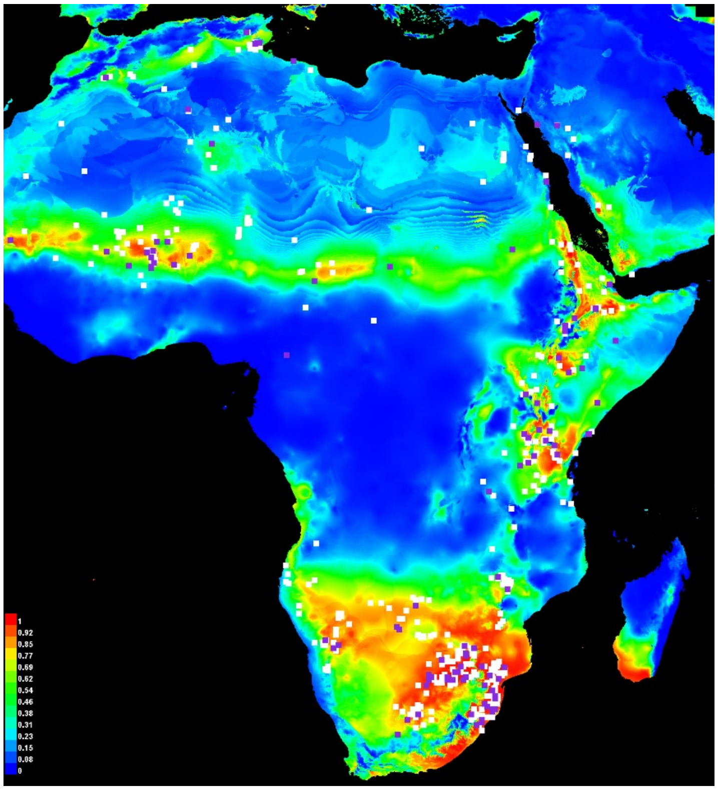

Figure 2-7. Species distribution model for *V. tortilis* using biomclim data and herbarium specimen locality data, (n = 402). White squares represent known GPS coordinates of the species, purple squares are plotted when a number of white squares overlap. The scale bar on the left shows the modelled probability of the species occurring in an area with red being 1 and dark blue being 0.

In conclusion, this chapter has presented the methodology used for this research and introduced the study species and their geographic ranges. The following chapter will present the findings derived from using this methodology, focusing on the growth and morphological response of the selected *Vachellia* species.

Chapter 3 – **Growth and morphological responses of *Vachellia* seedlings at sub-ambient CO_2 concentrations**

Introduction

In this Chapter, I assess the growth and morphological responses of the four selected *Vachellia* species seedlings at a range of sub-ambient [CO_2] from pre-glacial to ambient. Parameters measured include: changes in relative growth rates, biomass accumulation and allocation, as well as changes to spinescent defences of the seedlings.

Global atmospheric CO_2 levels have risen by over 50% since the last glacial maximum (LGM) from 18 Pa to 40 Pa. With the world's current reliance on the burning of fossil fuels these levels will continue to rise into the future (Steffen et al., 2007). CO_2 is historically a rare resource for plants, relative to light and water, and as it is the basic substrate for photosynthesis, changes in atmospheric [CO_2] can have significant effects on plant growth and performance (Sage, 1994). Research has shown that as [CO_2] increases the majority of plants become more efficient and productive, as well as more water and nutrient use efficient (Drake et al., 1997). The literature shows a large range of responses among species, as well as between functional groups (Ainsworth and Long, 2005; Sage, 1994). This range of responsiveness to *i*CO_2 has the potential to impact the way plants co-exist within ecosystems, especially in systems where community structure depends on competitive interactions.

CO_2-stimulated increases in productivity have been shown to strongly increase relative growth rates (RGRs) (Ainsworth and Long, 2005; Temme et al., 2015), producing taller, larger seedlings with better developed root systems (Kirschbaum and Lambie, 2015). Stimulated RGRs increase seedlings' access to resources such as light and water, further promoting growth through a positive resource feedback (Kirschbaum and Lambie, 2015). Increased RGR also decreases the time required for seedlings to reach disturbance tolerant size classes by reducing the time taken for the seedlings to become disturbance tolerant, the risk of experiencing a fatal mortality is reduced, increasing woody seedling recruitment (Ripley et al., unpublished).

Resource allocation is dependent on plant source-sink relationships and is likely to favour sinks at increased [CO_2] (Poorter et al., 2011; Temme et al., 2013). The increased carbohydrate production at [iCO_2] must be either used or stored to avoid hexose-mediated down regulation (Moore et al., 1999). As all the *Vachellia* species used in this study produce lignotubers (a storage organ located in the stem below the soil surface), they have the potential to use and store additional carbohydrates produced under iCO_2 (Wigley et al., 2009). Lignotubers enable stored resources to be used for plant re-growth after disturbance and are key to the survival of these species in a disturbance driven savanna (Kgope et al., 2010; Wigley et al., 2009). It is expected that the seedlings grown at iCO_2 will allocate surplus carbohydrates to the lignotuber thereby increasing the below ground biomass component of the seedlings. These carbohydrate stores, in turn, reduce starch-mediated down regulation while increasing resource provisioning to be remobilised in the event of a top-kill disturbance (Wigley et al., 2009).

Vachellia trees produce woody spines as a physical defence against herbivory. CO_2 fertilization and an additional supply of carbohydrates could promote the production of more spines, as well as the investment into individual spines making the seedlings less vulnerable to herbivory(Gowda, 1996). Spines reduce the bite size that herbivores take, thus reducing feeding efficiency and limiting the amount of growth material removed from the plant (Charles-Dominique et al., 2016; Gowda, 1996). These physical defences in *Vachellia* trees are induced by herbivory, the plants produce longer, thicker spines in response to increased herbivory, while spines remain less developed in areas with low herbivore densities (Midgley and Ward, 1996). As CO_2 increases carbohydrate production this increase in thorn production need not necessarily compromise carbon allocation to other resources such as reproductive structures and leaves.

This CO_2 study is intended to contribute to this understanding about the historic role that increasing CO_2 has played in bush encroachment. CO_2 stimulated growth would contribute to the development of root resources altering bottom-up (nutrient and water) savanna seedling limitations and allow C_3 seedlings to become more aggressive competitors relative to C_4 grasses (Bond and Midgley, 2000; Ward, 2010). CO_2 fertilization would also alter top-down control (fire and herbivory) of tree recruitment, as faster growth would allow plants to outgrow and escape disturbance bottlenecks and heighten their ability to re-sprout after disturbance (Kgope et al., 2010). Rising CO_2 increases the competitive ability of woody species in the savanna, while simultaneously decreasing plant susceptibility to disturbance (Bond and Midgley, 2000).

Methods

Four species from the *Vachellia* genus were grown from germinated seeds under a gradient of sub-ambient $[CO_2]$s, in three different experimental runs (see general methods in Chapter 2 for detailed species descriptions and a design for the CO_2 control system). Once grown for 60 days, seedlings were subjected to gas exchange measurements (described and presented in Chapter 4) and then harvested.

At harvest, seedlings were carefully removed from the pots to avoid damaging the roots and washed to remove the soil. The height of the seedlings was measured from the base of the stem to the top of the primary apical shoot. The seedlings were then dried at 60°C for 7 days. Once dry, plants were separated into above and below ground compartments and weighed. The spine lengths were measured after drying with Vernier callipers, clipped from the stems and weighed. Spine density was calculated by dividing the total number of spines by the total length of the seedlings above ground stem area. Spine mass fraction (SMF) was calculated by dividing the dry mass of the combined spines by the total dry mass of the seedlings.

Relative growth rate (RGR) was calculated from the height of the seedlings at the time of harvest: RGR= (plant height)/8weeks (Kirschbaum and Lambie, 2015). Mass fractions were calculated as the selected dry biomass component/total dry biomass, e.g. SMF = stem mass/total plant mass.

Statistical analysis

Linear regressions used as these are simpler to fit and non-linear regression would not have provided better fits overall. The linear regressions were fitted to the response variables against growth CO_2 concentrations. The R^2 and p values from the regressions were then used to determine the strength of responses and the relationship to growth $[CO_2]$.

For an overall genus response, the data from all species were combined and response variables were regressed against growth [CO_2] using Restricted Maximum Likelihood (REML) mixed effects models with [CO_2] as a fixed effect and species as a random factor. This model accounts for the inherent differences between species (Bates et al., 2015) and allowed the overall CO_2 effect to be determined. Analyses were conducted in R using the packages LME4 and Car.

Comparisons among species were performed with standardised major axis estimation (R package SMATR) which allows regression slopes and intercepts to be fitted to data from individual species and compared. SMATR uses multi-component comparisons and was implemented with the R package SMATR (Warton et al., 2012).

The response variables were log transformed to stabilize the variance and improve the data normality.

For the replicated experiment the seedlings were grouped into three CO_2 growth concentration groups 16, 31, and 39 Pa. The response variables were then compared between these groups by fitting an Analysis of Variance Model and comparing the Tukey Honest Significant Differences.

Results

Relative growth rates

The RGR of the seedlings responded strongly to CO_2, and when averaged across all species, increased by 50% over the CO_2 range from pre-glacial to ambient (**Figure 3-1**) These results showed similar trends for the seedlings grown under CO_2 gradient conditions and in the replicated experiment (**Figure 3-2**), verifying our experimental approach.

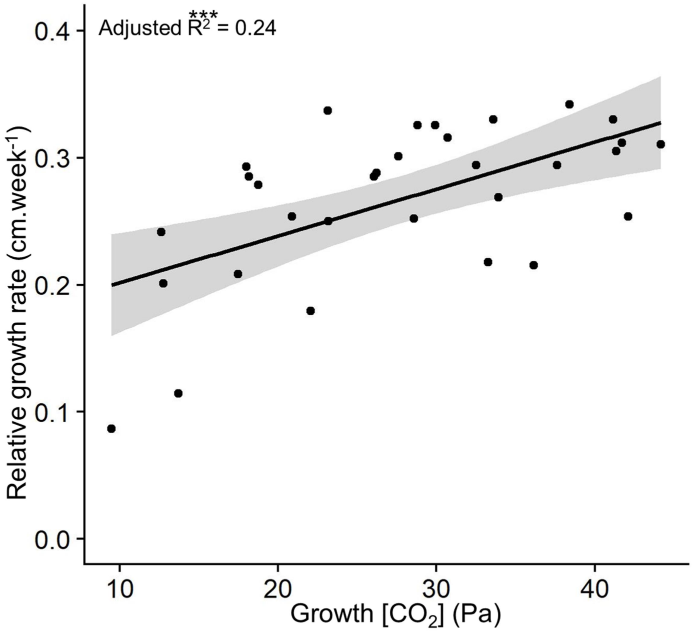

Figure 3-1. Combined species relative growth rate response to iCO_2 Grey shading = 95% prediction interval. Elevation and slope are calculated using SMATR (Warton et al., 2012). Symbols represent ▪p ≤ 0.1, *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001.

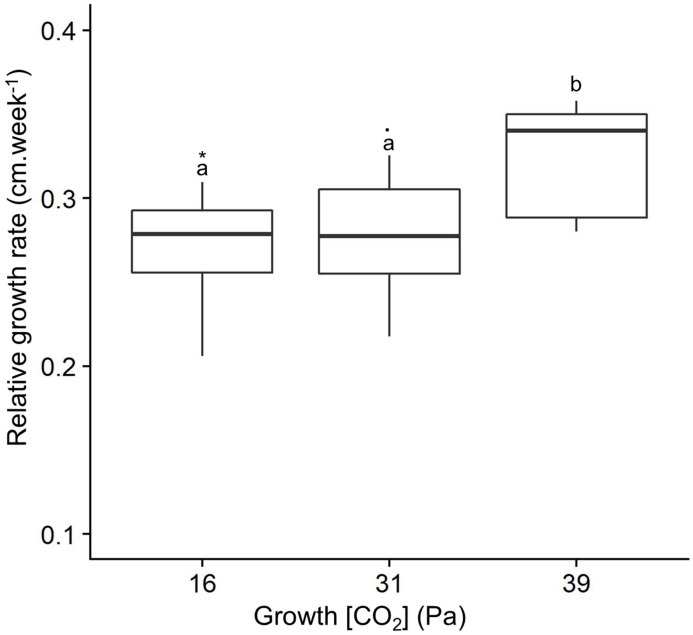

Figure 3-2. *V. karroo* seedlings relative growth rate response to growth at 3 distinct sub-ambient [iCO_2], with 7 chambers at each concentration. Significant differences reported as letters, symbols represent ▪p ≤ 0.1, *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001.

When compared on a species basis (**Figure 3-3**), SMATR analyses showed that the regression slopes were not different, implying uniform responses across species. Regression fits were significant for all species with the exception of *V. nilotica.* Species were, however, different in the elevation of RGR responses implying inherent differences in growth. Growth rates were higher for *V. karroo* and *V. robusta,* compared to *V. tortilis*.

