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Encroaching species are stronger anisohydric “water spenders” under elevated CO_2 conditions: Implications for savanna seedling establishment rates

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Preface:

The research contained in **"Encroaching species are stronger anisohydric "water spenders" under elevated CO_2 conditions: Implications for savanna seedling establishment rates"** was completed by the candidate while based in the Discipline of Botany, Department of Botany, Rhodes University, Makhanda, South Africa. The research was financially supported by National Ecological Research Council Grant NE/T000759/1 and NRF Thuthuka TTK200305508223.

The contents of this work have not been submitted in any form to another university and, except where the work of others is acknowledged in the text, the results reported are due to investigations by the candidate.

Signed: Brad Ripley

Date: 09 May 2024

Signed: Tiffany Pillay

Date: 09 May 2024

Plagiarism Declaration

I, Liam Macleod Reynolds, declare that:

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Abstract:

Plant water transport systems play a fundamental role in the productivity and survival of terrestrial plants due to the vascular architecture placing a physical limit on metabolic function. Savannas have high variability in rainfall, leading multiple studies to suggest that plant water-use strategies are key mechanisms affecting seedling establishment rates. Many savannas are seeing a directional shift towards an increase in the abundance of certain woody species through a process known as bush encroachment, which has been largely attributed to the fertilising effect of rising atmospheric [CO_2] on C_3 trees. These species are classified as encroachers. While there have been multiple studies investigating changes in the physiology of savanna species under elevated CO_2 ($e$$CO_2$), few have examined how climate and $e$$CO_2$ affects the fundamental water-use strategies in the seedling stage, a crucial demographic bottleneck. Here, this research provides valuable insights into the mechanisms behind bush encroachment in the context of $e$$CO_2$ using results from a pot experiment at the Rhodes University Elevated CO_2 facility and a field experiment. All species showed water use strategies characteristic of anisohydric "water-spenders", however, the vulnerability to embolism and rates of water-use were different between encroachers and non-encroachers. Encroachers are better at taking advantage of water pulses, particularly under $e$$CO_2$ and grass competition. This comes at the cost of higher xylem vulnerability during drought, leading to reductions in conductance when exposed to heavy water stress. The response of the photosynthetic parameters mirrored this, with encroaching species had higher rates of photosynthesis and photosystem II quantum yield than non-encroachers under the well-watered treatments. Field experiments revealed that small trees are particularly vulnerable to drought stress, when compared to medium and large trees. The outcomes of this complex response will largely depend on the extent of changes to biotic and abiotic factors across spatial and temporal zones caused by climate change. This research highlights potential hydraulic mechanisms contributing to the increase in bush encroachment, as well as providing important insights into the determinant factors that make a savanna species capable of encroachment.

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Chapter 1: Introduction

Climate change and savannas:

There has been a rise in atmospheric carbon dioxide concentration [CO_2] of around 7.5% in the last 15 years, bringing us to a current concentration of 422 ppm (NASA, 2024). Atmospheric [CO_2] is now at the highest it has been in the past 420 000 years (Petit et al., 1999). Alarmingly, these levels are expected to keep increasing during the next century if the burning of fossil fuels continues its current trajectory, reaching 1000 ppm by the year 2100 (Field et al., 2014). Rapidly increasing CO_2 levels contribute to significant global changes in climate, such as predicted increases in both vapour pressure deficits (Field et al., 2014) and an increase in air temperature from 2 to 5 °C by 2100 (Intergovernmental Panel on Climate Change, 2014). These changes will force ecosystems to adapt to warmer temperatures, increasing evaporative demands, and causing widespread changes in the length and severity of drought both on a regional and global scale. To predict shifts in ecosystem function and demographic distributions, it is imperative to understand how climatic conditions may affect physiological plant traits conferring drought tolerance.

Some of the ecosystems currently most affected by rising CO_2 levels in Southern Africa are the savannas (Bond & Midgley, 2000; Eamus & Palmer, 2007). Savannas are open canopy biomes characterized by the co-dominance of two contrasting life forms: woody trees and grasses (Sankaran et al., 2005). The balance between these lifeforms is maintained by the interplay of resource and disturbance mediated factors such as competition, rainfall, fire, and herbivory (Sankaran et al., 2005; Hempson et al., 2007; Riginos, 2009). Many savannas have experienced a phenomenon known as woody bush encroachment (Bond & Midgley, 2000; Bond, 2008). Woody bush encroachment is characterized by large-scale shifts in dominance and range expansion of certain woody plants, which suppress the grass layer resulting in negative biodiversity and socioeconomic outcomes (Bond & Midgley, 2000; Bond, 2008). These shifts are not only attributed to changes in top-down climatic drivers, such as atmospheric [CO_2], but also to bottom-up drivers, such as overgrazing, fire suppression, temperature, and drought (Venter et al., 2018; Quirk et al., 2019). For the purposes of this thesis, however, the focus with be on climatic drivers of woody bush encroachment. Adult savanna trees may persist and thrive due to their ability to root deep enough to access ground water supplies to avoid drought and minimize competition with grasses (Shadwell & February, 2017; Geißler et al., 2019), as well as reach the fire-suppression threshold by growing tall and the fire-resistance threshold by growing thick bark (Hoffmann et al., 2012). Conversely, seedlings are not yet able to avoid drought, competition for resources with grasses, or fire damage via these mechanisms (Chidumayo, 2013; Anderson et al., 2015; Morrison et al., 2019), thus making the seedling stage a crucial demographic bottleneck for woody species. While the CO_2 fertilisation effect is undeniably playing a role in exacerbating the rates of encroachment (Bond & Midgley, 2000; Eamus & Palmer, 2007), recent work has demonstrated how this effect can only be realized if both large-scale and local-scale drivers are not limiting (Raubenheimer & Ripley, 2022). This means that environmental conditions and levels of competition must be extensively investigated alongside the CO_2 response to accurately predict demographic shifts in savannas.

Plant physiological constraints and response to a changing climate:

One of the primary physiological traits relating to the survival and productivity of terrestrial plants is the functional characteristics of the vascular bundle (Tyree & Sperry, 1989; Brodribb, 2009). This is because the rate of water and nutrient transport places a physical limit on the rates of gas exchange and metabolic function that cannot be overcome (Brodribb, 2009; Tyree & Zimmermann, 2013). Vascular plants cannot photosynthesize and assimilate carbon without also losing a substantial amount of water through the stomata. Xylem is the primary type of transport tissue that allows plants to replace the water lost through photosynthesis and transpiration (Brodribb, 2009). The maximum rate at which the xylem can transport water is controlled by the morphology of the pit membranes and end plates, which scale directly with the dimensions of xylem conduits (Li et al., 2020; Venturas et al., 2017). The Hagen-Poiseuille law provides a useful conceptual framework to help understand how the anatomical structure of the xylem can affect water transport efficiency. It states that "the hydraulic conductance of a cylindrical pipe of uniform diameter increases with the fourth power of the diameter" (Tyree & Zimmerman, 2013). This law is best used as an approximation, however, as xylem conduits are neither cylindrical nor of uniform diameter throughout their length. Decreasing levels of soil moisture also increase the salt concentration of the soil, which further exacerbates the water potential stress between the soil and the plant (Zargar et al., 2017). Xylem conduits face the risk of embolism under conditions of drought due to the increasing negative pressure placed on the conduit walls. This leads to cavitation, which creates air blockages that prevent the xylem from transporting water and nutrients from the soil (Tyree & Zimmerman, 2013). Xylem cavitation can lead to wall collapse and even full hydraulic failure (Tyree & Zimmerman, 2013).

Stomata aid plants in regulating their water usage to prevent hydraulic failure. This regulates not only the water loss but also the uptake of CO_2, effectively controlling the rate of photosynthesis (Woodward, 1987; Xu et al., 2016). CO_2 can only reach the chloroplast, where it will be fixed to Rubisco, by diffusing through the boundary layer at the leaf surface, stomata, and intercellular air spaces that surround the chloroplast and each of these represents a specific resistance to diffusion

(Woodward, 1987; Xu et al., 2016). Numerous factors control stomatal opening, such as Ca^{2+} levels, hormones, and guard cell turgor (Assmann, 1999; Lawson et al., 2014). Environmental factors, such as water stress, temperature, and atmospheric [CO_2] also affect stomatal behaviour. Stomata can respond to both short-term (via stomatal closure) and long-term (via stomatal density and size) environmental changes, with both responses often occurring simultaneously (Purcell et al., 2018).

Furthermore, there is a close relationship between stomatal function and net carbon assimilation (Franks & Farquhar, 1999). At the leaf level, this relationship can be expressed through Fick's law (Eq. 1):

$A_{net} = g_{st}[CO_2](1 - [CO_2]_i/[CO_2])$,

where A_{net} is the net rate of photosynthesis, g_{st} is stomatal conductance, [CO_2] is the atmospheric carbon dioxide concentration, and $[CO_2]_i$ is the intercellular carbon dioxide concentration. The ratio of $[CO_2]_i/[CO_2]$ tends to remain constant across different levels of stomatal conductance (Voelker et al., 2016) indicating that the parameter responsible for most of the variability of A_{net} is g_{st} (Domec et al., 2017). A_{net} results in the production of sugars like glucose which are the plants' primary form of energy. Without adequate carbon assimilation, these reactions cannot proceed.

Under drought stress, reductions in carbon assimilation are not only attributed to the closure of stomata but also to decreased mesophyll conductance, impaired photosynthetic apparatus (changes or damage to PSI and PSII), enhanced metabolite fluxes, reductions in overall gas exchange and decreases in turgor pressure (Chaves et al., 2003; Jaleel et al., 2009; Sun et al., 2014). Enhanced metabolite fluxes caused by adverse conditions lead to the production of free radicals that severely limit the development and repair of photosynthetic apparatus (photoinhibition; described below) (García-Caparrós et al., 2021). Decreases in turgor pressure lead to reductions in cell growth (Coster et al., 1976). There is also evidence that exposure to drought stress leads to upregulated proteins associated with carbon metabolism in certain C_3 species, which protects the plant from oxidative stress by enhancing the carbohydrate conversion efficiency (Chang et al., 2019). Long-term exposure to drought is detrimental to the physiology of the plant, affecting the ultrastructure of the chloroplast itself, disrupting grana stacking, producing more compact grana lamellae, increasing disorganization in thylakoid membranes, and promoting starch granule accumulation (Chang et al., 2019).

One of the major contributors to the loss of photosynthetic capacity under high-stress environments is the increase in photoinhibition (Kok, 1956; Takahashi & Murata, 2008). Photoinhibition was first described in 1956 as the reduction in the photosynthetic capacity of a plant brought on by intense light (Kok, 1956). Photoinhibition occurs continuously as the plant is exposed to light; however, this damage is repaired by a series of chemical reactions in the chloroplast. The photosystem II (PSII) repair cycle consists of the degradation and subsequent synthesis of the D1 polypeptide protein, found in the reaction centre of PSII, followed by the re-activation of that reaction centre (Aro et al., 1993). Evidence in the literature suggests that this damage occurs in two steps. Firstly, high intensities of light cause damage to the oxygen-evolving complex, which involves a disruption of the manganese cluster and causes the release of manganese ions (Nishiyama & Murata, 2014). This excess of manganese ions then disrupts the supply of electrons to the PSII reaction centre, forcing PSII to remain in its oxidized state and causing damage to the D1 protein (Nishiyama & Murata, 2014). Under standard conditions, the rate of repair is enough to prevent a decrease in photosynthetic rates; however, if the rate of degradation of the D1 protein surpasses that of the rate of synthesis, then the symptoms of photoinhibition become apparent. Environmental stresses, such as drought and high temperatures, limit the amount of carbon available to the plant, decreasing the rate of repair of PSII (Takahashi & Murata, 2008). The primary mechanism that plants use to alleviate the effects of photoinhibition is non-photochemical quenching (Müller et al., 2001). This process involves returning the singlet excited state chlorophylls back to the ground state via internal conversion that dissipate excess excitation energy as heat or fluorescence. Most photosynthetic eukaryotes utilise non-photochemical quenching to prevent photosynthetic damage in conditions of excess light energy absorption (Bassi & Dall'osto, 2021).

Plant physiological traits are not only sensitive to drought but also changes in the atmospheric [CO_2]. Recent studies have shown that the [CO_2] response can vary considerably between different species. A literature review conducted by Huang & Xu (2015) revealed that most species mitigate the worst effects of heat and drought when exposed to *e*CO_2. This was an extensive literature review that examined multiple studies encompassing a wide range of species and ecosystems (Huang & Xu, 2015). More recent studies, conducted on *Glycine max* at 550ppm [CO_2] (Jin et al., 2018) and on *Pinus halepensis* at 936ppm [CO_2] (Gattmann et al., 2021) had species showing no mitigation effects of heat and drought with *e*CO_2, with no significant shifts in lethal hydraulic threshold or the time it takes to reach lethal hydraulic thresholds. These effects are largely attributed to adjustments in the anatomical structure of the xylem caused by long-term exposure to *e*CO_2 (Domec et al., 2009; Domec et al., 2017) and to the rates of leaf growth potentially being sufficient under *e*CO_2 to offset the water savings benefits (Jin et al., 2018; Gattmann et al., 2021). However, these anatomical changes were more common in long-term experiments (>10 years) (Kostiainen et al., 2014; Kim et al., 2015) than in short-term experiments (<2 years) (Gartner et al., 2003; Vaz et al., 2012). Some studies have even shown results that link decreases in hydraulic conductivity to aquaporin activity, rather than anatomy or conduit structure (Locke et al., 2013; Locke & Ort, 2015). Plant resistance to embolism also shows mixed results in response to eCO$_2$. With the observed changes in anatomy for many species grown with long-term CO$_2$ exposure, one may predict that the increases in conduit size and overall biomass allocation reduce the resistance to embolism (Domec et al., 2017), but few studies support this (Domec et al., 2010; Warren et al., 2011). A literature review done by Domec et al. (2017) stated that roughly half the literature on the effect of atmospheric [CO$_2$] on resistance to xylem embolism showed no significant results. Based on the studies sited by Domec et al. (2017) this appears to be a species-specific response, meaning each species of interest needs to be investigated individually.

Stomata show strong responses to instantaneous changes in atmospheric [CO$_2$], with CO$_2$ levels below ambient causing stomata to open and CO$_2$ levels above ambient causing stomata to close (Mott et al., 2008). Recent molecular work has suggested that angiosperms have a network of core and peripheral guard cell signalling pathways that drive the stomatal response to eCO$_2$ (Dubeaux et al., 2021). These studies only looked at the response to instantaneous changes in [CO$_2$], however, and long-term exposure to eCO$_2$ alters leaf anatomy and therefore the regulation of stomata and water loss (Gattmann et al., 2023). Certain species exposed to long-term eCO$_2$ have shown reductions in both stomatal density (number of stomata per unit leaf area) and stomatal index (proportion of epidermal cells that are stomata) (Woodward & Kelly, 1995), while others show no effect or, in rare cases, an increase in stomatal density (Pritchard et al., 1999; Domec et al., 2017). Many of these changes are attributed to the alterations in cell division and expansion that an increase in carbon dioxide availability causes (Pritchard et al., 1999). Large-scale meta-analyses of physiological responses to eCO$_2$ have reported that most species see a mean reduction in stomatal conductance of 16-19% (Ainsworth & Long, 2005; Ainsworth & Rogers, 2007).

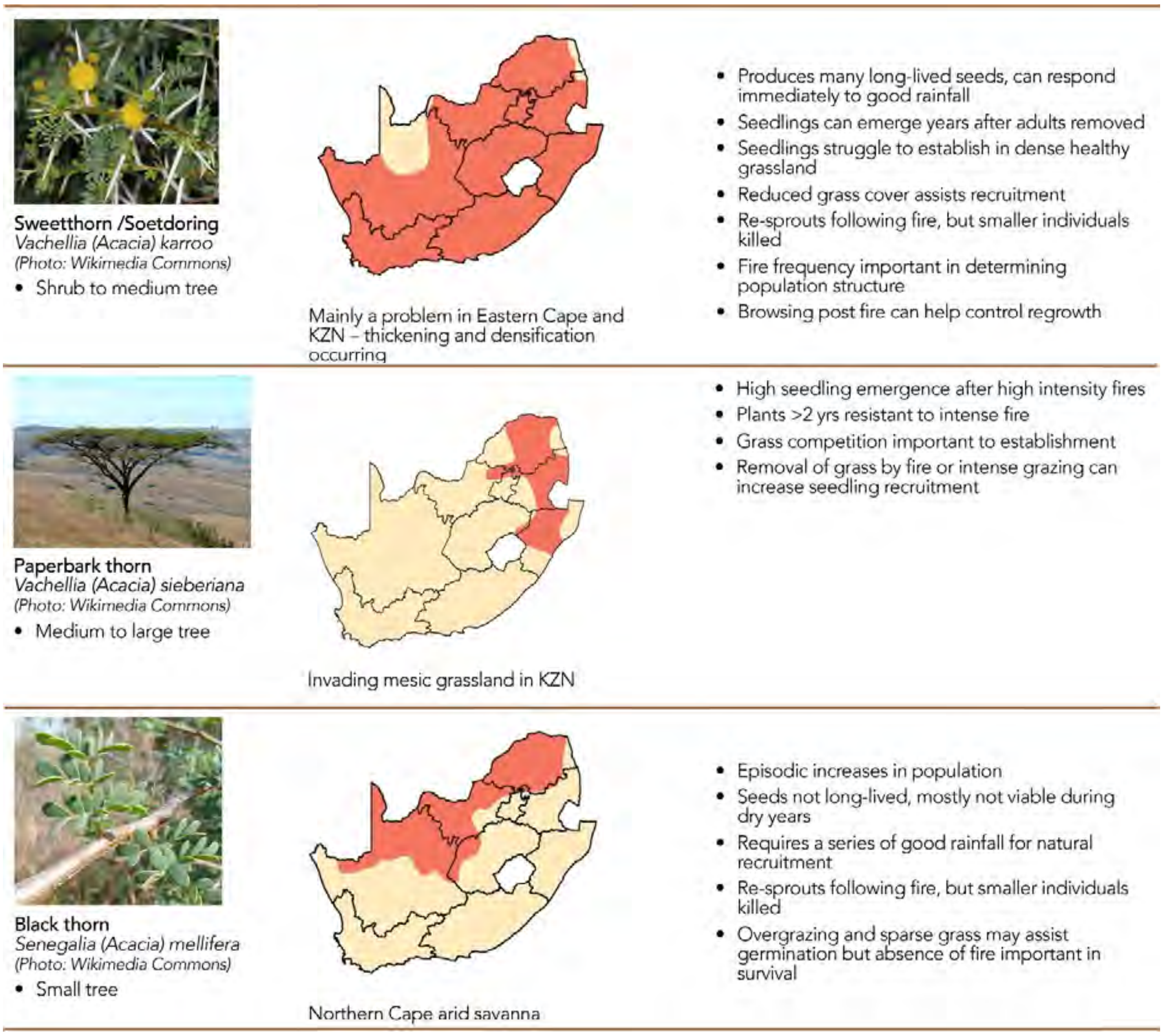
Given that C$_3$ photosynthesis is carbon-limited at current atmospheric [CO$_2$] (Long et al., 2004), rising [CO$_2$] is expected to increase photosynthesis and decrease photorespiration for C$_3$ plants (Ainsworth & Long, 2005). eCO$_2$ has also been shown to alleviate photoinhibition by allowing carbon metabolism to proceed, consuming light reaction products, and reducing the production of reactive oxygen species, therefore decreasing the requirement for the dissipation of excess energy as heat (Kitao et al., 2007). An extensive theoretical analysis by Von Caemmerer & Furbank (2003) suggested that an increase in [CO$_2$] from 380 ppm to 550 ppm might increase A_{net} by as much as 38%. Actual measured values of carbon assimilation in eCO$_2$ often fail to match the theoretical increase, largely due to photosynthetic downregulation (acclimation) associated with nutrient limitations in the soil (Ainsworth & Long, 2005; Ainsworth & Rogers, 2007; Leakey et al., 2009). Photosynthetic downregulation is attributed to either a decreased investment in ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) and maximum rate of carboxylation (V_{cmax}) (Ainsworth & Long, 2005), or to a decrease in the regeneration ability of Rubisco, which decreases V_{cmax} limited by the electron transport chain (J_{max}) due to either insufficient inorganic phosphate in the chloroplast for ATP synthesis or vast reductions in the capacity of electron transport (Ainsworth & Rogers, 2007; Zhu et al., 2012). Within C_3 plants, there is a range of responses to $e\mathrm{CO}_2$ between different species and growth forms. Trees, particularly fast-growing species with a large carbon sink capacity, generally see a much greater stimulation of carbon assimilation under long-term $e\mathrm{CO}_2$ than annuals or shrubs (Ainsworth & Long, 2005).