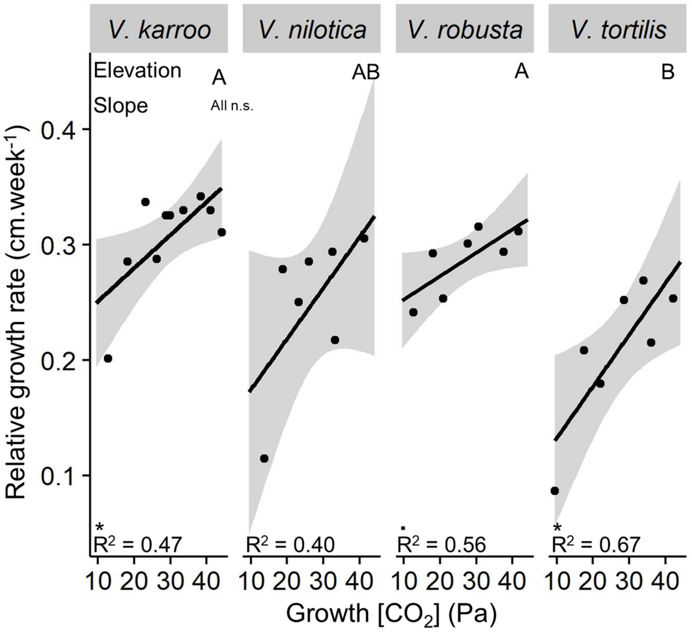

Figure 3-3. Species growth rate responses to growth [CO_2]. Grey shading = 95% prediction interval. Elevation and slope are calculated using SMATR (Warton et al., 2012) and significant differences in elevation reported as capital letters, differences in slope as small letters and n.s. as not significant. Symbols represent ▪$p \leq 0.1$, *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$.

Biomass production and allocation

The whole-plant dry biomass responded strongly to $i\mathrm{CO}_2$, with a combined species response showing an increase in biomass of 186% over the range of CO_2 concentrations used in the study (**Figure 3-4**; results were: $F_{1,30}=48$, $p< 0.0001$, adjusted $R^2=0.29$). This response in total biomass was mirrored in both the above and below ground compartments and $i\mathrm{CO}_2$ increased these biomasses by 183% and 220%, respectively, data not shown (Mixed effects model – aboveground biomass:

$F_{1,30}$= 43, $p< 0.0001$, adjusted R^2=0.78; below ground biomass: $F_{1,30}$= 37, $p< 0.0001$, adjusted R^2=0.68). There were, however, differences in the sizes of compartments with approximately 35% more biomass invested in above- than below ground biomass. As the CO_2 effect was similar in both compartments there was no significant change in the ratio of above- to below ground biomass with *i*CO_2.

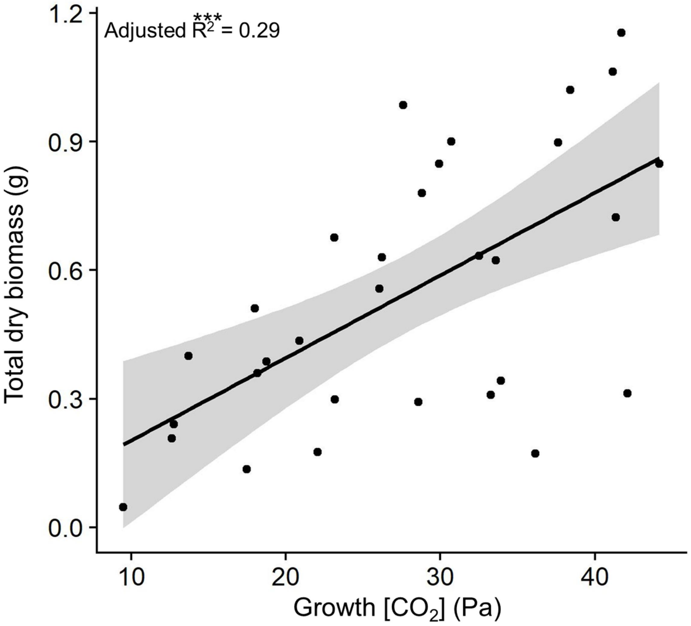

Figure 3-4. Combined species total biomass response to *i*CO_2. Grey shading = 95% prediction interval. Symbols represent ▪p ≤ 0.1, *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001.

When biomass responses were compared among species there were no differences in regression slopes for the, above- (**Figure 3-5A**) or below ground biomass (**Figure 3-5B**), suggesting similar *i*CO_2 responses across all species. Again, there were differences in slope elevation for the *i*CO_2 responses; *V. karroo*, *V.nilotica* and *V. robusta* had inherently larger above-ground biomass than *V. tortilis*. The situation was very similar for below ground biomass with the exception that *V.nilotica* root mass was not different from that of *V. tortilis*. In all cases the regressions were significant with the exception of the below ground response in *V. nilotica*.

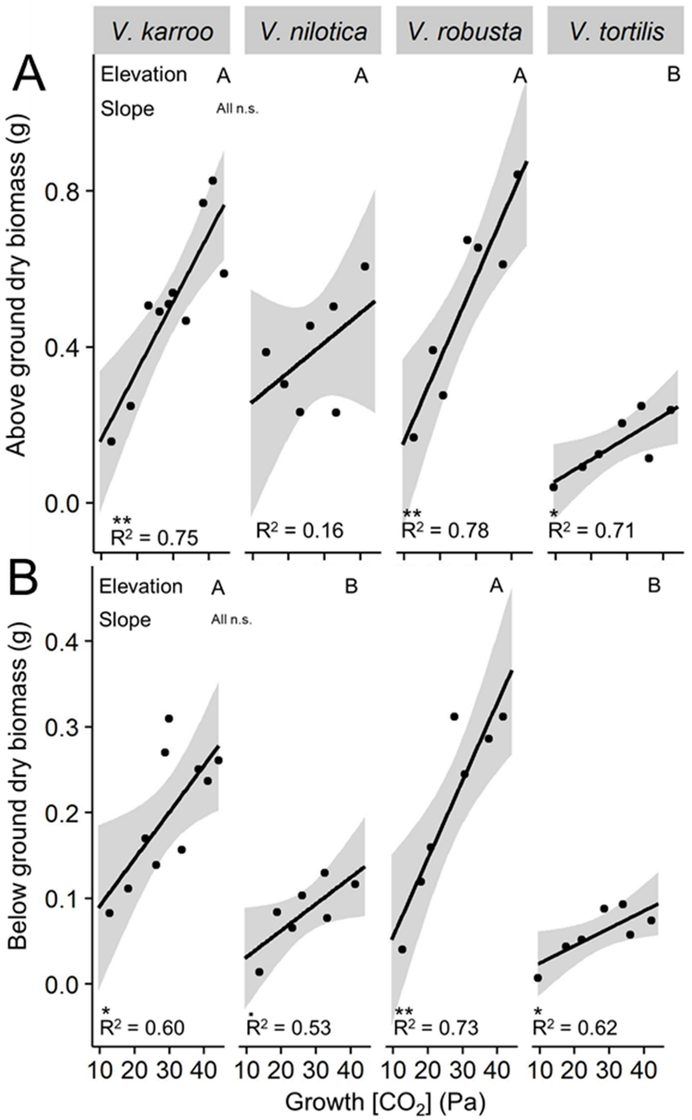

Figure 3-5. Species above (**A**) and belowground (**B**) dry biomass responses of the seedlings to growth [CO_2]. Grey shading = 95% prediction interval. Elevation and slope are calculated using SMATR (Warton et al., 2012) and significant results reported. Grey shading = 95% prediction intervals, and significant differences in elevation reported as capital letters, differences in slope as small letters and n.s. as not significant. Symbols represent ▪$p \le 0.1$, *$p \le 0.05$, **$p \le 0.01$, ***$p \le 0.001$.

The seedlings showed weak root to shoot ratio responses, with a shallow combined response significant to the 90 but not 95 confidence interval (**Figure 3-6**). This indicates that the seedlings do not alter biomass investment at *i*CO_2, but increase all biomass components equally, at least in the seedling size class.

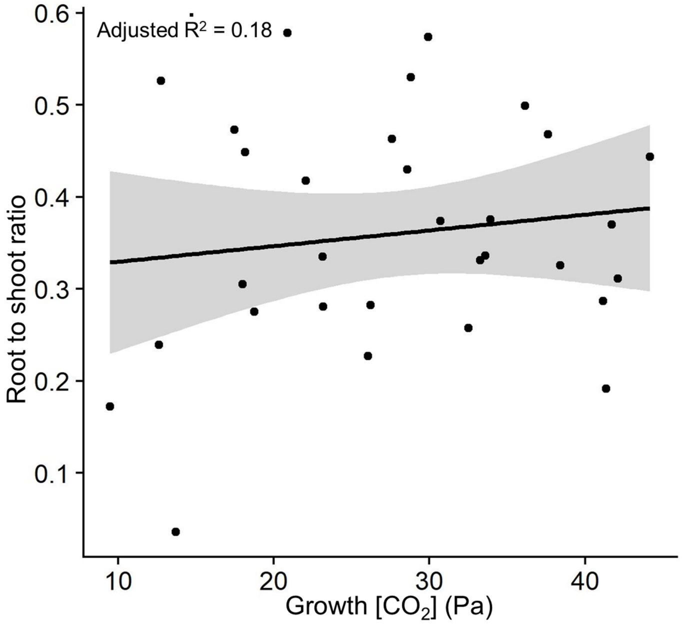

Figure 3-6. Combined species' root to shoot ratio response to *i*CO_2. Grey shading = 95% prediction interval. Symbols represent ▪p ≤ 0.1, *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001.

When species were compared individually (**Figure 3-7**) response slopes and elevations were not different. Although not significant *V. karroo* demonstrated a different trend to the other species, allocating more biomass to the shoot component at *i*[CO_2] in both the replicated (data not shown) and regression experiment, while the other species invested more biomass to the root component.

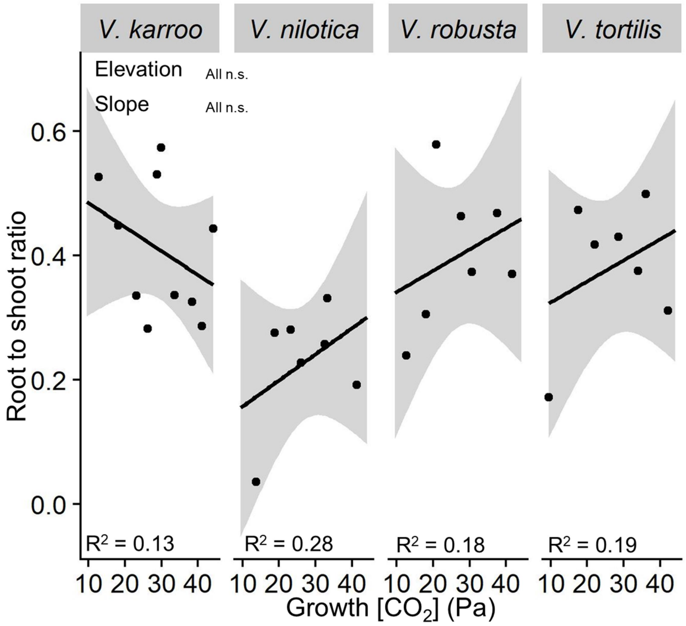

Figure 3-7. Species Root to shoot ratio response to growth [CO_2]. (Grey shading = 95% prediction interval. Elevation and slope are calculated using SMATR (Warton et al., 2012) and significant results reported. Grey shading = 95% prediction intervals, and significant differences in elevation reported as capital letters, differences in slope as small letters and n.s. as not significant. Symbols represent ▪p ≤ 0.1, *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001.

Physical defences

When species were combined, physical defences responded positively to *i*CO_2, increasing the average dry spine mass by 225% over the CO_2 gradient (**Figure 3-8;** Mixed effects model: $F_{1, 30}$= 21, p< 0.0001, adjusted R^2=0.32).

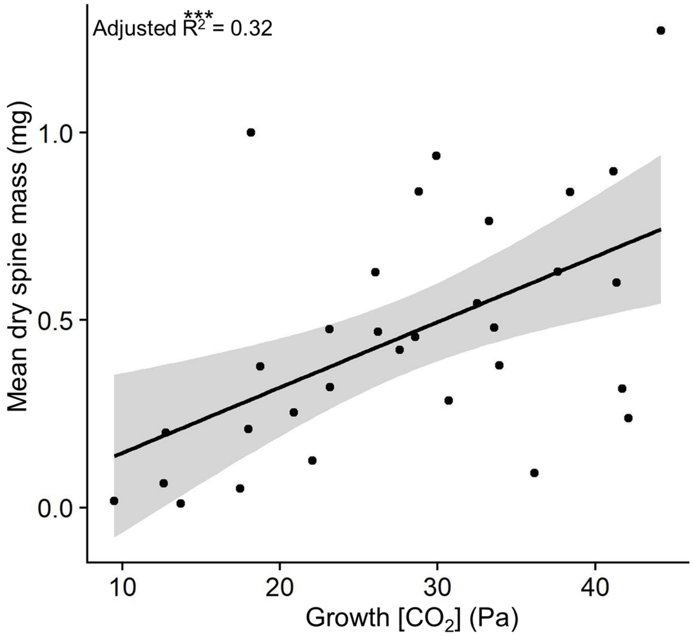

Figure 3-8. Combined species spine biomass response to *i*CO_2. Grey shading = 95% prediction interval. Symbols represent ▪$p \leq 0.1$, *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$.