Predicting savanna vegetation changes:

In the context of savanna ecosystems, one of the most significant bottlenecks for C_3 tree seedlings in the recruitment phase to overcome is the competition with C_4 grasses (Skarpe, 1992; Bond, 2008). C_4 species show a markedly different response to rising atmospheric [CO_2] than C_3 species due to differences in metabolism (Wand et al., 1999; Ward et al., 1999; Reich et al., 2018). Experimental work done on grasses shows that under current [CO_2], C_4 species are less carbon limited than C_3 species and are therefore less responsive to rising [CO_2] in the short term (Wand et al., 1999; Ainsworth & Long, 2005). Over long-term exposure, however, results are less predictable. One study found that C_4 species were out-competing C_3 species for the first three years of the experiment (Morgan et al., 2011), but that this relationship had reversed by the end of the sixth year (Mueller et al., 2016). Another experiment, conducted over 20 years, saw C_3 benefitting more from $e\mathrm{CO}_2$, as expected, for the first 12 years. Then, unexpectedly, over the next 8 years, the pattern reversed: biomass increases were significantly greater for C_4 species than for C_3 species (Reich et al., 2018). A four-year study conducted within a savanna ecosystem in the Kruger National Park showed that the competition between C_3 and C_4 species is also affected by drought, with higher levels of rainfall increasing the competitive pressure exerted by C_4 grasses on C_3 seedlings (February et al., 2013).

Woody plant encroachment has significant negative impacts in savannas globally. In South Africa, there are over forty species of trees that are considered encroachers, most of which are legumes in the genera *Vachellia* and *Senegalia*, including *V. karroo*, *V. sieberiana*, *V. tortillis* and *S. cafra* (Turpie et al., 2019). Identifying the encroachment probability of these species is challenging, as there are many potential culprit species that share many traits and generally have a similar response to the main drivers of woody bush encroachment. It is the species-specific nuances in relation to its environment that determines whether a species will encroach (O'Connor et al., 2014; Turpie et al., 2019). This means that developing a trait-based framework and understanding of the life-history of the plant is essential for landholders to manage woody bush encroachment. While certain species have been identified as the main encroachers, it is important to note that woody bush encroachment is not just the increase in density of certain species, but rather a change in balance of the broader phenotypes present within a particular ecosystem (Turpie et al., 2019). There are certain characteristics that makes a species more likely to encroach. Table 1 provides a summary of the distribution and characteristics of a few common *Vachellia* and *Senegalia* encroacher species taken from Turpie et al. (2019). It is important to note that these characteristics do not mean that a plant will encroach in all ecosystems and must therefore be looked at in relation to the environmental conditions where the plant occurs, which makes defining encroachers and non-encroachers difficult. (O'Connor et al., 2014; Turpie et al., 2019). For the purposes of this thesis, the classification of encroacher and non-encroacher was based on the work of Turpie et al. (2019).

Table 1: Characteristics of some of the species most commonly associated with bush encroachment throughout its range in South Africa (Turpie et al., 2019)



Sweetthorn /Soetdoring *Vachellia (Acacia) karroo* *(Photo: Wikimedia Commons)* • Shrub to medium tree	Mainly a problem in Eastern Cape and KZN – thickening and densification occurring	• Produces many long-lived seeds, can respond immediately to good rainfall • Seedlings can emerge years after adults removed • Seedlings struggle to establish in dense healthy grassland • Reduced grass cover assists recruitment • Re-sprouts following fire, but smaller individuals killed • Fire frequency important in determining population structure • Browsing post fire can help control regrowth
Paperbark thorn *Vachellia (Acacia) sieberiana* *(Photo: Wikimedia Commons)* • Medium to large tree	Invading mesic grassland in KZN	• High seedling emergence after high intensity fires • Plants >2 yrs resistant to intense fire • Grass competition important to establishment • Removal of grass by fire or intense grazing can increase seedling recruitment
Black thorn *Senegalia (Acacia) mellifera* *(Photo: Wikimedia Commons)* • Small tree	Northern Cape arid savanna	• Episodic increases in population • Seeds not long-lived, mostly not viable during dry years • Requires a series of good rainfall for natural recruitment • Re-sprouts following fire, but smaller individuals killed • Overgrazing and sparse grass may assist germination but absence of fire important in survival

Elevated atmospheric [CO_2] is thought to be one of the main driving forces behind woody bush encroachment (Bond & Midgley, 2000; Eamus & Palmer, 2007), however, there is evidence of considerable variation in the extent of bush encroachment across space (O'Connor et al., 2014; Skowno et al., 2017). Numerous other factors relating to climate change, such as temperature and drought, have not been as extensively studied, calling into question the validity of such a high focus on CO_2 effects (Venter et al., 2018; Quirk et al., 2019). The highly erratic, disturbance-prone nature of savannas means that there is a high variability in environmental stresses both between and within landscapes (Bond et al., 2003). A recent study done by Raubenheimer & Ripley (2022) shows that C_3 savanna tree species can only realize the benefits of CO_2 enrichment when other growth conditions are not limiting. These growth conditions include large-scale climatic and environmental drivers (e.g. temperature, water availability, and nutrients) and local-scale drivers (e.g. fire, herbivory, and competition) (Raubenheimer & Ripley, 2022). Savanna trees must overcome these environmental bottlenecks to properly establish themselves in the typically grass-dominated ecosystem. Water stress is one of the strongest of these environmental bottlenecks, with mesic and semi-arid savannas showing markedly different responses to changing climate (Fensham et al., 2009; Skowno et al., 2017). More xeric savannas have much higher rates of full plant mortality caused by xylem cavitation (Rice et al., 2004) following the exhaustion of soil water reserves during extended droughts (Bowman & Prior, 2005). Some studies suggest that this will counteract the rates of bush encroachment during periods of extended drought when the soil water is exhausted (Fensham et al., 2009), yet the observed rates of bush encroachment in more xeric savannas remain high (Belayneh & Tessema, 2017). A study investigating the rates of woody bush encroachment across rainfall gradients in the Eastern Cape found that woody canopy cover doubled at the more xeric site from 1949 – 2013 (Skowno, 2018). While extreme drought may lead to large-scale diebacks in tree cover (Fensham et al., 2009; Domec et al., 2017; Case et al., 2020; Swemmer, 2020), there is evidence that moderate drought benefits C_3 seedling recruitment by reducing grass competition within savannas (February et al., 2013). This story is likely to change with rising [CO_2], if increasing carbon supply alleviates much of the drought effects in C_4 species (Ward et al., 1999).

Open-top carbon dioxide enrichment chambers (OTC) provide a method to grow plants under simulated climatic conditions to test physiological responses (Leadley & Drake, 1993). These chambers allow us to test multiple interacting effects, such as increased temperature, water stress and grass competition, alongside carbon enrichment and how they affect plant physiology (Bremer et al., 1996; Lawson et al., 2001; Sun et al., 2017; Raubenheimer & Ripley, 2022). There are numerous studies that suggest that the seedling stage is a strong demographic bottleneck for savanna C_3 tree species (Chidumayo, 2013; Anderson et al., 2015; Morrison et al., 2019), thus the focus of these experiments will be on the physiological responses within this age class. This thesis will focus on generating a link between hydraulic and photosynthetic functions under potential future climates and investigate whether this relationship is different for encroaching and non-encroaching species. Assumptions of water use partitioning between different size classes in an Eastern Cape savanna ecosystem will then be tested to identify the stage at which these woody species can fully establish and begin accessing groundwater to escape their demographic bottleneck.

In semiarid ecosystems, there are often extended periods of drought between rain events (Scanlon et al., 2006) resulting in the deep-rooted soil-plant systems being highly efficient with rain use (Seyfried et al., 2005). Data from recent papers show that within Southern African savannas, large trees are reliant on groundwater to get through periods of drought (Kulmatiski & Beard, 2013; Shadwell & February, 2017; Geißler et al., 2019). Within the C_4 grass layer, however, shallow rooting depths of establishing seedlings mean that most of their water is obtained from the upper soil layer (Scholes & Archer, 1997; Ward et al., 2013). Consequently, competition for water occurs primarily in the recruitment phase for woody C_3 species. There is also evidence that, due to the larger transpirational surface that they possess, C_3 trees and shrubs may take up rainwater (soil water of potential groundwater recharge) more effectively than the C_4 grasses, where rooting zones overlap (Meinzer et al., 2005; Geißler et al., 2019). This makes studying the response to changing climate between C_4 grasses and C_3 seedlings in the recruitment phase of particular importance for understanding encroachment rates. The natural abundance of oxygen ($\delta^{18}O$) and hydrogen ($\delta^{2}H$) stable isotopes in plant xylem water provides a useful method to determine the source of water, as root water uptake is considered a non-fractionating process (Dawson & Ehleringer, 1991). Gradients in $\delta^{2}H$ and $\delta^{18}O$ isotopic signatures form across soil layers of different depths, making it possible to determine which water source(s) is currently being exploited by the plant (Ehleringer & Dawson, 1992). These gradients form through seasonal differences in the input of moisture into the soil (Friedman et al., 1964; Rozanski et al., 1982), evaporation in the uppermost layers of soil (Allison et al., 1983; Barnes & Allison, 1983), or to differences in isotopic composition between the bulk soil water and the ground water (Fritz & Fontes, 1980; Gat, 1980). Isotopic signatures from precipitation and groundwater often have very distinct signals, allowing for plant water sources to be determined by testing the isotopic signatures of the xylem water (Ehleringer & Dawson, 1992). Once established, woody plants tend to use water opportunistically to reduce water stress, using water from the upper layers of soil during the wet season and switching to ground water at greater depths during the dry season (Dawson & Pate, 1996; Xu et al., 2011).

To test for differences in plant water source, an experiment was conducted at a field site as Endwell Safaris, located in the Smaldeel region of the Eastern Cape province of South Africa (32°44'15.7"S

26°29'06.6"E) (Figure 1). The land is used predominantly for livestock farming, particularly cattle and goats (Martens et al., 1996; Smit, 2004). Wild ungulates that are common on the farm include blesbok (*Damaliscus pygargus phillipsi*), common duiker (*Sylvicapra grimmia*), impala (*Aepyceros melampus*), kudu (*Tragelaphus strepsiceros*), and waterbuck (*Kobus ellipsiprymmus*). The dominant tree species in this area is *Vachellia karroo* (Acocks, 1988). Other common species include *Brachylaena elliptica*, *Grewia occidentalis*, *Gymnosporia buxifolia*, *Olea europaea* subsp. *Africana*, *Scutia myrtina*, *Searsia longispina*, and *Ziziphus mucronata* (Mucina & Rutherford, 2007). The site has a mean annual precipitation of 700 – 800 mm (Cole et al., 2018) and rainfall is generally higher in the summer months (Martens et al., 1996). Trevanian, Glenrosa and Mispah are the most predominant soil types in the area (Martens & Morris, 1994). The vegetation in Smaldeel is classified as a semi-arid savanna, with mixture of trees and a discontinuous grass layer. More specifically, it is a combination of Bedford Dry Grassland and Bisho Thornveld (Mucina & Rutherford, 2007).

Figure 1: Map of South Africa, with an arrow pointing to the field site at Endwell Safaris (32°44'15.7"S 26°29'06.6"E).

Predicting shifts in ecosystem dynamics in response to *e*CO_2 is complex and requires interspecific comparisons under multiple environmental conditions (Skowno et al., 2017; Raubenheimer & Ripley, 2022). In this research, the focus is on the hydraulic and photosynthetic response of common savanna encroacher and non-encroacher seedlings to drought and grass competition in the context of rising [CO_2], by conducting a pot experiment using Open Top Chambers (OTC). Water use partitioning between small, medium, and large trees was then investigated by conducting a field experiment using stable isotopes to determine if different size classes of an encroaching woody tree display differing water use (ground versus precipitation) during periods of low and high precipitation. *Vachellia* and *Senegalia* species (formerly *Acacia*), are categorized as anisohydric (Cory et al., 2022). These species have more variable water potentials (Ψ) and keep their rates of gas exchange high during periods of drought when compared to isohydric species (Sade et al., 2012). Anisohydric plants perform better during conditions of moderately stressful drought but face the risk of full hydraulic failure under conditions of intense drought (Alvarez et al., 2007; Sade et al., 2009; Kumagai & Porporato, 2012).

I hypothesize that (1) $e\mathrm{CO_2}$ will increase the hydraulic conductance and xylem vulnerability for encroaching species, making them stronger anisohydric "water spenders", (2) $e\mathrm{CO_2}$ will increase photosynthesis and photochemical quantum yield for encroaching species under well-watered conditions, consistent with the hydraulic results, and (3) small savanna trees will not have access to groundwater reserves during periods of increased water stress, making them more susceptible to reaching critical levels of hydraulic failure than more established trees.

Thesis outline:

In Chapter 2, I aim to test hydraulic mechanisms behind bush encroachment in the context of *e*CO_2 and drought. I conducted an experiment which tested xylem vulnerability and hydraulic conductance for seedlings of six woody tree species, characterized as either “encroaching” or “non-encroaching”. I predicted that all species would show water use strategies characteristic of anisohydric “water-spenders”, and that vulnerability to embolism and rates of water-use would be highest for encroachers.

In Chapter 3, I aim to test water use implications for photosynthesis and photochemical energy dissipation under *e*CO_2. I conducted an experiment which tested stomatal conductance, photosynthesis, photosystem II quantum yield for seedlings of six woody tree species, characterized as either “encroaching” or “non-encroaching”. I predicted that encroaching species would see a greater increase in photosynthetic rate and photosystem II quantum yield with *e*CO_2 in well-watered conditions, with non-encroaching species maintaining higher rates of photosynthesis and photochemical energy dissipation into drought.

In Chapter 4, I aim to quantify the relationship between tree size and water source for *Vachellia karroo*, one of the most significant encroaching species in South African savannas. I conducted an experiment which tested plant source water using the relative abundance of hydrogen (δ^2H) and oxygen ($\delta^{18}O$) stable isotopes in xylem water. I predicted that large trees would be much less reliant on rainwater (soil water of potential groundwater recharge) than small trees.

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Chapter 2: Effects of Elevated CO_2 and Drought on Savanna Tree Seedling Water Use Strategies

Abstract:

Plant water-use strategies are key mechanisms affecting seedling establishment rates within savanna ecosystems. Many of these ecosystems are undergoing a directional shift towards an increase in the abundance of woody species through a process known as bush encroachment. While multiple drivers have been identified, the fertilising effect of elevated atmospheric CO_2 (*e*CO_2) on C_3 trees is implicated as one of the most important determinants of encroachment. While there is extensive literature on the physiological responses of common savanna species to *e*CO_2, most of these studies focused on the stomatal and photosynthetic responses, while few studies have examined how climate and *e*CO_2 affects the fundamental water-use strategies in the seedling stage which represents a crucial demographic bottleneck. Here, this research provides valuable insights into the mechanisms behind bush encroachment in the context of *e*CO_2 using results from a pot experiment conducted at the Rhodes University Elevated CO_2 facility. Seedlings of six woody tree species, characterized as either "encroaching" or "non-encroaching" were grown under full factorial treatments of *e*CO_2 and drought. Xylem vulnerability and hydraulic conductance was measured. While all species showed water use strategies characteristic of anisohydric "water-spenders", the vulnerability to embolism and rates of water-use were different between encroachers and non-encroachers. Encroachers had higher shoot hydraulic conductance than non-encroachers in all treatments where water was not limiting but reached the point of 50% xylem embolism significantly faster than non-encroachers under *e*CO_2. This means that encroachers are better at taking advantage of water pulses, particularly under *e*CO_2 and grass competition. This comes at the cost of higher xylem vulnerability during drought, leading to reductions in shoot hydraulic conductance when exposed to heavy water stress. The implication of these findings is that the conductance rates of encroachers are likely to increase in most projected future scenarios, except under severe water stress. This research highlights potential hydraulic mechanisms contributing to the increase in bush encroachment, as well as providing important insights into the determinant factors that make a savanna species capable of encroachment.

Keywords: xylem vulnerability, hydraulic conductance, embolism, woody bush encroachment, elevated CO_2, drought

Introduction:

Plant water transport systems play a fundamental role in the productivity and survival of terrestrial plants (Tyree & Sperry, 1989). Savanna ecosystems have a high variability in rainfall and water stress, leading multiple studies to suggest that plant water use strategies and xylem vulnerability are key mechanisms affecting seedling establishment (Johnson et al., 2011; Cory et al., 2022; Uni et al., 2023). Seedlings do not have the deep root systems of adult trees and are therefore much more susceptible to water stress during periods of drought (McDowell et al., 2008).The primary type of tissue that plants use to transport water is known as xylem. The size and structure of these conduit vessels determine the maximum rate of flow (Tyree & Zimmerman, 2013). The vascular systems of plants exert a resistance to flow on the water being transported through it. Plants overcome this by exerting tension on the water column, which is measured as a decrease in leaf water potential (Ψ) (Van Den Honert, 1948). Water potential gradients determine the rate of water flow through a set resistive hydraulic pathway in a way analogous to the way that electrical resistors affect the flow of current across electrical potential gradients (Cowan, 1972). This relationship can be represented by the equation (Eq. 2):

$E = (\Psi_{\text{soil}} - \Psi_{\text{leaf}})K_{\text{plant}}$

Where E is the rate of evapotranspiration, Ψ_{soil} is the water potential of the soil, Ψ_{leaf} is the water potential of the leaf, and K_{plant} is the hydraulic conductance of the full plant (Brodribb, 2009). Under severe drought conditions, these water transport systems face the risk of embolisms, caused by wall collapse and the cavitation of water columns, which can lead to full hydraulic failure (De Guzman et al., 2016; Tyree & Zimmerman, 2013). While plant hydraulic conductivity and xylem vulnerability are strongly regulated by genetic control (Choat et al., 2012), recent studies have demonstrated xylem plasticity shows considerable variation within species, beyond genetic control (Stiller, 2009; Wang et al., 2016).

One of the major factors in climate change that can influence how plants respond to water stress is atmospheric [CO_2]. Elevated CO_2 ($e$$CO_2$) has been shown to have mixed results on stem xylem and water usage, with some studies showing $e$$CO_2$ significantly increasing hydraulic conductance and xylem vulnerability (Atkinson & Taylor, 1996; Kostiainen et al., 2014; Kim et al., 2015), and others showing no or insignificant effects (Kilpelainen et al., 2007; Kostiainen et al., 2004; Vaz et al., 2012). For the studies that did find changes in hydraulic parameters when exposed to $e$$CO_2$, it was found that embolising pressure was reduced in *Quercus ilex*, *Cornus florida*, and *Liquidambar styraciflua* (Domec et al., 2010; Warren et al., 2011). While studies that looked at hydraulic conductivity generally found a tendency towards an increase in specific hydraulic conductivity with $e$$CO_2$ (Domec et al., 2017), certain examples showed that increases in leaf biomass could offset the increases in specific hydraulic conductivity to create an overall decrease in whole-plant conductivity (Heath et al., 1997) or that specific hydraulic conductivity itself decreased alongside total vessel area in the petiole (Eguchi et al., 2008). While many of these studies indicate changes in hydraulic properties conferring higher drought resistance, it is important to note that these benefits may be offset by the increased severity of drought stress projected future climates (Field et al., 2014; Intergovernmental Panel on Climate Change, 2014). An extensive literature review by McDowell et al. (2011) found that mechanisms behind drought-associated vegetation mortality are likely to be amplified under the projected warmer, drier climate.

Multiple studies have demonstrated that the seedling stage for savanna species is a demographic bottleneck (Chidumayo, 2013; Anderson et al., 2015; Morrison et al., 2019), making it a crucial stage to understand when looking at the mechanisms behind woody bush encroachment. Most savanna species are drought deciduous, dropping their leaves during the dry season to minimise the effects of prolonged exposure to low soil moisture (Dahlin et al., 2017). This means that the wet season is crucial to the survival and establishment of savanna seedlings. *Vachellia* and *Senegalia* species are strongly anisohydric "water spenders" while in the seedling stage, however, there are differences in their rates of water usage between species (Cory et al., 2022; Uni et al., 2023). The differences in rates of water use between these species have been shown to reflect their topographical distributions (Uni et al., 2023). *Themeda triandra*, the species selected for the grass competition treatment is also considered an anisohydric "water-spender" (Williams et al., 1998). This anisohydric strategy allows seedlings and grasses to maximise their carbon assimilation, and therefore growth to escape their bottlenecks, during pulses of water availability at the cost of increased drought vulnerability during the dry season.

In this study, the effects of *e*CO_2 and drought on seedlings of six savanna trees, classified as encroachers or non-encroachers will be investigated. This experiment aims to test whether encroaching species have differing water usage strategies from non-encroaching species, and how this relationship is affected by exposure to *e*CO_2. To test this, the following questions are addressed:

(1) Do encroaching species have a higher hydraulic conductance and xylem vulnerability than non-encroaching species, making them stronger anisohydric water-spenders? Encroaching species should have stronger anisohydric properties than non-encroaching species.

(2) What is the effect of *e*CO_2 on the hydraulic parameters of encroaching species and non-encroaching species? *e*CO_2 should increase the higher hydraulic conductance and xylem vulnerability more for encroachers than non-encroachers.

(3) Is the predicted *e*CO_2 response dependant on water availability? The *e*CO_2 response should be heavily dependent on water availability, with encroachers facing higher xylem vulnerability and greater reductions in hydraulic conductance during drought than non-encroachers.