At a species level there were no significant differences in slope indicating similar *i*CO_2 responses (**Figure 3-9**). There were, however, significant differences in elevation of slopes with *V. karroo* having a significantly higher spine mass relative to *V. robusta* and *V. tortilis*. The number of spines also increased significantly with [CO_2], however, when calculated per stem length (spine density) the response was no different between species (mean = 0.518 +- 0.274 SD, spines per cm of stem, data not shown), suggesting that the higher number of spines produced was simply an allometric effect that scaled with taller plants at higher [CO_2]s.

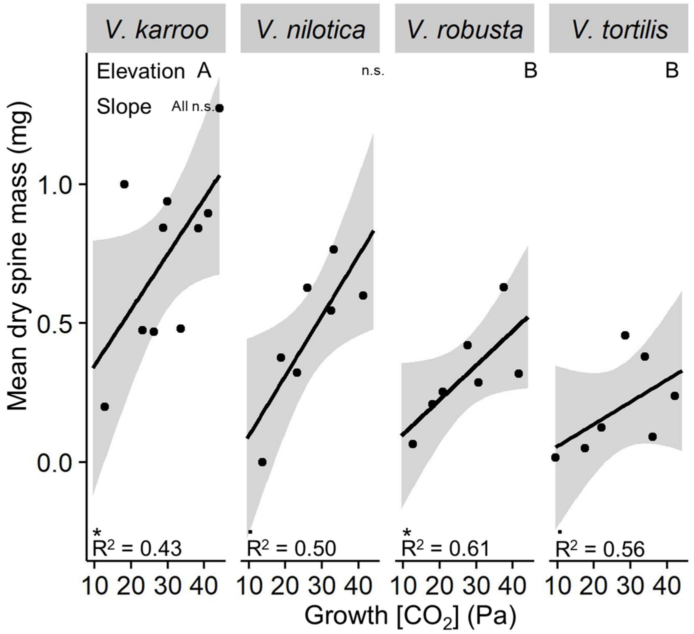

Figure 3-9. Species mean spine biomass response to growth [CO_2]. Grey shading = 95% prediction interval. Elevation and slope are calculated using SMATR (Warton et al., 2012) and significant results reported. Grey shading = 95% prediction intervals, and significant differences in elevation reported as capital letters, differences in slope as small letters and n.s. as not significant. Symbols represent ▪p ≤ 0.1, *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001.

The increase in spine mass was, however, proportionately larger than the increase in plant biomass such that the SMF increased with *i*CO_2 (**Figure 3-10**).

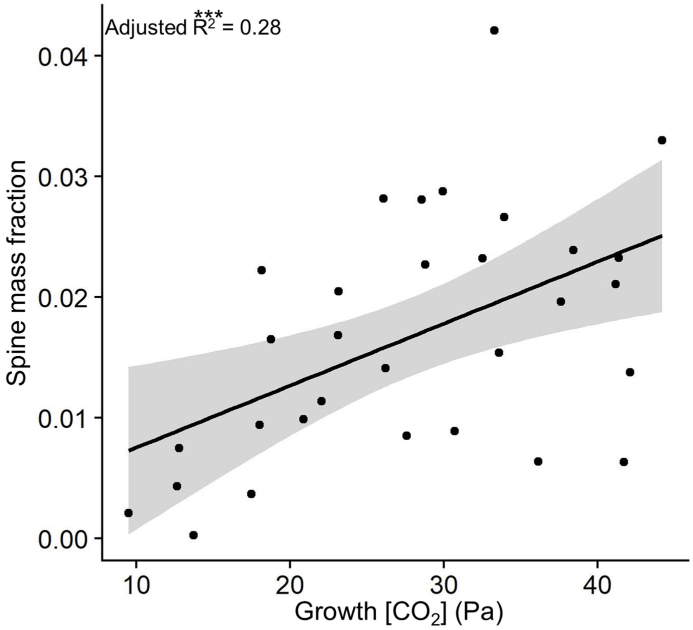

Figure 3-10. Combined species' spine mass fraction (spine dry biomass/total plant dry biomass) response to *i*CO_2. Grey shading = 95% prediction interval. Symbols represent ▪$p \leq 0.1$, *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$.

Once again, when species were compared (**Figure 3-11**) response slopes were not different; however, differences in elevation showed that *V. karroo* had a higher SMF than *V. nilotica*, while *V. robusta* and *V. tortilis* did not respond significantly.

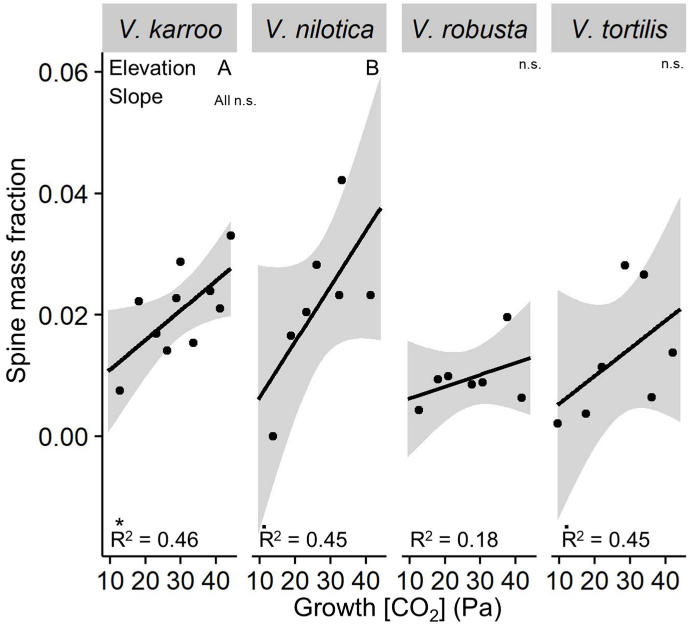

Figure 3-11. Species Spine mass fraction (spine dry biomass/ total plant dry biomass) response to growth [CO_2]. (Grey shading = 95% prediction interval. Elevation and slope are calculated using SMATR (Warton et al., 2012) and significant results reported. Grey shading = 95% prediction intervals, and significant differences in elevation reported as capital letters, differences in slope as small letters and n.s. as not significant. Symbols represent ▪$p \le 0.1$, *$p \le 0.05$, **$p \le 0.01$, ***$p \le 0.001$.

Discussion

These results support the hypothesis that all the investigated *Vachellia* species responded positively and similarly to *i*CO_2, although species did show some intrinsic differences in growth that were unaffected by [CO_2]. The responses measured for seedlings of these species were larger than those reported in the literature for other species of similar C_3 woody seedlings. The increase in RGR measured in this study was 61% bigger than the 23% increase reported for 28 different species of seedlings grown under sub-ambient CO_2 concentrations and summarised in a meta-analysis (Temme, 2015). Like RGR, biomass increases of up to 186% were also higher than the 80% change reported by Temme (2013) and were bigger than previously recorded responses for *V. karroo,* where sub-ambient CO_2 treatments were applied for two seasons (Kgope, 2010) In this study *Vachellia* responses remained linear up to current atmospheric [CO_2] (40Pa), showing that the stimulatory effect of CO_2 has not yet begun to saturate, with important implications for growth under future increasing atmospheric CO_2 conditions (Kgope, 2010).

CO_2-stimulated RGR (which was based on plant height) combined with bigger above- and below ground biomass showed that, with *i*CO_2, the rate of canopy and root system development was increased (Temme *et al*., 2015). This would have important consequences for light, water and nutrient foraging. Improved access to resources would, in turn, promote faster growth, resulting in positive feedback and potentially improving early seedling establishment (Kirschbaum and Lambie, 2015). Improved water acquisition is of particular importance for savanna species where seedling mortality is frequently the result of desiccation (O'Connor, 1995; Polley et al., 1999). Improved plant water status through better water acquisition would be complemented by reduced leaf level water use, a characteristic of plants grown at elevated CO_2 concentrations and a subject addressed in Chapter 4.

Additionally, the faster RGRs and biomass accumulation would reduce the time the seedlings spend in the fragile seedling size classes (Bond and Midgley, 2000). Large portions of the seedling size class are killed by damage from herbivory, fire and desiccation before they can reach persistent size classes. Mortality does, however, decrease rapidly once a certain size threshold has been reached (Staver et al., 2009; Perumal, 2016; Polley et al., 1999). Through field studies a seedling tolerance threshold has been determined. This particular threshold is reached when the seedling has sufficient resources below ground to regrow damaged above-ground structures (Perumal, 2016; Ripley et al., un published). CO_2-facilitated increases in productivity and biomass accumulation of the seedlings, could reduce the time spent in the fragile seedling size class, resulting in a larger portion of the population establishing and persisting in savanna habitats (Kgope et al., 2010; Wigley et al., 2009).

The seedlings' biomass was not equally allocated; the above-ground component was, on average, 35% larger than the below ground biomass contrary to previous findings, where *V. karroo* increased its biomass allocation to below ground structures as CO_2 increased (e.g. Kgope et al., 2010, Wigley et al*.,* 2009), Furthermore, the biomass partitioning between these compartments was unaffected by growth [CO_2]. Shifts in plants' allocation patterns are understood to be a key component in maintaining efficient source-sink relationships and avoiding photosynthetic down regulation (Paul and Foyer, 2001; Sage, 1994). The response of photosynthesis and down regulation in *Vachellia* seedlings is examined in Chapter 4, but appeared not to be related to altered allocation in these species. It is possible that young seedlings, like those investigated in this study, are not sink limited in these early stages of growth and the need for shifts in allocation may only develop in older stages when resources or sinks become limiting (Körner, 2006).

The seedlings' physical defences responded to CO_2, increasing the average spine mass and proportion of biomass allocated to spines. The increase in size of thorns may act as an improved deterrent to herbivores (Gowda, 1996). Thicker spines might be more visible as well as more rigid thus improving the seedlings' physical defences against vertebrate herbivores. Ultimately, this could mean the plants become more effective at reducing herbivore damage at an earlier age (Gowda, 1996). The increased biomass allocation to spines may also demonstrate their value as carbon sinks, preventing the build-up of hexose concentrations, and thus limiting downregulation (Curtis and Wang, 1998), this does, however, require further investigation. Spine density, or the number of spines per unit stem length, did not increase with *i*CO_2, and, as spines are produced at leaf nodes, may be allometrially linked to branch length and not able to respond to CO_2 fertilisation (Midgley et al., 2001).

Conclusion

Seedlings of the four species of *Vachellia* investigated showed similar strong growth responses to *i*CO_2. RGRs and biomass accumulation increased and this increase was of greater magnitude than has been recorded for other species of C_3 seedlings subjected to studies using similar [CO_2]s over comparable growth durations. Contrary to other studies, no changes in; biomass allocation to roots and shoots, or mass fraction were observed. Physical defenses also responded to *i*CO_2, with the seedlings producing larger spines but without an increase in spine density. Collectively these responses are likely to improve seedling establishment and their tolerance of herbivory and disturbance, while also potentially reducing levels of herbivory through the production of more deterrent spines. Improved seedling establishment may have knock-on effects on tree recruitment although these causal links are yet to be determined.

Having discussed, in more detail, the seedlings; growth, allocation, and physical defences responses to *i*CO_2, the Chapter 4 will analyse the seedlings photosynthetic, water use and leaf level responses to *i*CO_2.

Chapter 4 – **Leaf-level responses of *Vachellia* seedlings to growth at sub-ambient CO_2 concentration**

Introduction

In this chapter, I interrogate the photosynthetic and leaf-level morphological mechanisms that underpin the growth responses observed in Chapter 3. Of specific interest are: i) how photosynthetic rates are stimulated with increasing [CO_2] and how this relates to down regulation, ii) the CO_2 effects on stomatal conductance, stomatal limitation and resultant effects on intercellular CO_2 concentrations, iii) the stomatal effects on plant water use, both at the leaf and whole plant scale and iv) leaf morphological changes in response to [CO_2].

For woody C_3 plants rising atmospheric CO_2 increases the [CO_2] at the site of RuBP carboxylation, promoting the carboxylation function of Rubisco, while decreasing the oxygenation function (Sage, 1994). This increase in photosynthesis relative to photorespiration can result in increased net CO_2 assimilation rates by as much as 40% with a doubling of CO_2 concentration (Ainsworth and Long, 2005). This phenomenon is not linear because Rubisco becomes CO_2 saturated, hence the response to historic changes in [CO_2] from 18 to 40 Pa result in much larger photosynthetic responses than those predicted for the future, where concentrations will increase as high as 80 Pa by the turn of the century (Steffen et al., 2007).