Methods:

OTC experiment:

To test for the effect of [CO_2], competition, and drought on the hydraulic parameters, six C_3 species (*Vachellia karroo, V. robusta, V. sieberiana, V. tortillis, Senegalia cafra,* and *S. burkei*) were grown from seed. Encroachment status was determined by the work conducted by Turpie et al. (2019), which is based on the life-history of the plant. Encroachers are *V. karroo*, *V. sieberiana* and *V. tortillis*. Non-encroachers are *V. robusta*, *S. burkei* and *S. Cafra*. Hydraulic conductance measurements were conducted on *V. karroo*, *V. robusta*, *V. sieberiana*, and *S. cafra*. Xylem vulnerability measurements were conducted on *V. karroo*, *V. robusta*, *V. sieberiana*, *V. tortillis*, and *S. burkei*. All of these species were grown at the Rhodes University Elevated CO_2 facility (RUECF). The plants were grown at either elevated CO_2 concentrations ($e$$CO_2$ at 550 ppm) or ambient CO_2 concentrations ($a$$CO_2$ at 400 ppm) for the duration of the experiment, which lasted 22 months. These CO_2 levels were monitored by open-path CO_2 analysers every second (GMP343, Vaisala, Finland) and managed by a proportional-integral-derivative procedure that controlled the flow of CO_2 (2873 proportional valve, Burkert, Germany). A three-phase fan was positioned at the canopy level that could blow CO_2-enriched air out of a perforated, circular diffuser around the chamber. This allowed proper ventilation throughout the chamber, as well as ensuring an even distribution of CO_2. Despite this, the temperature in the chambers was 4 – 5 °C higher than ambient (Ripley et al., 2022). The CO_2 levels were maintained by sixteen open-top CO_2 chambers (OTC) at the Rhodes University Elevated CO_2 facility. Plant specimens used for hydraulic conductance ranged in age from 10 to 13 months. Plant specimens used for xylem vulnerability ranged in age from 19 to 22 months. Treatment codes are GTD (grass competition + drought), GTW (grass competition + well-watered), TD (no competition + drought), and TW (no competition + well-watered). Plants that were under the droughted treatment only received water under natural rainfall events, while well-watered treatments were watered frequently using either drip irrigation or hand watering. Plants under the grass competition treatment were planted with *Themeda triandra*, a common savanna C_4 species. Grasses were established first for a full growing season before seedling were added. *T. triandra* specimens were grown from tillers collected at Burnt Kraal, Makhanda, Eastern Cape (33°16’40” S 26°29’20” E).

Xylem vulnerability (P_{50}):

Xylem vulnerability measurements were conducted on *Vachellia karroo*, *V. robusta, V. sieberiana, V. tortillis,* and *Senegalia burkei*. *S. cafra* was not investigated due to issues with germination that prevented sufficient individuals from establishing to run the experiment. Plant shoots were collected early morning (8:30-9:00 am). Samples were cut near the base of the stem and immediately placed into a bucket of water with a plastic bag covering the leaves in order to minimise the rate of desiccation during transport. Once enough samples to fill the pneumatron (described below) were collected, they were transported back to the lab. At the lab, all samples had their water potential (Ψ) readings taken using a PMS pressure chamber (Model 1505D). The bark and phloem were then removed from the bottom 1 cm of the stem to connect to the pneumatron. Contact adhesive was used to ensure that the seal between the stem and tubing was airtight.

All xylem vulnerability measurements were made using the pneumatic method on a device called the pneumatron (see Appendix A; Figure 23 [suppl.]) (Pereira et al., 2020; Trabi et al., 2021). The device consists of (a) a vacuum pump to generate the partial vacuum, (b) a solenoid valve to control which sample the vacuum is applied to, (c) a pressure transducer to monitor the rate of pressure change, and (d) an Arduino Uno microcontroller to collect data and control the system (Pereira et al., 2020). Measurements from the pneumatron are based on the volume of air diffused (AD in microlitres), which is estimated by measuring the pressure change inside the tubing of a known volume for a set time period (40 seconds here). This measurement is based on the theoretical assumption that as the xylem vessels become embolised, more openings for air to diffuse through the plant material are created, and hence the vacuum returns to atmospheric pressure at a faster rate. To compare measurements, AD is converted into percentage air diffused (PAD). This process happens in two main steps. Firstly, the vacuum pump and relevant solenoid valves are activated by the microcontroller. The vacuum pump reduces the pressure by ~70 kPa before the microcontroller switches it off and closes the solenoid valve connecting to the pump. As this vacuum is created inside the tubing, it slowly begins to return to atmospheric pressure with the air being sucked through the plant material attached to the device. This change in pressure, taken between the start (2 seconds) and the end (40 seconds) of the measurement is what is used to calculate AD, and thus PAD. After this first step, the solenoid valve is opened to allow the pressure to return to atmospheric pressure. The setup used in this experiment was based off the M-Pneumatron as described by Pereira et al. (2020), which allows multiple samples to be attached at once. Using this configuration, each sample was measured in sequence, before a lag time of 15 minutes was allowed before the next programmed measurement. Samples typically took 2 days to fully dehydrate. After the initial Ψ reading, repeat readings were made at intervals of 2, 5, 12, and 24 hours after the measurement was started to produce vulnerability curves of PAD vs. Ψ. All Ψ readings were taken using a PMS pressure chamber (Model 1505D). Contact adhesive was used to seal the cuts where leaves were excised for Ψ readings to prevent artificial embolisms. From these vulnerability curves, the point that is usually taken to compare between different species and treatments is the water potential leading to 50% reduction in PAD (P_{50}) (Pereira et al., 2020; Trabi et al., 2021).

Hydraulic conductance (*K*):

All plant material used in the hydraulic conductance measurements was collected between October 2022 and December 2022. Plant shoots were cut near the base of the shoot and immediately recut underwater roughly 5 cm from the base of the stem. The shoots were then bagged in clear plastic bags with their base submerged in filtered water. The shoots were then transported to the labs and kept in a 4 °C cold room (for up to 7 days) until the hydraulic conductance measurement could be conducted. A single leaf was cut off the remaining plant material and placed into a plastic bag with a damp paper towel for Ψ measurements. The Ψ samples were placed into a cooler box with ice bricks for transport back to the lab and were immediately measured upon return to the lab. All samples were collected between 9:30 AM and 3:00 PM on days with no rain.

Hydraulic conductance (*K*) measurements were made using the method proposed by Kolb et al. (1996). This method involves using a vacuum pump to pull a KCl solution of 0.01 M through the stem section from a balance. Before hydraulic conductance measurements, shoots had to be recut at the base and defoliated while submerged in a KCl solution using a fresh razor blade. Stems were connected via PVC tubing to a 0.01 M KCl filled reservoir on a balance (BEL Engineering MARK 205A with a resolution of 0.0001 g) while keeping the defoliated shoot submerged in KCl. Once connected to the reservoir, stems were placed inside a hard-walled chamber and sealed with a rubber bung. The opposite end of this chamber was connected to a vacuum pump and a mercury manometer. The flow rate from the balance into the stem was recorded every 5 seconds until a stable rate had been achieved. Using a series of pressures below atmospheric (10 kPa, 20 kPa, and 30 kPa), stem conductance was calculated from the regression of flow rate into the leaf against the pressure it was measured at ($kg.s^{-1}.MPa^{-1}$). The cross-sectional area at the base of the stem was measured to present conductance values per unit stem area ($kg.s^{-1}.MPa^{-1}.m^{-2}$).

Statistical analysis:

The water potential of 50% xylem embolism (P_{50}) was compared between encroachment status and CO_2 treatment using a mixed effects model comparison with encroachment status and CO_2 treatment as fixed effects and sampling date as a random factor. Sidak post-hoc tests were then performed to test for significance between each individual treatment. To test for species effects within each encroachment status, a mixed effects model comparison with species and CO_2 treatment as fixed effects and sampling date as a random factor was run for each encroachment status. There were no grass competition treatments for P_{50} measurements. Hydraulic conductance (*K*) was compared between different encroachment status, CO_2 treatment, drought treatment, and grass competition using a mixed effects model comparison with encroachment status, CO_2 treatment, drought treatment, and grass competition as fixed effects and chamber number as a random factor. To test for species effects within each encroachment status, a mixed effects model comparison with species, CO_2 treatment, drought treatment, and grass competition as fixed effects and chamber number as a random factor was run for each encroachment status. Shapiro-Wilk tests were conducted on all the data to test for normality. Due to the number of interacting factors and the limited sample size, no *post-hoc* tested were performed for measurements of *K*. The statistical analysis was conducted in R version 4.0.5 (R Development Core Team, 2023). All mixed effects model comparisons were made using lme4 R package (Bates, 2010).

Results:

Both encroachment status (χ^2 = 28.48, *df* = 1, *P* = <0.005) and CO_2 treatments (χ^2 = 16.87, *df* = 1, *P* = <0.005) had significant effects on the xylem vulnerability of the species tested. Sidak *post hoc* tests revealed that encroaching species under *e*CO_2 were statistically different from all other groups (Figure 2). At both CO_2 concentrations, encroaching species exhibited lower resistance to embolism when compared with non-encroaching species (Figure 2). Under *a*CO_2, the encroaching species reached 50% xylem embolism at -3.55 MPa ± 0.31 compared to non-encroaching species at -3.85 MPa ± 0.36. Under *e*CO_2, encroaching species reached 50% xylem embolism at -3.09 MPa ± 0.16 compared to non-encroaching species at -3.57 MPa ± 0.25. There was no significant difference between species within each encroachment status (Figure 3). For non-encroachers, there were no significant effects on P50 from either species (χ^2 = 1.36, *df* = 1, *P* = 0.243) or CO_2 treatment (χ^2 = 1.12, *df* = 1, *P* = 0.291). For encroachers, there was no significant effects from species (χ^2 = 1.60, *df* = 1, *P* = 0.449), but there was a significant CO_2 treatment effect (χ^2 = 28.07, *df* = 1, *P* = <0.005).

The water potential of 50% xylem embolism (P_{50}) was increased from -3.55 MPa ± 0.31 to -3.09 MPa ± 0.16 for encroaching species and from -3.85 MPa ± 0.36 to -3.57 MPa ± 0.25 for non-encroaching species under *e*CO_2 (Figure 3). All species began embolising once the shoot Ψ declined to -2 MPa. Species grown under *e*CO_2 reached their maximum rate of embolisms at -5 MPa, and species grown under *a*CO_2 reached their maximum rate of embolisms at -6 MPa (Figure 4).

Hydraulic conductance (*K*) was significantly affected by encroachment status (χ^2 = 32.36, *df* = 1, *P* = <0.005), grass competition (χ^2 = 67.20, *df* = 1, *P* = <0.005), and watering treatment (χ^2 = 86.13, *df* = 2, *P* = <0.005), but not by CO_2 treatment (χ^2 = 2.09, *df* = 1, *P* = 0.148). All well-watered treatments showed encroachers with a significantly higher *K* than non-encroachers (Figure 5). *e*CO_2 increased *K* for encroaching species under well-watered competition treatments and decreased *K* for encroaching species under droughted competition treatments (Figure 5). *e*CO_2 did not significantly change *K* for any of the other treatments. Within the non-encroachers, there were significant effects of species (χ^2 = 9.61, *df* = 1, *P* = <0.005), drought treatment (χ^2 = 13.29, *df* = 1, *P* = <0.005), and grass competition (χ^2 = 6.30, *df* = 1, *P* = 0.012), but not CO_2 treatment (χ^2 = 1.37, *df* = 1, *P* = 0.242). Within the encroachers, there were significant effects of drought treatment (χ^2 = 24.44, *df* = 2, *P* = <0.005) and grass competition (χ^2 = 21.96, *df* = 1, *P* = <0.005), but not species (χ^2 = 2.94, *df* = 1, *P* = 0.086) or CO_2 treatment (χ^2 = 1.73, *df* = 1, *P* = 0.189).

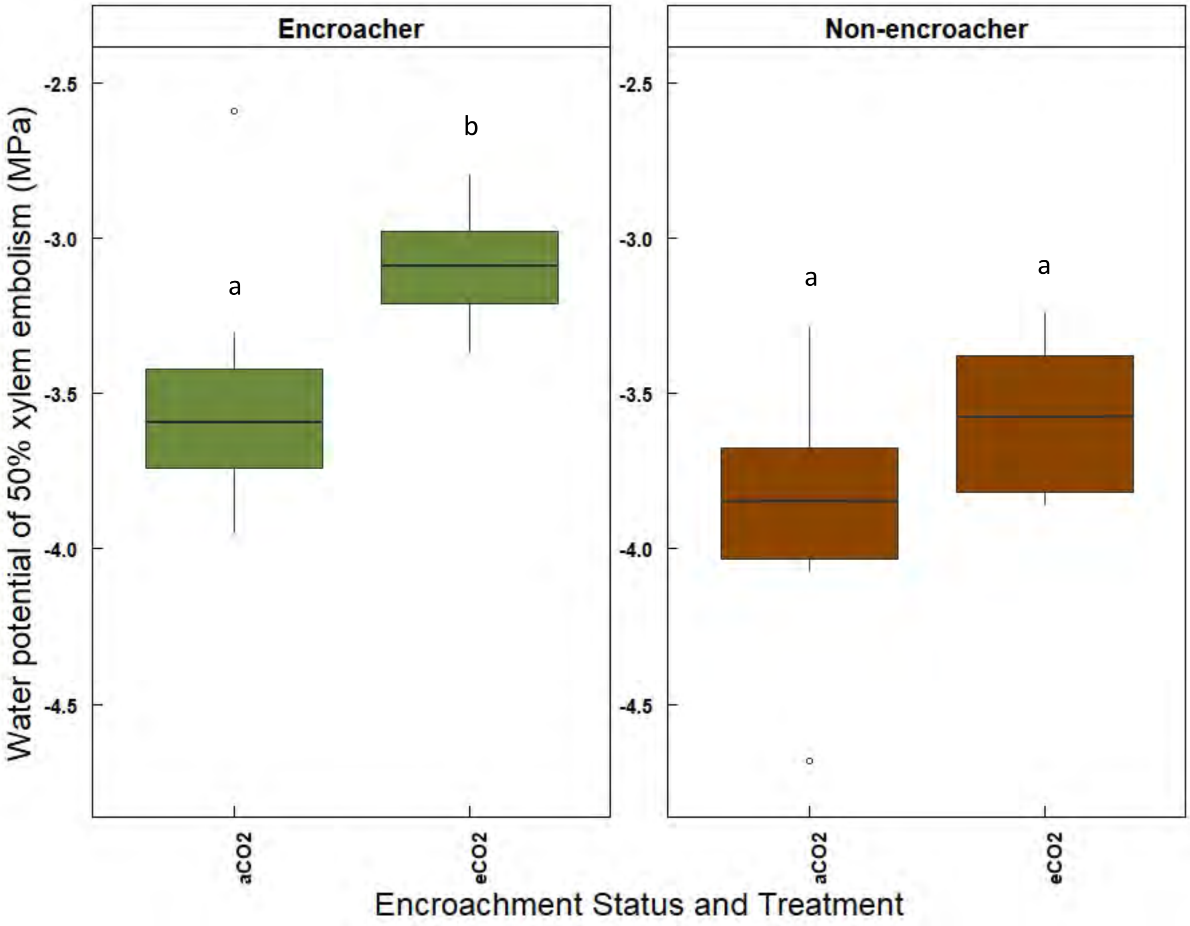

Figure 2: Boxplots showing the water potential of 50% xylem embolism for encroaching (ENC) and non-encroaching (NON) species at ambient and elevated CO_2 concentrations. Encroaching species are *Vachellia karroo*, *V. sieberiana*, and *V. tortillis*. Non-encroaching species are *V. robusta* and *Senegalia burkei*. The letters on the plot represent the results of the Sidak post hoc test. Groups sharing the same letter are not significantly different, while those with different letters are significantly different at a significance level of $P < 0.05$. N = 12.

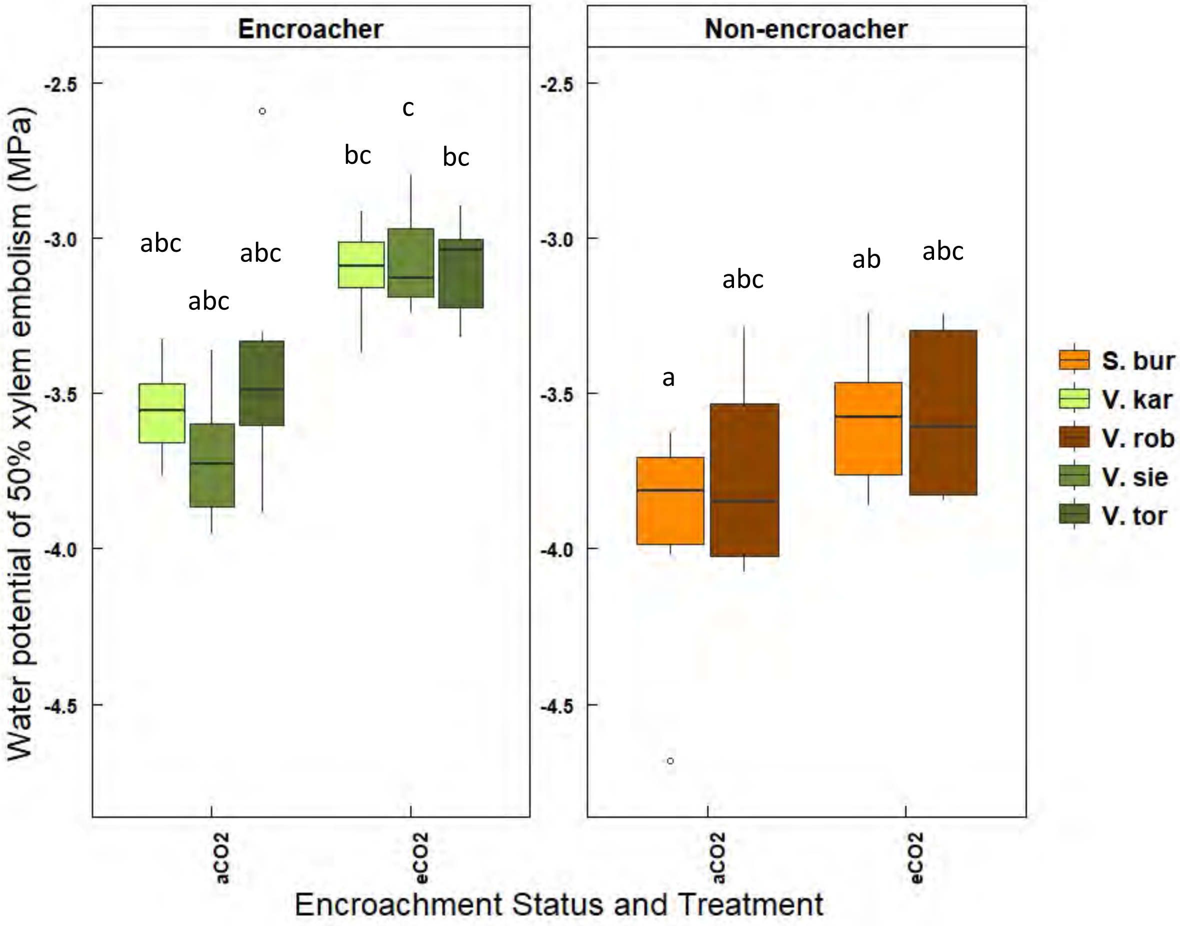

Figure 3: Boxplots showing the water potential of 50% xylem embolism for encroaching (ENC) and non-encroaching (NON) species at ambient and elevated CO_2 concentrations. This is the same data as Figure 2, except with the species split. Encroaching species are *Vachellia karroo*, *V. sieberiana*, and *V. tortillis*. Non-encroaching species are *V. robusta* and *Senegalia burkei*. The letters on the plot represent the results of the Sidak post hoc test. Groups sharing the same letter are not significantly different, while those with different letters are significantly different at a significance level of $P < 0.05$. N = 6.