Long term plant responses to [CO_2] are frequently not as strong as those predicted using instantaneous leaf-level measurements. This is caused by a down regulating response when sources or sinks become limiting, supressing the influence of [CO_2] (Anderson et al., 2001; Oosten and Besford, 1996). The level of down regulation varies widely depending upon access to resources, carbohydrate sinks and interspecific differences (Paul and Foyer, 2001; Way et al., 2015). The *Vachellia* species selected in this study are all capable of developing lignotubers, which are variable and effective carbohydrate sinks (Wigley et al., 2009), and are nitrogen fixers (Sprent and Parsons, 2000), thereby reducing their dependence on soil nitrogen. Such adaptions may predispose the *Vachellia* seedlings to avoiding or reducing the requirement for down regulation, leading to strong sustained CO_2 responses.

Photosynthetic enzymes are nitrogen-dense structures. The reduction in leaf enzyme concentrations associated with [*i*CO_2], results in a large improvement in plant nitrogen use efficiency (Drake et al., 1997). A more nitrogen efficient seedling will be more likely to establish itself in marginal or nutrient-poor areas, and for the same amount of nitrogen, the seedlings can develop a larger canopy and root area (Drake et al., 1997). Nutrient deficiencies and the inability to balance carbon and nitrogen demands are a cause of photosynthetic down regulation (Körner, 2006). A CO_2 stimulated increased nutrient use efficiency (NUE) could offset nutrient deficiencies, creating an important interaction between CO_2 and nutrient supply. CO_2-stimulated increases in plants the leaf C:N ratio can also reduce the forage quality of the plants, and can have consequential effects on herbivory (Ward, 2010).

Increasing [CO_2] has direct immediate effects on stomatal conductance; as [CO_2] increases, stomatal conductance decreases in a linear fashion – ultimately reducing water loss through stomata (Franks and Beerling, 2009). This effect has important consequences for plant-water relations as *e*CO_2 can result in more productive plants that require decreased soil water availability (Drake et al., 1997). Whole plant water use efficiency can also have important consequences for savanna tree seedlings where water availability is frequently a limiting factor (Kriticos, 2003). The observed decrease in leaf conductance is relatively instantaneous but when [CO_2] is elevated over longer periods, months to years, it can result in leaf morphological changes that also affect leaf conductance such as a reduction in stomatal density and size (Franks et al., 2012; Franks and Beerling, 2009), and alterations to mesophyll conductance (Flexas et al., 2008).

Savanna dynamics are centred on tree-grass interactions and how these respond to top-down or bottom-up drivers. These include competition for nutrients and water (bottom-up) and responses to fire and herbivory disturbances (top-down) (Ratnam et al., 2011). In both instances, plant size is an important determinant of competitive success and disturbance tolerance (Bond, 2008). For tree recruitment there are several important bottlenecks related to plant size. The first of these is seedling establishment, where plants must attain sufficient size to tolerate disturbance and acquire resources (Wigley et al., 2009). Hence, any factor that promotes growth, such as [CO_2], will have potential impacts on woody seedling recruitment and thus savanna dynamics. The second is the recovery and survival of saplings and adult trees after reoccurring stress and disturbance events. Increased productivity with increasing [CO_2] and the increased allocation of resources below ground allows savanna tree species to recover more effectively after disturbance than historically possible at lower atmospheric [CO_2]s, increasing woody species in savanna ecosystems (Bond, 2008; Kgope et al., 2010).

Methods

Four plant species from the *Vachellia* genus were grown from seed under a range of sub-ambient [CO_2]s, from 18 Pa - 40 Pa. A single individual was grown at each of ten [CO_2]s (see general methods in Chapter 2 for detailed species descriptions and a design for the CO_2 control system). After 60 days' of growth, gas exchange measurements were taken and then the seedlings were harvested (see chapter 3).

Gas exchange

After 60 days of growth, the well-watered seedlings were enclosed in a LI-6400-05 conifer chamber (Li-Cor Inc., Lincoln, NE, USA) connected to a LI-6400 photosynthesis system (Li-Cor Inc., Lincoln, NE, USA). The chamber and seedlings were light saturated using an array of blue/red LEDs and incandescent plant growth spotlights. Light saturation was confirmed by increasing or decreasing the light intensity by turning off individual spotlights and confirming that photosynthetic rates were unaffected. Measurements were taken between 09:00 and 14:30 while the seedlings were most photosynthetically active.

In order to determine the response of photosynthesis to [CO_2], A/Ci responses were constructed by exposing seedlings to 14 [CO_2]s ranging from 5 Pa - 140 Pa, using the protocol of Long and Bernachii (2003). This involves a stepwise change in [CO_2], initially decreasing concentrations (40, 30, 20, 10 5 Pa), returning seedlings to 40 Pa and then increasing concentrations (40, 50, 60, 70, 100 Pa). Leaf chamber temperature was set at 25^0C and leaf temperature was measured at the abaxial leaf surface via a thermocouple. Leaf temperatures ranged from 25°C – 28°C and VPD was kept between 2.3 kPa and 2.8 kPa throughout the gas exchange measurements.

On completion of A/Ci curves, seedlings were harvested and their wet leaf areas measured using a calibrated photographic method, where leaves are placed on a horizontal light box and photographed beside a known scale. The images were then processed in WinDIAS (Version 3), the scale calibrated and the accurate leaf area calculated. CO_2 response curves were fitted according to the equations of von Caemmerer *et al.* (1980) using the R package Plantecophys (Duursma, 2015) in R studio (Version 1.0.143). The bilinear method was used to fit the Farquhar-Berry-von Caemmerer model of photosynthesis to measurements of photosynthesis and intercellular [CO_2]. The bilinear method linearizes the *Vcmax* and *Jmax*-limited regions, and applies linear regression twice to estimate at first *Vcmax* and *Rd*, and then *Jmax* (Duursma, 2015).

The leaf WUE was calculated as photosynthetic rate/transpiration rate. Relative stomatal limitation (R_{SL}) was calculated as (*A* at Ci – *A* at Ca)/(*A* at Ca), While the whole plant water use was calculated by multiplying the total canopy area by the leaf level transpiration rate, while this method has many assumptions it was only used to compare different responses between the seedlings within the same study.

Stomatal density and size and leaflet morphology

The stomatal density and size were assessed on three randomly selected leaflets per seedling. A negative impression of the leaf surface was created by painting the surface with a thin layer of varnish that, once dried, was peeled off and mounted on microscope slides. These impressions were then visualised and photographed at 40x magnification using an Olympus BX61 fluorescence microscope. Each image was analysed using image J (version 1.41).

Stomatal density was calculated by manually counting all of the stomata present in a full field of view by using the cell counter add-in for image J and then dividing by the leaf area assessed. Stomatal size (**Figure 4-1**) was calculated by measuring the length of the stomatal guard cells for 10 randomly selected stomata within the field of view and then calculating as an average on three leaflets.

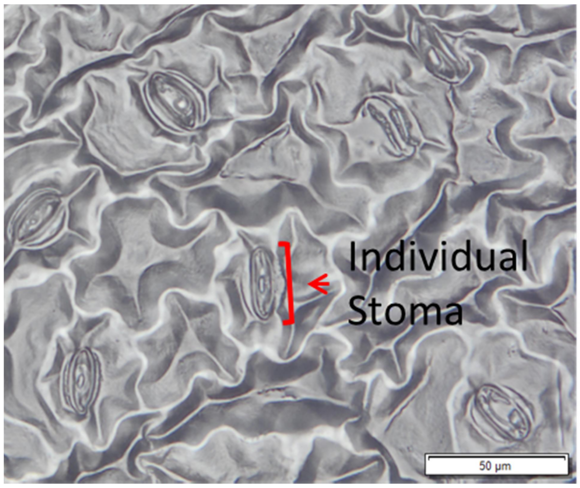

Figure 4-1. Photo of the negative impression taken from the surface of a *V. karroo* leaflet.

The mass and area of the seedlings' secondary pinnuales, hereafter referred to as leaflets, were measured. Due to their small size, a group of 10 or more leaflets was randomly selected per seedling by shaking out the sample envelope. The total mass and area was determined using a photographic process with a calibrated scale and then weighing the sample on a balance (Adventure pro, d = 0.0001, Ohaus Corporation, pine Brook, USA). The total was then divided by the number of leaflets in the sample to reach a 'per leaflet' measurement.

Statistical analysis

Linear regressions were fitted to the response variables against growth CO_2 concentrations. The R^2 and p values from the regressions were then used to determine the strength of responses and relationship to growth [CO_2].

For an overall genus response, the data from all species were combined and response variables were regressed against growth [CO_2] using Restricted Maximum Likelihood (REML) mixed effects models with [CO_2] as a fixed effect and species as a random factor. This accounts for the inherent differences between species (Bates et al., 2015) and allowed the overall CO_2 effect to be determined. Analyses were conducted in Rstudio version 1.1.453 using the packages LME4 and Car.

Comparisons between species were performed with standardised major axis estimation (SMATR) which allows regression slopes and intercepts to be fitted to data from individual species and compared. SMATR uses multi-component comparisons and was implemented with the R package SMATR (Warton et al., 2012).

The response variables were log transformed to stabilize the variance and improve the data normality.

Results

Photosynthetic performance and function responses

When averaged across the study species, the seedlings' maximum photosynthetic rate (*Amax*) at growth CO_2 increased by 130% over the CO_2 range from pre-glacial to ambient (**Figure 4-2 A**; Mixed effects model: $F_{1, 30}$= 11.3, p< 0.001, R^2= 0.21). However, when the photosynthetic rates were measured at a [CO_2] of 40 Pa, the rates showed a negative relationship to growth [CO_2], decreasing by 23% and demonstrating photosynthetic down regulation (**Figure 4-2 B**; Mixed effects model: $F_{1, 30}$= 12.8, p< 0.001, adjusted R^2= 0.21).

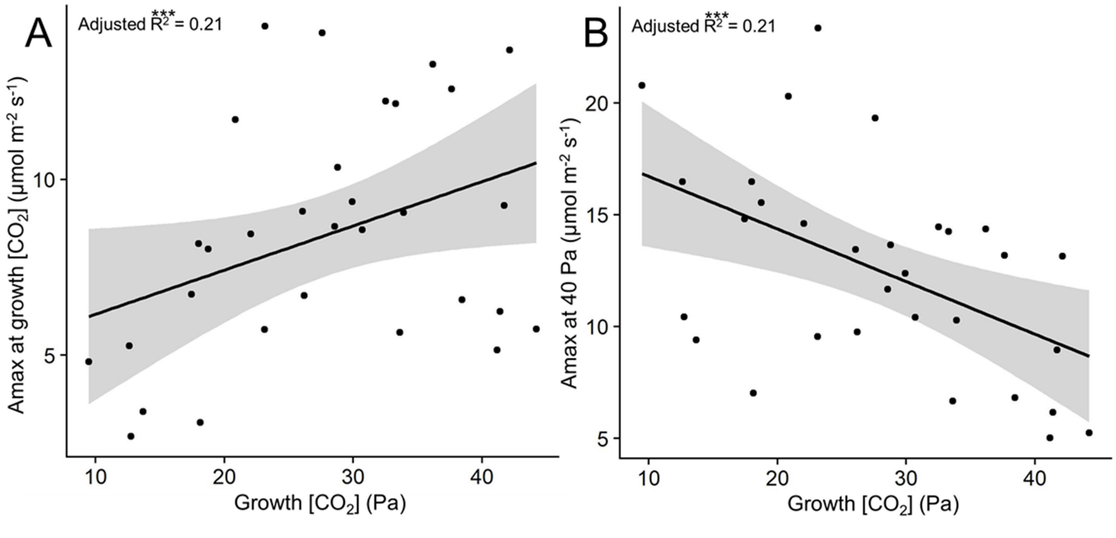

Figure 4-2. Combined species *Amax* measured at growth [CO_2] (**A**) or at 40 Pa (**B**) plotted against growth [CO_2]. Grey shading = 95% prediction interval. Symbols represent ▪p ≤ 0.1, *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001.

When compared at a species level the SMATR analysis showed that the regression slopes were not significantly different between species for *Amax* at either growth CO_2 (**Figure 4-3 A**) or at a fixed [CO_2] of 40 Pa (**Figure 4-3 B**). There were, however, significant differences in the elevation of responses between species; in particular *Amax* at growth CO_2 was lower for *V. karroo* than for either *V. robusta or V. tortilis.* Similarly, when *Amax* was calculated at 40 Pa*, V. karroo* had the lowest inherent *Amax* rates in comparison to *V. tortilis,* while the elevations of the other species were not significantly different.