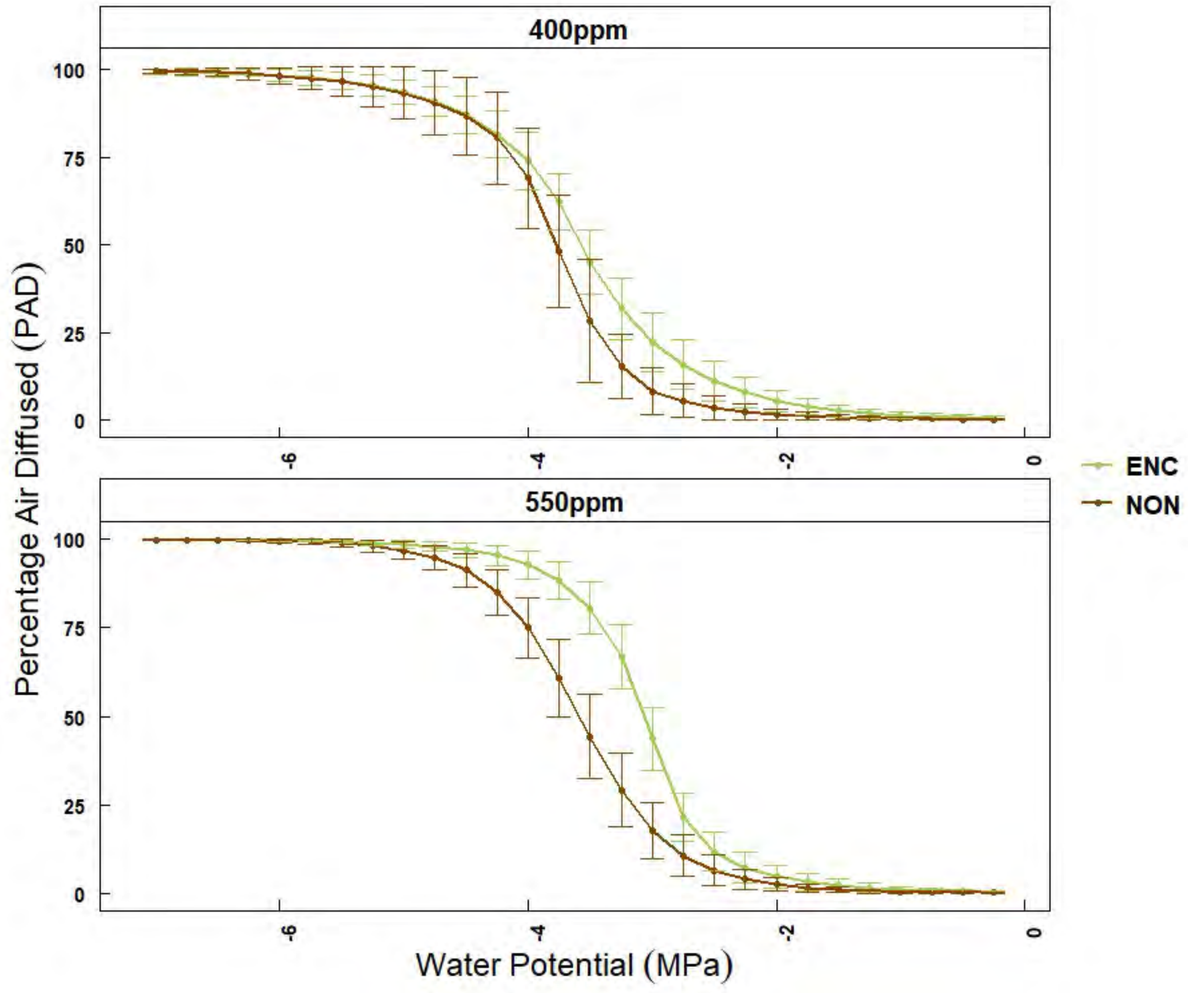

Figure 4: Xylem vulnerability curves for encroaching (ENC) and non-encroaching (NON) species at ambient and elevated CO_2 concentrations. Encroaching species are *Vachellia karroo*, *V. sieberiana*, and *V. tortillis*. Non-encroaching species are *V. robusta* and *Senegalia burkei*. Means ± CI is shown. N = 12.

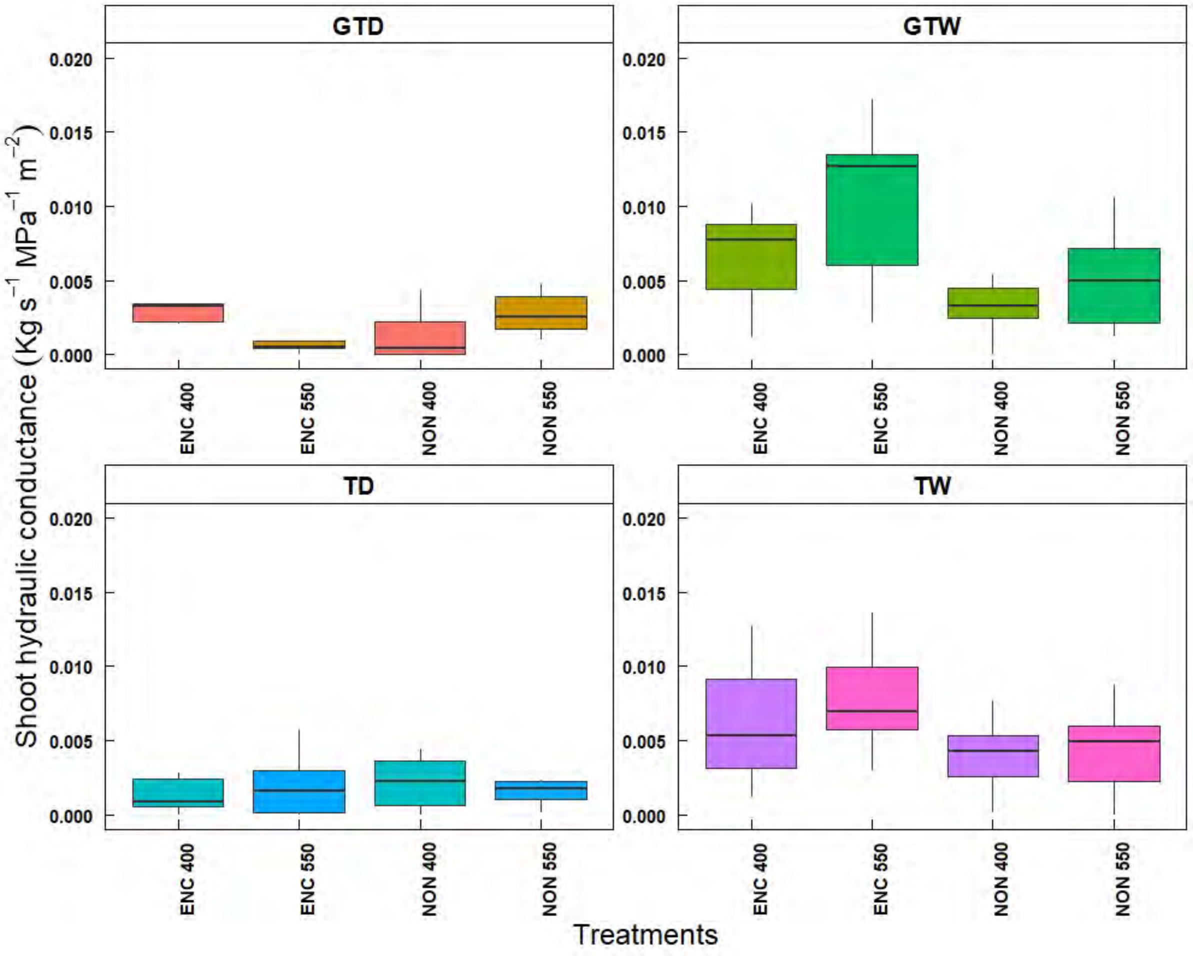

Figure 5: Boxplots showing native hydraulic conductance (*K*) for encroaching (ENC) and non-encroaching (NON) species under different drought and competition treatments at ambient and elevated CO_2 concentrations. N = 6.

Discussion:

While there have been multiple studies trying to understand the underlying mechanisms behind the phenomenon of woody plant encroachment (e.g., O'Connor et al., 2014; Skowno et al., 2017; Venter et al., 2018; Raubenheimer & Ripley, 2022), few have examined how the fundamental water strategies in the seedling stage respond to changing climate (Cory et al., 2022; Uni et al., 2023). The seedling stage is a crucial demographic bottleneck to the establishment, and therefore the distribution and density, of woody savanna species (Skarpe, 1992; Bond, 2008). Hence, testing physiological differences between species at this stage is crucial for identifying underlying physiological mechanisms leading to woody bush encroachment.

Using a selection of widespread savanna species (*V. karroo*, *V. Robusta*, *V. Sieberiana*, *V. tortillis*, *S. cafra,* and *S. burkei*), the effects of future CO_2 concentrations on fundamental water use strategies of the seedlings were tested. The results show that encroaching species (*V. karroo*, *V. sieberiana* and *V. tortillis*) are stronger anisohydric "water spenders" particularly under growth at *e*CO_2, rapidly using water during pulses of moisture availability at the cost of a higher risk of embolisms. This anisohydric behaviour was also observed by Cory et al. (2022), who found that first-year seedling establishment for savanna species was dependent on high carbon uptake during pulses of water availability. This study found that in all well-watered treatments, encroaching species had a higher hydraulic conductance (*K*) than non-encroaching species. In the presence of *e*CO_2, encroaching species with grass competition treatments increased *K* under well-watered conditions and decreased *K* under drought conditions. This is potentially due to increased water availability in pots with grass competition, as grasses can slow the rate of soil desiccation despite accessing soil moisture themselves (Blight, 2005; L. Wang et al., 2008). Plants without grass competition did not see significant variation in *K*. This indicates that the presence of competition increases the anisohydric properties of the seedlings tested, as they compete for water. The xylem vulnerability experiment showed that encroaching species' water systems are much more vulnerable to drought under *e*CO_2, suggesting the water transport systems had failed in the GTD treatment, leading to the observed decline in *K* under *e*CO_2. Having a high *K* often leads to a trade off with drought resistance (De Guzman et al., 2016). More conductive plants are often able to maintain higher rates of photosynthesis, and hence growth, by transporting more water and soil nutrients up until the point of drought, where their xylem begin to embolise. Conversely, plants with a lower *K* are not able to maintain as high of a rate of photosynthesis under well-watered conditions but suffer significantly less of a reduction in function when drought occurs. The results here show that the hydraulics of encroaching species are set to maximise the plant's gas exchange to allow for rapid growth during pulses of water availability, which helps it escape the seedling stage (Brodribb, 2009).

Xylem vulnerability for the savanna species tested was significantly affected by both the encroachment status and the CO_2 concentration. All the encroaching species demonstrated a uniformly lower resistance to embolism (P_{50}) under both *a*CO_2 and *e*CO_2. All species showed an increase in the stem Ψ at which the maximum embolism was reached under *e*CO_2. These results are consistent with the experiment run by Nackley et al. (2018), which found that *e*CO_2 significantly benefitted the growth and establishment of *Vachellia karroo* seedlings, except in the case of prolonged drought. A recent paper by Swemmer (2020) revealed that mortality was higher for tall individuals for most species, but not for any of the *Vachellia* or *Senegalia* species studied. Based on these results, it is likely that the anisohydric properties present in the seedling stage are present when the plant is fully grown. More research is needed to fully test this.