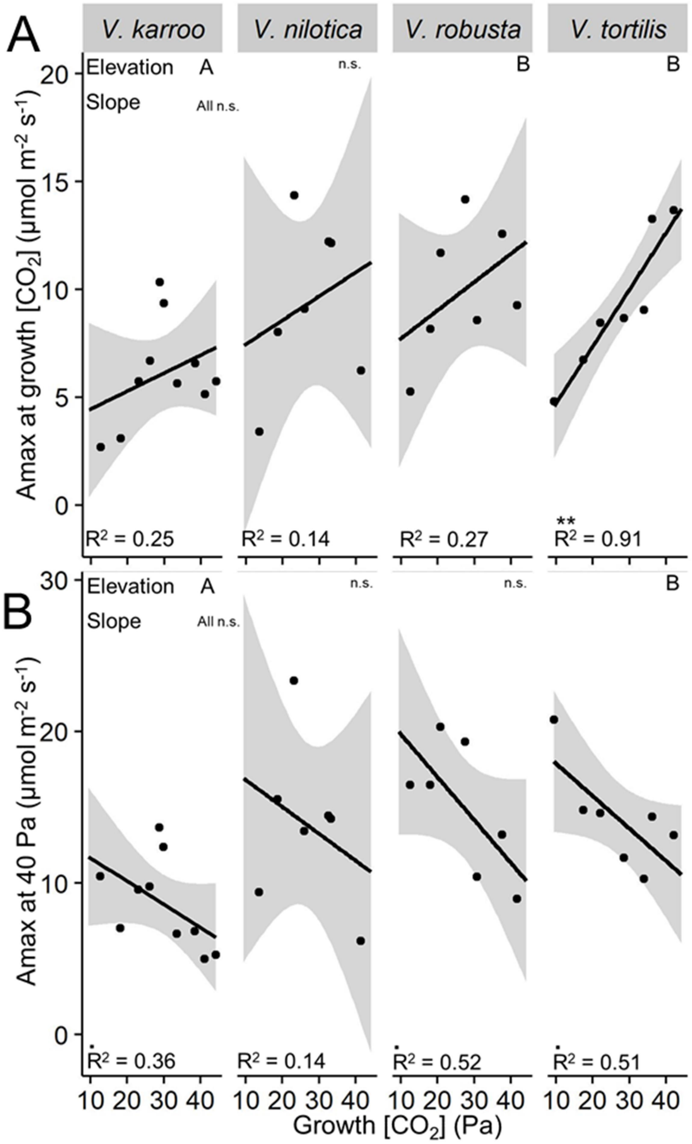

Figure 4-3. Individual species *Amax* measured at growth [CO_2] (**A**) or at 40 Pa (**B**) plotted against growth [CO_2]. (Grey shading = 95% prediction interval. Elevation and slope were calculated using SMATR (Warton et al., 2012) and significant differences in elevation reported as capital letters,

differences in slope as small letters and n.s. as not significant. Symbols represent ▪$p \leq 0.1$, *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$.

The seedlings' maximum rate of carboxylation (*Vcmax*), and maximum rate of electron transport (*Jmax*) both showed significant negative responses to increasing growth [CO_2] (**Figure 4-3**), explaining the downward trends observed for *Amax* measured at 40 Pa. The combined (**Figure 4-4)** seedling response in *Vcmax* decreased by 34% as the CO_2 increased towards ambient (Mixed effects model: $F_{1, 30}=15.5$, $p< 0.0001$, adjusted $R^2=0.28$), while *Jmax* had a weaker negative response and declined by 28% as [CO_2] was increased (Mixed effects model: $F_{1, 30}= 9.7$, $p< 0.01$, adjusted $R^2= 0.24$).

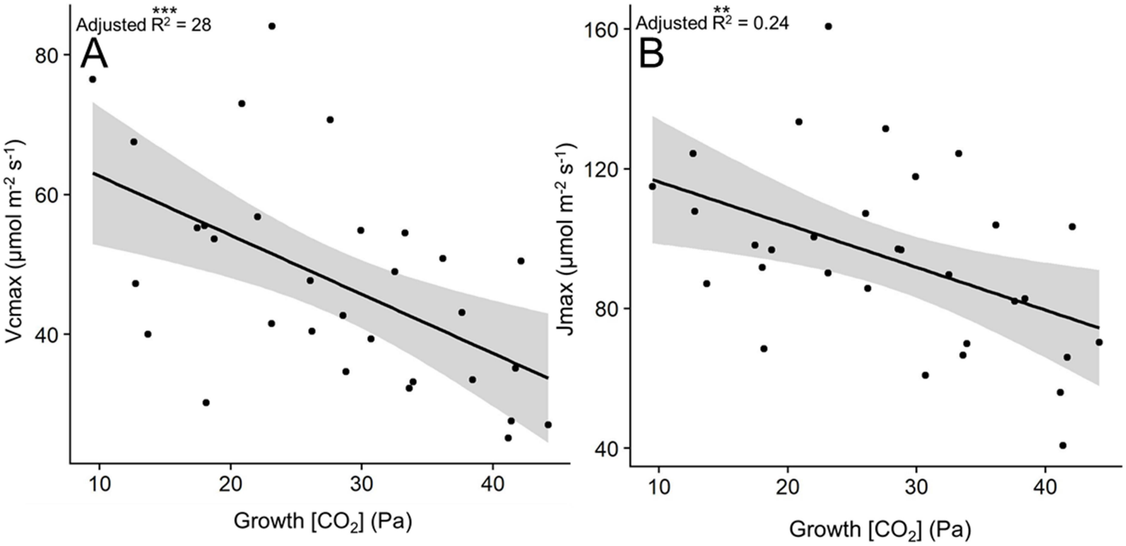

Figure 4-4. Combined species *Vcmax* (**A**) and *Jmax* (**B**) plotted against growth [CO_2]. Grey shading = 95% prediction interval. Symbols represent ▪$p \leq 0.1$, *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$.

The individual species response in *Vcmax* (**Figure 4-5 A)** and *Jmax* (**Figure 4-5 B)** also showed negative trends with *i*CO_2, but interspecific differences in slopes were not significant. The only interspecific difference noted was that for elevation of the *Vcmax* response for *V. karroo,* which was lower than that for *V. nilotica*.

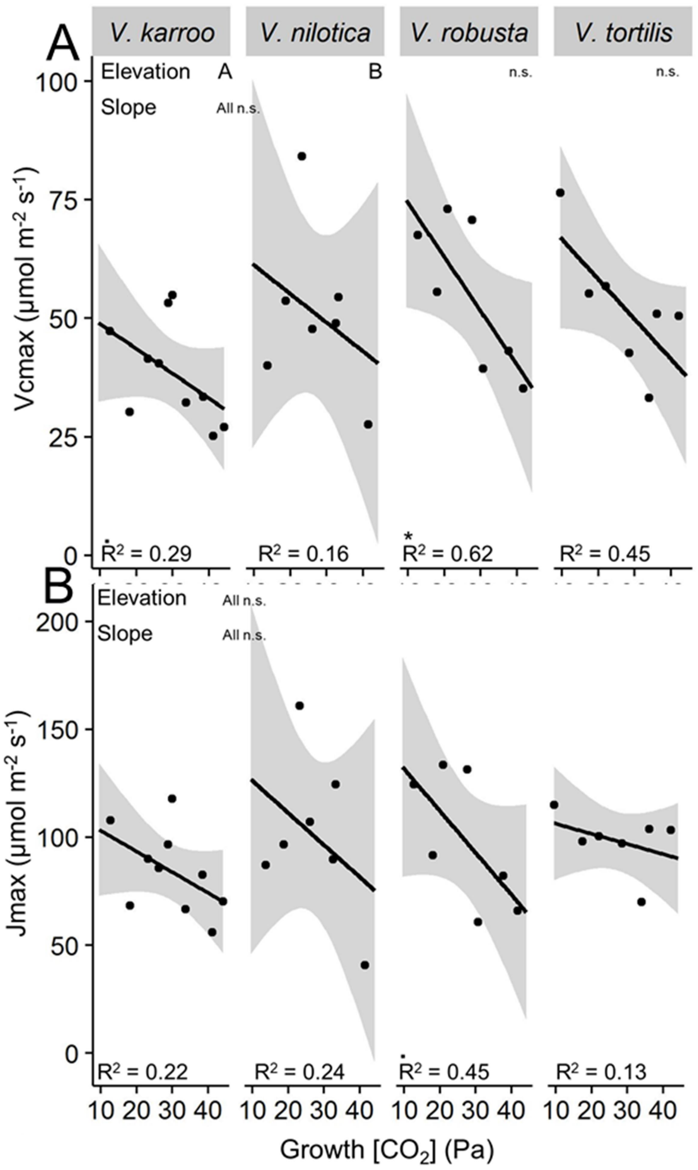

Figure 4-5. Individual species *Vcmax* (**A**) and Jmax (**B**) plotted against growth [CO$_2$]. Grey shading = 95% prediction interval. Elevation and slope were calculated using SMATR (Warton et al., 2012) and significant differences in elevation reported as capital letters, differences in slope as small letters and n.s. as not significant. Symbols represent ▪p ≤ 0.1, *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001.

Growth and dry biomass relationships to photosynthetic rates

When combined across species, biomass and RGR were not correlated to *Amax* measured at the growth [CO_2] (data not shown). On a species level, however, there were some significant correlations between photosynthetic rate and productivity. For *V. karroo* and *V. tortilis,* RGR was both positively correlated to *Amax*$_{growth}$, but the slope was not different between species (**Figure 4-6 A**). The same was true for dry biomass, except that this relationship also held for *V. robusta* (**Figure 4-6 B**). Elevations of slopes were different between species showing that species like *V. karroo* had intrinsically higher dry biomass and growth rates for any given photosynthetic rate.

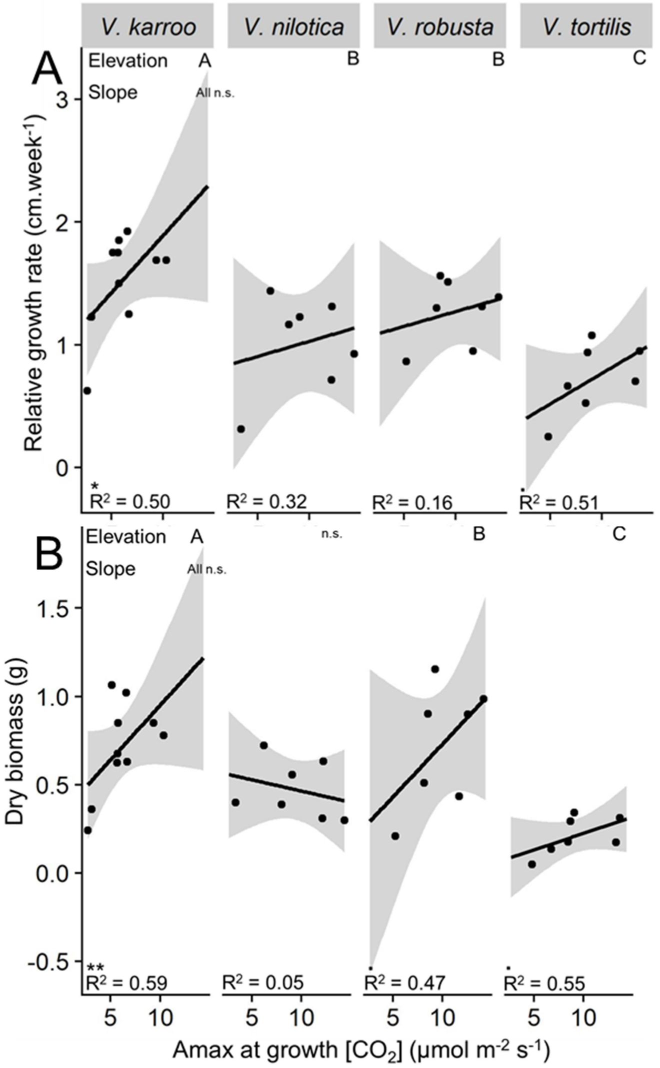

Figure 4-6. Individual Seedling relative growth rate (**A**) and dry biomass (**B**) responses to Amax at growth [CO_2]. Grey shading = 95% prediction interval. Elevation and slope are calculated using

SMATR (Warton et al., 2012) and significant differences in elevation reported as capital letters, differences in slope as small letters and n.s. as not significant. Symbols represent ▪$p \leq 0.1$, *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$.

Water use and leaflet morphological responses

Stomatal limitation

The seedlings' stomatal limitation - averaged across all species - decreased by 60% with an increase in CO_2 from pre-glacial to ambient (**Figure 4-7**; Mixed effects model: $F_{1, 30}$= 13.7, p< 0.001, adjusted R^2=0.29).

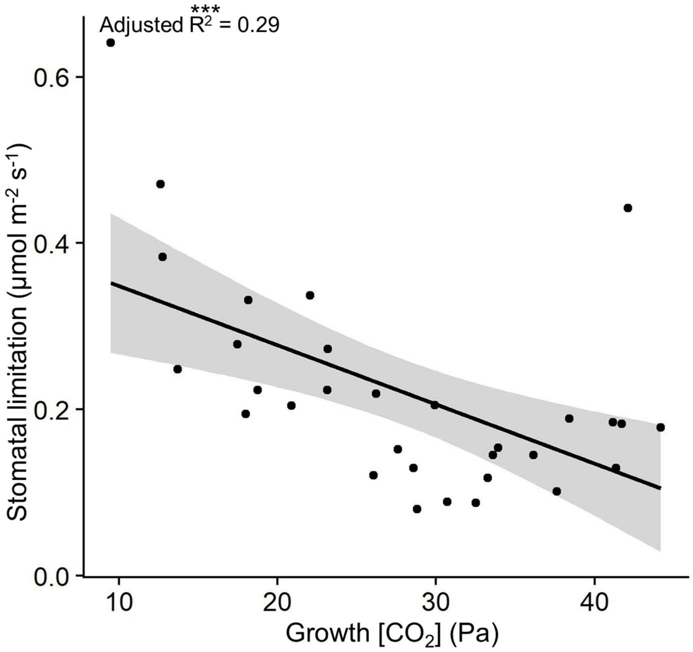

Figure 4-7. Combined species stomatal limitation response to growth [CO_2]. Grey shading = 95% prediction interval. Symbols represent ▪$p \leq 0.1$, *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$.

When analyses were done on individual species, there were no differences in slope or elevation of the stomatal limitation response to *i*CO_2 between species (**Figure 4-8**). This finding indicates that photosynthesis was limited by stomatal conductance to the same degree in all species and that this response is affected in a similar way by *i*CO_2.

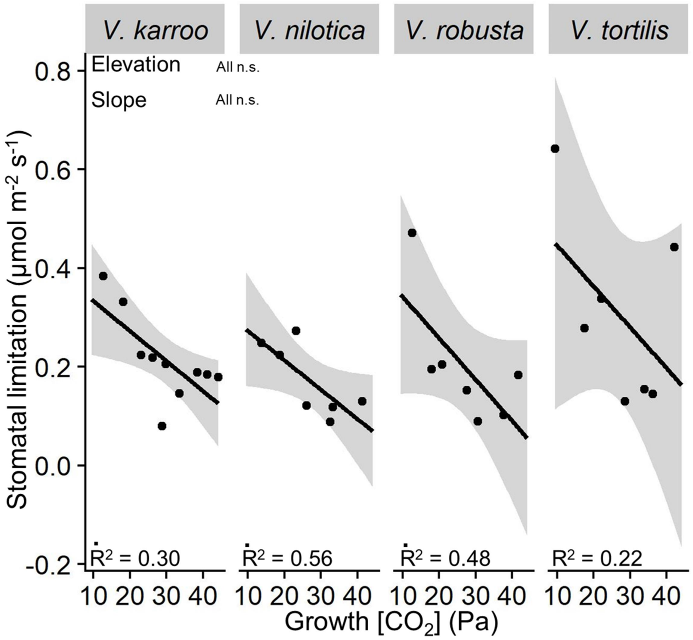

Figure 4-8. Individual species stomatal limitation responses to growth $[CO_2]$. Grey shading = 95% prediction interval. Elevation and slope are calculated using SMATR (Warton et al., 2012) and significant differences in elevation reported as capital letters, differences in slope as small letters and n.s. as not significant. Symbols represent ▪$p \le 0.1$, *$p \le 0.05$, **$p \le 0.01$, ***$p \le 0.001$.

Leaf water use efficiency

When data was combined across species, water use efficiency increased with *i*CO_2, with seedlings becoming an average of 218% more efficient at high CO_2 (**Fig 4-9**).

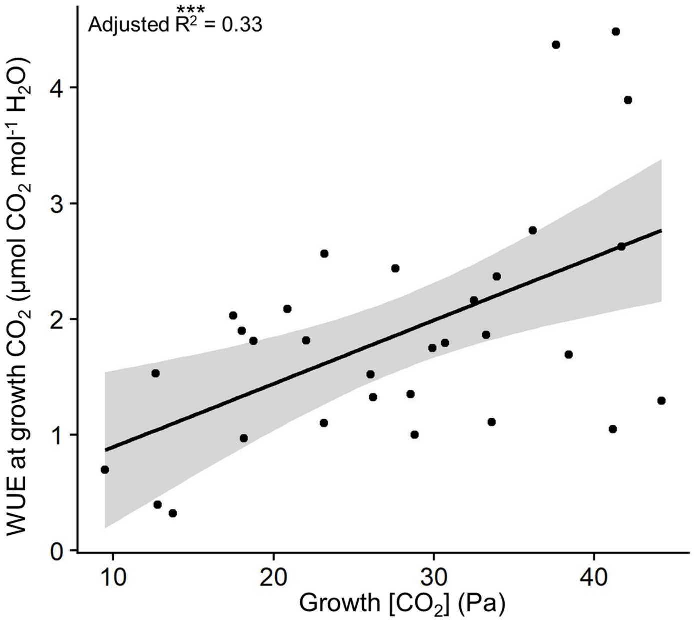

Figure 4-9. Combined species leaf water use efficiency (WUE) response to growth [CO_2]. Grey shading = 95% prediction interval. Symbols represent ▪p ≤ 0.1, *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001).

When compared on a species basis, there were no differences in the slopes of the WUE to iCO_2 responses between species (**Figure 4-10**). The *V. karroo* response did, however, have a lower elevation than any of the other species, indicating that its WUE was inherently lower than that of the other species.

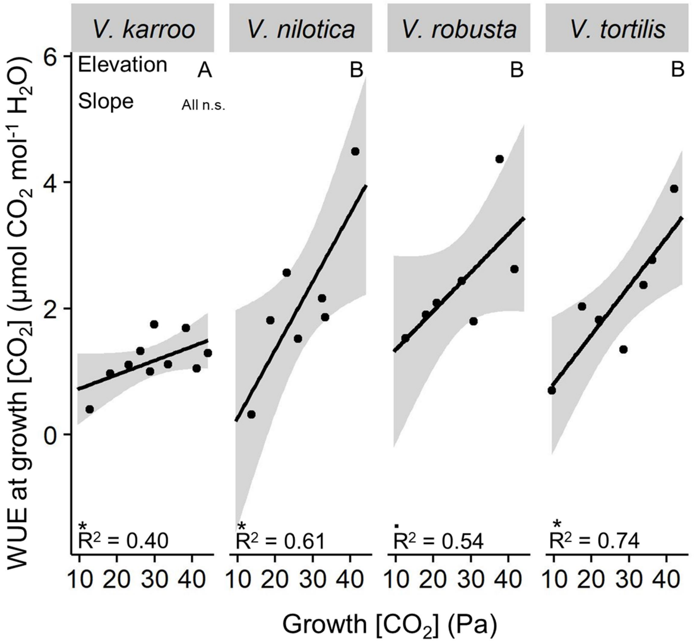

Figure 4-10. Individual species water use efficiency (WUE) responses to growth [CO_2]. Grey shading = 95% prediction interval. Elevation and slope were calculated using SMATR (Warton et al., 2012) and significant differences in elevation reported as capital letters, differences in slope as small letters and n.s. as not significant. Symbols represent ▪$p \leq 0.1$, *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$.

Whole plant instantaneous water use

Whole plant instantaneous water use was calculated by scaling leaf transpiration rate to the leaf area of entire canopies and hence accounts for the growth effect that CO_2 has on canopy area. When this data was combined across species, whole plant water use increased with *i*CO_2 by an average of only 80%, which was ~ 140% lower than the WUE effect noted on a unit leaf area basis.

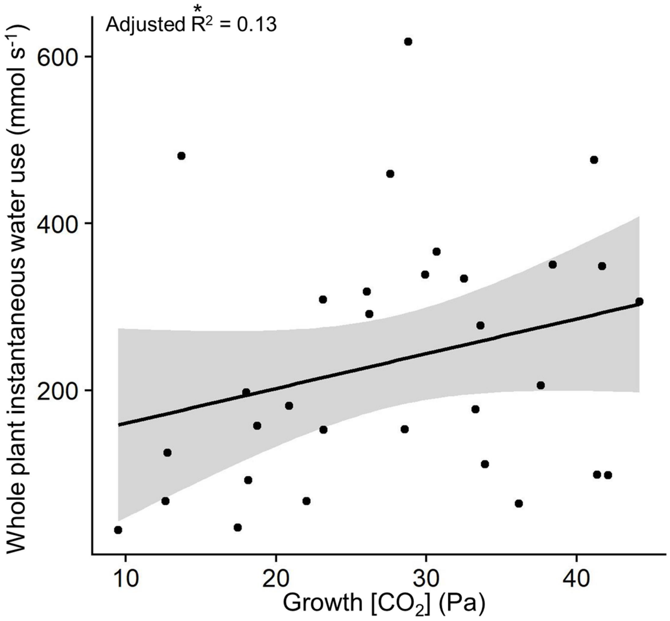

Figure 4-11. Combined species whole plant water use response to growth [CO_2]. Grey shading = 95% prediction interval. Symbols represent ▪p ≤ 0.1, *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001.

When compared on a species basis, there were no significant differences in the slopes of the whole plant water use to *i*CO_2 responses between species (**Figure 4-12**). Although some interesting trends were shown, *V. nilotica* used 60% less water at *i*CO_2, while the other three species all increased water use by between 124 and 135%. *V. tortilis* showed a significantly lower elevation than any of the other species, indicating that its whole plant water use was inherently lower than that of the other species.

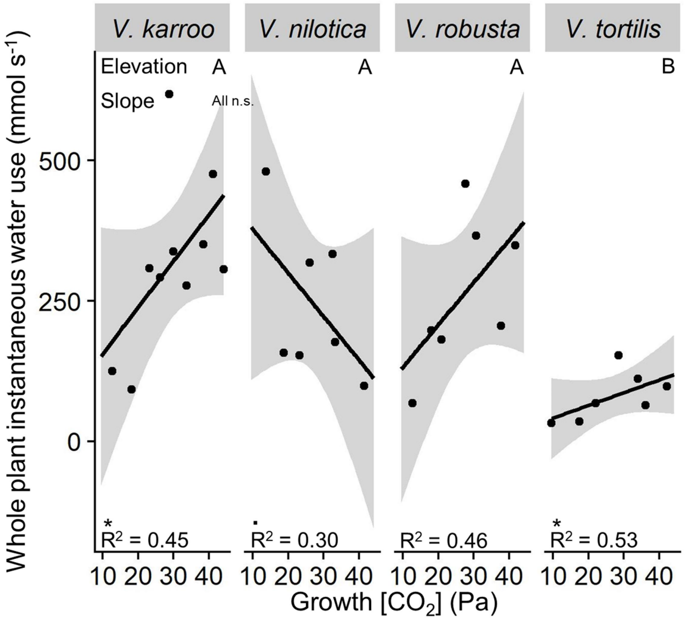

Figure 4-12. Individual species whole plant water use responses to growth [CO_2]. Grey shading = 95% prediction interval. Elevation and slope were calculated using SMATR (Warton et al., 2012) and significant differences in elevation reported as capital letters, differences in slope as small letters and n.s. as not significant. Symbols represent ▪$p \leq 0.1$, *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$.

Leaflet morphological responses

When species responses were combined, leaflet area and leaflet mass did not respond positively to *i*CO_2 (Mixed effects model, leaflet area: $F_{1, 30}$= 4.5, $p< 0.05$, adjusted R^2=0.07; leaflet mass: $F_{1, 30}$= 7.0, $p< 0.01$, adjusted R^2=0.09, results not shown). However, when analysed on a species basis, the leaflet area for *V. karroo* increased with *i*CO_2, while that of *V. tortilis* decreased (**Figure 4-13 A**). The same was not true for the responses of leaflet mass with no variation amongst the species. Differences in elevation showed that leaflet area of *V. karroo, V. tortilis* and *V. robusta* were intrinsically different. Leaflet mass showed a similar response, although the elevation for *V. nilotica's response* grouped with that of *V. tortilis.*

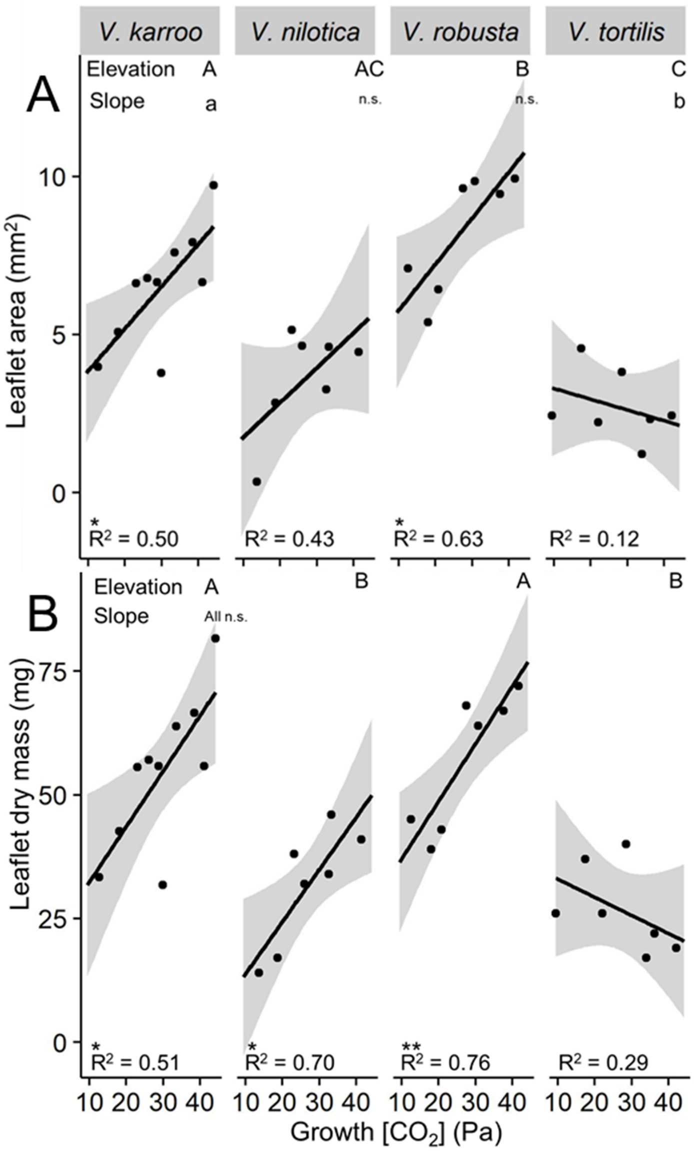

Figure 4-13. Individual species leaflet area (**A**) and mass (**B**) responses to growth [CO_2]. Grey shading = 95% prediction interval. Elevation and slope were calculated using SMATR (Warton et al., 2012) and significant differences in elevation reported as capital letters, differences in slope as small letters and n.s. as not significant. Symbols represent ▪$p \leq 0.1$,*$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$.

Discussion

The results support the hypothesis that the investigated *Vachellia* seedlings respond strongly to *i*CO_2, with positive increases in photosynthetic rates, growth and productivity as well as leaf level water use efficiency (WUE), all while maintaining relatively low rates of photosynthetic down regulation. While the species did show some interspecific differences they responded largely as a functional group despite being from different ecological niches. The responses of the *Vachellia* seedlings remained linear up to current atmospheric [CO_2]s showing that the stimulatory effect of CO_2 has not yet begun to be saturated at current ambient concentrations ,and support the findings of similar studies on *Vachellia* species (Kgope et al., 2010) . These findings have important implications for growth under future increasing atmospheric CO_2 conditions and imply a continued stimulation of growth in seedlings (Kgope et al., 2010; Kriticos, 2003).

The seedlings' photosynthetic rates increased by 130% over the CO_2 gradient from glacial to ambient; this was caused by a reduction in photorespiration and increased Rubisco substrate saturation, thereby promoting more efficient photosynthetic reactions (Sage, 1994). The $Amax_{\text{growth}}$ noted for the combined *Vachellia* species was larger than the response noted in the meta-analyses of Temme and Ainsworth (Ainsworth and Long, 2005; Temme et al., 2013). The strong positive photosynthetic responses agree with results from similar CO_2 studies and confirm the *Vachellia* seedlings' strong response to *i*CO_2 (Kgope et al., 2010; Schortemeyer et al., 1999; Temme et al., 2015). The observed increases in photosynthesis (*Amax*) did not correlate directly with the seedlings' increase in RGR and biomass discussed in Chapter 3. This indicates that the relationship between photosynthetic CO_2 fertilization and seedling morphology is more complex, with CO_2 fertilization being a combination of photosynthetic and morphological responses (Oosten and Besford, 1996).

The strong increases in $Amax_{\text{growth}}$ noted for *Vachellia* seedlings occurred in spite of an average 23% down regulation of photosynthesis with *i*CO_2. These measures of down regulation were lower than those reported by studies using similar time frames and species (Ainsworth and Long, 2005; Drake et al., 1997) and did not vary significantly between the species studied. Photosynthetic down regulation is triggered either by resource limitations or inadequate carbohydrate sinks (Oosten and Besford, 1996; Way et al., 2015). As the studied seedlings are capable of developing below ground lignotubers for storing carbohydrates and are nitrogen-fixing, it is possible that these adaptions allow for sustained photosynthesis with increasing [CO_2], by avoiding down regulation triggered either by resource limitations or inadequate carbohydrate sinks (Oosten and Besford, 1996; Way et al., 2015). It is also likely that seedlings, in general, have lower down regulation than mature size classes, because seedlings are in an exponential growth stage with no competitive interactions and initially low resource limitations (Kirschbaum and Lambie, 2015; Way et al., 2015).

While down regulation reduces the photosynthetic rate, it can increase the plant's nitrogen use efficiency by 15 – 19% with a doubling of CO_2. This is a result of a reduction in nitrogen-dense photosynthetic enzymes within the leaves which corresponds to the increased per-enzyme efficiency at eCO_2 (Drake et al., 1997). The increased nitrogen efficiency could partially offset the negative effects of down regulation and increase the potential growth potential for plants in nitrogen-limited competitive ecosystems (Ainsworth and Long, 2005). Nitrogen limitations can also stunt sink development which, in turn, triggers down regulation, so the increased NUE causes a slight negative feedback loop with down regulation, at least with regards to carbohydrate sink development, although this remains to be experimentally tested for the selected *Vachellia* species (Drake et al., 1997).

One of the strongest responses of the seedlings was in leaf level water use efficiency, which increased by 200% at iCO_2 when averaged across all species. This resulted from a decreased stomatal conductance and thus transpiration rate, and an increase in photosynthetic rate (Drake et al., 1997; Morgan et al., 2001). For the seedlings studied transpiration rate remained fairly constant across the CO_2 gradient; however, the significant increases in photosynthetic rates meant that more CO_2 was sequestered per water molecule lost. This will not change the seedlings tolerance to drought or desiccation, however it will increase the area of canopy which the seedlings can support at iCO_2 with the same amount of water, increasing the photosynthetic potential of the seedling (Drake et al., 1997; Morgan et al., 2004). When scaled to a canopy level the leaf level increases in WUE did not reduce total plant water use as a result of larger leaf canopies produced at iCO_2, with all the seedlings except *V. nilotica*, which had the lowest increases in canopy area, using more water at *i*CO_2.

A range of similar CO_2 studies have demonstrated significant changes to stomatal morphology at *i*CO_2, with the leaf epidermis adapting to optimize gas exchange at the [CO_2] at which the leaf was grown (Ainsworth and Rogers, 2007; Franks and Beerling, 2009). However, the tested seedlings showed no significant changes to stomatal density or size over the CO_2 gradient measured. There are a number of reasons why the seedlings' stomata did not respond. The CO_2 gradient the seedlings were grown under, for example, is smaller than other studies which showed strong stomatal responses (Ainsworth and Rogers, 2007). There is also a potential chamber effect caused by the OTP chambers causing a very humid microclimate (Ainsworth and Long, 2005). This coupled with the frequent watering may have removed selective pressures such as desiccation, to optimize stomatal morphology (Franks and Beerling, 2009).

Leaflet area and mass increased strongly for all of the seedlings except *V. tortilis*. *e*CO_2 increases the plant's access to carbon while photosynthetic acclimation reduces the nutrient requirement per leaf area (Cramer et al., 2009; Drake et al., 1997). The combination of increased carbon availability and a reduced nutrient requirement lowers the investment cost of producing larger leaves (Temme et al., 2013). The larger leaf area will increase the amount of light intercepted by the plant, potentially increasing photosynthetic potential or fitness of seedlings germinating in light limited areas, such as under the closed canopies of the parent plants (Drake et al., 1997).

Conclusion

The four species investigated showed strong photosynthetic responses to *i*CO_2, strongly increasing photosynthetic rates, as well as increases in WUE and leaflet dimensions, while experiencing a relatively low photosynthetic down regulation. The positive photosynthetic rates did not correlate directly with the increases in RGR or biomass accumulation discussed in Chapter 3. This demonstrates that CO_2 responsiveness is a complex response which occurs based on a combination of different photosynthetic and morphological responses, and not just as a factor of elevated photosynthetic rates at *e*CO_2. The observed combination of morphological and photosynthetic responses at higher levels of CO_2 resulted in *Vachellia* seedlings that are more efficient and productive than those at low CO_2. This could have had impacts on the way in which C_3 woody seedlings have been responding to environmental stresses and disturbances and, ultimately, having strong impacts on savanna ecology.

Having discussed, in more detail, the *Vachellia* seedlings' photosynthetic, water use, and leaf level responses, the following chapter will combine the results from Chapter 3 and 4 to explore how increasing CO_2 has possibly been facilitating the encroachment of *Vachellia* species into historically open savannas.

Chapter 5 – **Increasing atmospheric CO_2 as a facilitator for the woody encroachment of *Vachellia* species into historically open savannas**

This research demonstrates that simulating the changes in atmospheric [CO_2] from pre-glacial to current concentrations has a marked effect on the growth of four species of the woody savanna tree genus *Vachellia*. This growth stimulation was in excess of the values previously recorded in other comparable experiments (Ainsworth and Long, 2005; Kgope et al., 2010; Temme et al., 2015), although it is not possible to ascertain whether this is the result of using young seedlings for this assessment or due to the *Vachellia* genus being particularly responsive to CO_2 fertilization. The four species investigated in this study have all been implicated in bush encroachment in their respective habitats, and are considered to be pioneer species, a functional type that is inherently fast growing (Hean and Ward, 2012; Moleele et al., 2002; Wand et al., 1996; Witkowski and Garner, 2000). Interspecific differences in growth rates and biomass production were not apparent in these young seedlings.

Chapter 3 shows that the seedlings' relative growth rates (RGR's) and biomass increased strongly with *i*CO_2, although no changes in above- and below ground biomass allocation were observed. Similar studies have shown significant changes to allocation in larger size classes of trees, with a larger proportion of biomass invested in below ground structures at *e*[CO_2] (Kgope et al., 2010; Ward, 2010). The *Vachellia* seedlings did however increase the mass fraction and average mass of spines in response to *i*CO_2, although there were no changes in spine density. The spines at *e*CO_2 with the increased carbon investment would be more rigid and visible, potentially acting as more of a deterrent against vertebrate browsers (Gowda, 1996).

Chapter 4 focused on photosynthetic, water use, and leaf-level photosynthetic responses of the seedlings to *i*CO_2. The seedlings all displayed increases in photosynthetic productivity at *i*CO_2 despite significant but low levels of photosynthetic down regulation. The most extreme responses seen were the leaf-level increases in WUE, which were on average 200% higher in the seedlings grown at ambient [CO_2]. This was as a result of markedly increased photosynthetic rates rather than decreases in transpiration. When scaled to a plant scale, the larger canopies produced at *i*CO_2 offset the increase in leaf level water use efficiency, resulting in seedlings at eCO_2 with larger canopies losing more water per plant than plants grown at lower concentrations. The seedlings' leaflet area and mass were also notably higher with *i*CO_2. Interestingly there were no morphological changes to stomatal density or size, despite similar studies having reported changes in response to *e*[CO_2] (Ainsworth and Rogers, 2007; Franks et al., 2012).

Species range and context

The four species of *Vachellia* reported on in this thesis occur across a large geographical range with differing climatic envelopes. Maxent was used to correlate climatic factors to species distributions and showed that distributions were more restricted by rainfall than temperature, while seasonality had varying effects depending on which species was considered (**Chapter 2,** and **Table 5.1**). Such climatic differences would exert distinct selective pressures driving the evolution of specific physiological differences and trait evolution. These trait combinations then affect the way in which the species respond to *i*[CO_2], as well as natural disturbance regimes (Osborne et al., 2018; Temme et al., 2015).

Savanna ecosystems are highly variable and are regulated through different combinations of stresses and disturbances depending on the biotic and abiotic factors prevalent at specific geographic locations (Beerling and Osborne, 2006). Species adapted for mesic savannas usually have high productivities, but suffer frequent top-kill events due to high herbivore densities, resulting in fast growing woody species that store large amounts of resources below ground to re-sprout (Wigley et al., 2009). On the other hand, species adapted for arid savannas generally have slower growth rates but have evolved strategies to reduce water loss and survive extended drought periods, such as water efficient leaf structures and stomatal morphology (Eamus and Palmer, 2007). I will discuss the observed seedling responses to *i*CO_2 in the context of their climatic ranges, and examine how these responses might affect the species' potential to become encroaching in the savannas they inhabit.

Species-specific responses

The traits and physiological adaptations which species have been selectively evolving for thousands of years will predispose them to certain CO_2 responses (Atkin et al., 1999; Poorter and Navas, 2003; Temme et al., 2015). The results show that there were both uniform responses and interspecific differences. The overall stimulation of photosynthetic rate, growth and biomass accumulation with *i*CO_2 was a ubiquitous feature and would confer an advantage through increased tolerance of disturbance (Bond and Midgley, 2000). However, interspecific differences in growth responses and resource efficiency would confer varying advantages that may relate to the climatic niches of species (Atkin et al., 1999). These responses will affect the way in which the *Vachellia* trees interact with their habitats as CO_2 increases, specifically in the competition- and disturbance-maintained savanna ecosystems (Bond and Midgley, 2000).

V. karroo inhabits broad regions of southern Africa, but does not extend as far north as the equator. The modeled ranges (**Chapter 2,** and **Table 5.1**) showed the strongest relationship to be with temperature seasonality and the precipitation of the driest quarter, likely showing intolerance for severe drought, including seasonal droughts. The more mesic savannas where *V. karroo* occurs are generally highly productive and maintained through high herbivore densities and frequent fire events (Bond et al., 2003). The observed CO_2 responses found in this study support a fast growing competition-based growth strategy, which will enhance seedling success in these savannas. Despite having the lowest inherent instantaneous photosynthetic rates, *V. karroo* had the strongest increase in RGR and biomass accumulation. This could be supported by a larger investment in above ground biomass than the other species, increasing access to light resulting in a positive feedback between CO_2 and growth (Ainsworth and Long, 2005; Kirschbaum and Lambie, 2015). *V. karroo* showed the lowest increases in leaf level WUE, and when water use was scaled to the canopy level the seedlings used more water at *i*CO_2, due to the larger leaf canopy areas produced when more CO_2 is available.

V. nilotica has large distribution, with the Maxent model showing a potential wide range across Africa, both north and south of the equator, although restricted to more mesic areas than *V. tortilis* (**Chapter 2,** and **Table 5.1**). *V. nilotica* responded to *i*CO_2 by increasing both growth and water use efficiency, having similar increases in

RGR, biomass, and physical defensive structures as *V. karroo* while still maintaining strong increases in WUE similar to those measured for *V. tortilis*. *V. nilotica* is the most widely dispersed and globally reported upon encroaching species investigated in this study (Kriticos, 2003; Witkowski and Garner, 2000). The combined strong responses in RGR, biomass accumulation and physical defenses may explain why this species is such a successful invader across such a large and climatically variable part of Africa.

V. tortilis had the widest range of the study species, inhabiting large areas of Africa both north and south of the equator, and extending far into the most arid deserts on the continent (**Chapter 2,** and **Table 5.1**). The *e*CO$_2$ growth responses of *V. tortilis* were more conservative than those of *V. karroo* or *V. nilotica*, with slower RGRs than the other species. However, *V. tortilis* showed the largest increases in leaf level WUE and spine allocation, suggesting it can support larger canopy areas in arid habitats, and increased herbivory tolerance in post-glacial environments. This may have been an important advantage in arid areas where the loss of biomass through herbivory comes at a significant cost to fitness and survival (Ward et al., 2014; Wiegand et al., 2006). The strong water use responses shown by *V. tortilis* may increase its success as an encroacher into areas which were historically too arid (Kriticos, 2003; Lemke and Brown, 2012).

V. robusta's modeled range was confined to the mesic areas of the east coast of Africa and was strongly determined by annual precipitation and precipitation of the driest quarter, while not being very affected by temperature (**Chapter 2,** and **Table 5.1**). *V. robusta* seedlings responded strongly to *i*CO$_2$, with high RGR and biomass accumulation, and were largely allometric, without changes to mass fractions or allocation patterns. CO$_2$ stimulated increases in WUE were similar to *V. nilotica and V. tortilis.* Hence there was no obvious pattern of CO$_2$ response that could explain why this species is cited less often as an encroacher than the other study species. It has been noted that the species prefers more shaded areas (Coates Palgrave, 2005) and the effect of $_i$CO$_2$ on increased productivity under shade conditions may offer a potential explanation.

Table 5-1 Summary results for the percentage influence which the climatic models calculate for the *Vachellia* species as well as the seedlings response (R^2) to the CO_2 gradient,

Climatic range determinant- (% influence on distribution)				
	V. karroo	*V. nilotica*	*V. robusta*	*V. tortilis*
Annual Temperature	19.1	2.4	4.4	7.8
Ann precipitation	0.7	17.6	35.5	36.0
Mean temperature of the driest quarter	13.3	1.9	2.3	15.7
Mean temperature of the wettest quarter	0.04	1.9	7.6	8.4
Precipitation of the driest quarter	31.4	5.3	26.0	6.4
Precipitation of the wettest quarter	1.0	50.0	7.9	21.0
Precipitation seasonality	2.0	0.9	0.07	1.1
Temperature seasonality	32.5	20.4	16.3	3.5
Seedlings responses (R^2) to an increase in [CO_2] Symbols represent ▪$p \leq 0.1$, *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$.				
Relative growth rate	0.47*	0.40	0.56▪	0.67*
Above ground biomass	0.75**	0.16	0.78**	0.71*
Below ground biomass	0.60*	0.53▪	0.73**	0.62*
Root to shoot ratio	0.13	0.28	0.18	0.19
Mean spine mass	0.43*	0.50▪	0.61*	0.56▪
Amax at growth CO_2	0.25	0.14	0.27	0.91**
Amax at 40 Pa	0.36	0.14	0.52▪	0.51▪
Vcmax	0.29▪	0.16	0.62*	0.45
Jmax	0.22	0.24	0.45▪	0.13
Stomatal limitation	0.30▪	0.56▪	0.48▪	0.22
Leaf level WUE	0.40*	0.61▪	0.54▪	0.74*
Whole plant water use	0.45*	0.30▪	0.46	0.53*
Leaflet area	0.50*	0.43	0.63*	0.12
Leaflet mass	0.51*	0.70*	0.76*	0.29

CO_2 fertilization as a facilitator for woody encroachment

Globally, woody species are becoming more dominant in historically open savannas increasing by an average of −0.13 to 1.28% per year in African savanna systems, with a rapid increase in encroachment occurring after the Industrial Revolution when humans began increasing atmospheric [CO_2] (Skowno et al., 2017; Venter et al., 2018; Ward, 2005). Historically, overgrazing and a suppression of fires were cited as the primary causes. While these are strong drivers of encroachment, there are a wide range of triggers and drivers which have been found by studies, such as changes in rainfall patterns, seasonality, herbivore composition, and increasing atmospheric CO_2 (Ward, 2005). The drivers or triggers of woody encroachment either promote woody species recruitment or promote escape events where suppressed individuals are able to escape bottlenecks and reach larger size classes, giving the impression of a rapid increase in density of woody species (Bond, 2008; O'Connor et al., 2014; Ward, 2005).

Recruitment bottlenecks limit plants from reaching larger size classes either through high mortality rates or, in the case of savannas, through long-term trapping or suppression of seedlings and saplings within the grass layer (Bond, 2008; Higgins et al., 2000). Two primary bottlenecks act on the *Vachellias*. The first is the very high early seedling mortality from herbivory and trampling, and the second is fire and desiccation which kills off a large proportion of the population before it can reach disturbance- tolerant size classes (Staver et al., 2009; Perumal, 2016; Polley et al., 1999). Once the seedlings have sufficient resources stored to survive disturbances they are able to persist within the ecosystem. Despite surviving disturbances they may, however, become trapped within the grass layer, where frequent top-kill events through fire, grazing and drought cause enough damage to prevent them from outgrowing disturbance traps and reaching larger size classes (Bond, 2008; Bond and Midgley, 2000; Staver et al., 2009).

A study by Perumal (2016) identified two distinct *Vachellia* seedling size classes with differing tolerances of herbivory. Those with a stem basal dimeter less than 9 mm had a 77% chance of mortality after a disturbance event, while the seedlings with a basal dimeter greater than 9 mm were able to survive multiple defoliation events and recover (Perumal, 2016). The 9 mm basal dimeter threshold represents the point at which the seedlings have accumulated sufficient resources in below ground storage organs to regrow damaged aboveground structures after a disturbance event (Wigley et al., 2009). The average time taken to reach this size class was calculated to be 16 weeks at current atmospheric [CO_2] (Perumal, 2016). Based on the *Vachellia* seedlings' demonstrated increases in RGR, biomass accumulation, and resource use efficiency as atmospheric CO_2 increased to current levels, it is estimated that seedlings at historical [CO_2] of 20 Pa would have taken up to 24 weeks to reach the disturbance tolerant size classes. This suggests significantly greater exposure to fatal disturbances and a strong recruitment bottleneck effect on woody savanna seedlings in historical atmospheres with [CO_2] of 20 Pa.

Increasing CO_2 resulted in higher RGR's, instantaneous leaf level water use efficiency and, based on photosynthetic acclimation, would have caused a significant increase in nutrient use efficiency (NUE) through the reduction in photosynthetic enzymes required in the seedlings' leaves (Drake et al., 1997). These CO_2-facilitated increases in water and nutrient use efficiency could extend the effective habitat ranges which the *Vachellias* can colonize by increasing the threshold for nutrient, light, and water limitations in terms of establishment and survival (Kriticos, 2003). Evidence of this can be seen in the pattern of encroachment that occurs in semi-arid areas. The *Vachellias* were historically confined to ravine areas where water availability would have been higher, but in recent history they began to spread out of the ravines and colonize historically grassy flats (Klopper, 2015). As CO_2 increases the *Vachellias* WUE, the seedlings can support larger canopy areas with the same amount of water, resulting in higher growth potentials in drier areas, promoting an expansion into the historically open arid plains between the ravines. This has been demonstrated in Australia where *V. nilotica* is an invasive encroaching species, as [iCO_2] alters the tolerance of climates and increases the potential ranges that *V. nilotica* can encroach into (Kriticos, 2003).

Species of the *Vachellia* genus are nitrogen fixers through a symbiotic relationship with Rhizobia soil bacteria, in which the bacteria which are housed within root nodules are supplied with carbohydrates in return for the fixation of nitrogen (Crews, 1999). Through anaerobic respiration, the bacteria break down the carbohydrates and produce nitrates which the plant can absorb and use (Cramer et al., 2010; Sprent and Parsons, 2000). None of the seedlings in this study developed visible root nodules, indicating a lack of or suppression of the nitrogen-fixing symbioses. This could be due to the young age of the seedlings in the study, or the nutrient-rich topsoil in which they were grown reducing the benefit of the relationship (Crews, 1999).or no inoculates available but this is unlikely as we used savanna soil. As CO_2 increases, the nitrogen-fixing symbioses could have significant benefits for the plant. Firstly, higher CO_2 increases carbon availability thereby reducing the cost/trade-off of exuding carbohydrates into the soil for the rhizobia to use (Cramer et al., 2010). Secondly, carbohydrate sinks can become limiting for plants as CO_2

increases, triggering starch-mediated down regulation (Moore et al., 1999). The plants could use the rhizobia as a carbohydrate sink, reducing down regulation and simultaneously increasing nitrogen availability.

My research focused on *Vachellias* in the seedling size classes but similar research on older *Vachellias* (*V. karroo* and *V. nilotica*) showed that *i*CO_2 increased the efficiency with which the saplings can re-sprout after a clipping treatment simulating a top kill disturbance (Kgope et al., 2010). While this will not impact the population's morality rate as even the saplings at low CO_2 survived the clipping events, it will increase the rate at which saplings can outgrow disturbance traps (Bond and Midgley, 2000; Kgope et al., 2010). This will increase the proportion of the population escaping disturbance traps such as grazing and fire to reach mature size classes, promoting woody encroachment through the formation of dense closed canopies. Such closed canopies will suppress the understory C_4 grasses and increase the proportion of *Vachellias* in reproductively-mature size classes (Bond and Midgley, 2000).

A meta-analysis on plant reproduction at elevated [CO_2] demonstrated average increases of 18% in the quantity of seeds produced and a 25% increase in average seed mass (Jablonski et al., 2002). The *Vachellias* have been consistently shown to be strong responders to *i*CO_2 (John et al., 2000; Kgope et al., 2010; Temme et al., 2015), making it very likely that they could have reproductive responses similar to or stronger than the results from the meta-analysis of Jablonski et al. (2002). This means that the density of the *Vachellia* seedbanks may be increasing annually with [CO_2] and the larger seed mass will improve seedling provisioning and establishment success (Marty and Bassirirad, 2014). This reproductive response to *e*CO_2 adds a factor to our proposed increase in seedling survival, making more viable seedlings available which have a higher probability of reaching disturbance tolerant size classes.

CO_2 fertilization responses are further increased through positive feedbacks. The observed increases in photosynthetic performance and plant growth lead to larger canopy and root areas which, in turn, increase the plants' access to light, water and soil nutrients, providing the resources required for further growth (Kirschbaum and Lambie, 2015; Wolfe et al., 1998). The increased access to resources further promotes and supports growth, causing a positive feedback enhancing the overall plant responses to *i*CO_2 (Kirschbaum and Lambie, 2015)..

In conclusion, I demonstrate that despite *V. karroo*, *V. nilotica*, *V. robusta* and *V. tortilis* being selected from different ecological areas and having different climatic niches the four species responded in a similar manner to changes in CO_2 and can be treated as a functional type. Increases in [CO_2] from pre-glacial to ambient can act as a facilitator for woody encroachment in historically open savannas by the C_3 woody trees. This is caused by faster growth and increased plant size that would promote seedling establishment and survival into mature disturbance-tolerant size classes. The combination of the CO_2-induced changes in growth strategy, leaf level responses, and photosynthetic efficiency result in *Vachellia* seedlings which are more equipped to survive early seedling disturbances, potentially increasing their recruitment to older size classes. Our study supports suggestions that increasing CO_2 has been a major driver in the observed increase in encroachment seen since pre-industrial times, and will continue to promote woody encroachment into savannas into the future as [CO_2] continues to rise. Further work on how this CO_2 stimulation interacts with resource supply and disturbance is needed to evaluate a more complete picture of how climate change and associated increases in atmospheric CO_2 concentrations may have driven woody encroachment.

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