By experimentally testing differences in water use strategies between common encroaching and non-encroaching species in potential future drought and CO_2 conditions, this study provides valuable insights into how encroacher establishment may be affected by changing climate. All encroaching and non-encroaching species showed water use strategies indicative of anisohydric "water-spenders" (Cory et al., 2022), however the vulnerability to embolism and rates of water use were different. It is important to note here that although we found characteristics indicative of "water spenders", without the stomatal control part of the measurement we were not able to measure the straight rates of water use. Nevertheless, these results show that encroachers are better at taking advantage of water pulses, particularly under *e*CO_2 and grass competition treatments. This comes at the cost of a higher vulnerability to embolisms during drought, leading to vast reductions in conductance when exposed to heavy water stress. Thus, encroaching species in the seedling stage can be expected to increase their competition with "water-spender" grasses (Williams et al., 1998) and non-encroaching species in most predicted future scenarios, except under intense water stress. The research presented here highlights hydraulic mechanisms contributing to the observed increase in woody bush encroachment, as well as providing important insights into why certain savanna species are encroachers and others are not. This work contributes to the existing understanding of the complex mechanisms and interactions behind woody bush encroachment, as well as indicating environmental factors that may determine which landscape are most affected under potential future climates.

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Chapter 3: Water Use Implications for Photosynthesis and Photochemical Energy Dissipation in Savanna Tree Seedlings under Drought, Competition and Elevated CO_2

Abstract:

There is extensive evidence that the enrichment of atmospheric CO_2 is one of the strongest driving forces behind woody bush encroachment in savannas. C_3 plants are carbon-limited under ambient CO_2 (*a*CO_2), leading to increases in carbon assimilation and decreases in stomatal conductance under elevated CO_2 (*e*CO_2). Plants may only realise the benefits of these changes in environmental conditions when other factors such as water, light or nutrient availability are not limiting. Savanna species considered to be encroaching generally have the strategy of fast growth by maximising carbon assimilation and hydraulic conductance at the cost of reduced drought resistance. While there is extensive literature on the physiological responses of common savanna species to *e*CO_2, few extensively examined how water limitations affect this response and how it relates the hydraulic properties of the species. In areas experiencing drought stress, plant growth would control for the development of photosynthetic and hydraulic systems in a way that ensures resource allocation is balanced between these processes. Here, this research provides valuable insights into the mechanisms behind bush encroachment in the context of *e*CO_2 using results from a pot experiment at the Rhodes University Elevated CO_2 Facility. Seedlings of six encroaching or non-encroaching woody tree species were grown under full factorial treatments of *e*CO_2, competition, and drought. Photosynthetic rate (A), stomatal conductance (g_{st}) and photosystem II quantum yield (ΦPSII) were measured. It was found that encroaching species had higher rates of A and ΦPSII than non-encroachers under the well-watered treatments. Just as predicted by the hydraulic response, this advantage was lost under drought conditions, where there was no significant difference between encroachers and non-encroachers. Both encroachers and non-encroachers had an increased slope of the A to g_{st} with *e*CO_2, and under both [CO_2] encroachers had a larger slope than non-encroachers. This implies that encroaching species can assimilate more carbon for a given stomatal conductance than non-encroaching species and that *e*CO_2 enhances this effect. This research links hydraulic mechanisms contributing to the increase in bush encroachment to the physiological responses under these conditions, as well as providing important insights into the determinant factors that make a savanna species a successful encroacher.

Keywords: photosynthesis, stomatal conductance, photosystem II quantum yield, woody bush encroachment, elevated CO_2, drought

Introduction:

Future climate predictions anticipate changes in the duration, frequency, and intensity of droughts alongside the increases in atmospheric CO_2 concentration (Intergovernmental Panel on Climate Change, 2014). These changes in water availability have already been reported to affect the productivity of various terrestrial plant species (Mubarik et al., 2021; Wang et al., 2022a). This is of particular importance for encroaching savanna trees implicated in a directional increase in density at the cost of the grass layer in grassy and savanna biomes worldwide. To predict shifts in ecosystem function and demographic distributions, it is imperative to understand how physiological traits are affected by environmental conditions relating to water availability in the context of rising CO_2 (Li et al., 2020). The major response of plants to elevated CO_2 (eCO_2) in the absence of drought is through increased photosynthesis and reduced stomatal conductance, which has downstream effects on whole plant function (Long et al., 2004). Stomata show very strong responses to instantaneous changes in atmospheric [CO_2], with CO_2 levels below ambient causing stomata to open and CO_2 levels above ambient causing stomata to close (Mott et al., 2008). By reducing stomatal conductance under eCO_2, many species (particularly C_4 species) are able to maintain high rates of photosynthesis into light to moderate drought (Wang et al., 2022b). There is an intrinsic link between stomatal function and net carbon assimilation (Franks & Farquhar, 1999). At the leaf level, this relationship can be expressed through Fick's law (Eq. 1), which indicates that the parameter responsible for most of the variability of A_{net} is g_{st} (Domec et al., 2017; Voelker et al., 2016). Under more severe water limitations, however, this relationship breaks down leading to vast reductions in photosynthetic rate and increases in photoinhibition (Takahashi & Murata, 2008). Photoinhibition is the reduction in the photosynthetic capacity of a plant brought on by intense light (Kok, 1956). Plants alleviate this stress by dissipating much of the incoming light as heat or fluorescence (non-photochemical energy dissipation) (Müller et al., 2001).

C_3 plants are carbon-limited under current atmospheric [CO_2], leading to increases in carbon assimilation and decreases in stomatal conductance under eCO_2 (Long et al., 2004; Ainsworth & Long, 2005). These are some of the most studied responses to eCO_2, however, experimental data does not always match the theoretical predictions, partially due to the variability of the photosynthetic parameters with environmental conditions and partially due to the process of photosynthetic downregulation (Ainsworth & Long, 2005; Ainsworth & Rogers, 2007; Leakey et al., 2009). Photosynthetic downregulation is attributed to either a decreased investment in ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) and maximum rate of carboxylation (V_{cmax}) (Ainsworth & Long, 2005), or to a decrease in the regeneration ability of Rubisco, which decreases V_{cmax} limited by the electron transport chain (J_{max}) due to either insufficient inorganic phosphate in the chloroplast for ATP synthesis or vast reductions in the capacity of electron transport (Ainsworth & Rogers, 2007; Zhu et al., 2012). Annuals, grasses, and shrubs are most susceptible to photosynthetic downregulation, as trees generally have a larger carbon sink and see a much greater stimulation of carbon assimilation under *e*CO_2 (Ainsworth & Long, 2005).

While photosynthetic and stomatal parameters are largely subject to the prevailing environmental conditions, it is the structure and function of the water transport systems that ultimately constrain these metabolic processes (Tyree & Sperry, 1989; Brodribb, 2009). Given the economics of carbon gain vs the structural investment costs, evolution should favour the coordination of these processes (Brodribb, 2009). The water transport systems of plants constitute a significant proportion of the total biomass (Mencuccini, 2003), and even more investment in the xylem plumbing of the leaves, stems and roots is required to increase hydraulic conductance (*K*) (Tyree & Zimmerman, 2013). Plants are only able to realise the benefits of these investments when other environmental factors unrelated to water supply are limiting, such as light or nutrient availability. It can therefore be predicted that plant growth would control for the development of photosynthetic and hydraulic systems in a way that ensures resource allocation is balanced between these processes. All studies conducted on the relationship between carbon assimilation and hydraulic conductance in C_3 species show either a linear (e.g., Franks, 2006) or a saturating curve (e.g., Brodribb et al., 2007). The results from Chapter 2 (Figures 2 & 5) showed that encroaching species gained advantages to hydraulic conductance and xylem vulnerability under *e*CO_2, except in the case of high drought stress (Figures 2 & 5). There is also evidence that the growth rate of encroacher seedlings is suppressed by the presence of grass competition (Macias et al., 2014; Pillay & Ward, 2021). Additionally, any benefits from the fertilisation effect of *e*CO_2 are largely negated by presence grass competition (Raubenheimer & Ripley, 2022). This adds further evidence to the theory that encroachers are only able to realise the benefits of *e*CO_2 when the availability of resources such as water, light and nutrients are not limited or under competitive pressure (Reich et al., 2014; Raubenheimer & Ripley, 2022).

The aim of this study is to compare photosynthetic parameter responses of encroaching versus non-encroaching savanna seedlings grown at *e*CO_2 and under drought versus well-watered conditions, with and without grass competition. Based on this, encroaching species should see a greater increase in photosynthetic rate and photosystem II quantum yield with *e*CO_2 than non-encroaching species in well-watered treatments. This should be accompanied by reductions in stomatal conductance. This relationship should be heavily affected by drought and grass competition. Non-encroaching species should maintain higher rates of photosynthesis and photochemical energy dissipation with heavy water stress and grass competition when compared to encroaching species.

Methods:

OTC experiment:

To test for the effect of [CO_2] and drought on the gas exchange parameters, four C_3 species (*Vachellia karroo, V. robusta, V. seiberiana,* and *Senegalia cafra*) were grown from seed. Encroachment status was determined by the work conducted by Turpie et al. (2019), which is based off the life-history of the plant. Encroachers are *V. karroo* and *V. sieberiana*. Non-encroachers are *V. robusta* and S. cafra. The plants were grown at either elevated CO_2 concentrations (*e*[CO_2]) (550ppm) or ambient CO_2 concentrations (*a*[CO_2]) (400ppm) for the duration of their lives. These CO_2 levels were maintained by open top chambers (OTC) at the Rhodes University Elevated CO_2 facility (see Chapter 2). Plant specimens ranged in age from 10 to 13 months. The plants were subjected to drought and grass competition treatments. Treatment codes are GTD (grass competition + drought), GTW (grass competition + well-watered), TD (no competition + drought), and TW (no competition + well-watered). Plants that were under the droughted treatment only received water under natural rainfall events, while well-watered treatments were watered frequently. Plants under the grass competition treatment were planted with *Themeda triandra*, a common savanna C_4 species. Grasses were established first for a full growing season before seedling were added. *T. triandra* specimens were grown from tillers collected at Burnt Kraal, Makhanda, Eastern Cape (33°16’40” S 26°29’20” E). All measurements on this plant material were made from October 2022 – December 2022.

Photosynthetic parameters:

Photosynthesis (*A*), stomatal conductance (g_{st}), and photosystem II quantum yield (ΦPSII) measurements were all made on fresh green leaves immediately before the plant was cut for hydraulic conductance and leaf water potential measurements. All measurements were taken between 9:00 AM and 3:00 PM. *A* and g_{st} measurements were made using an infrared gas analyzer (Li-Cor 6800, Li-Cor Biosciences, USA). Leaf-to-air vapour pressure deficit was kept below 1.5 kPa, block temperature was set at a constant of 30°C and PAR was set at a constant 1500 μmol m^2 s^{-1}. Instantaneous measurements *A* and g_{st} were made at both *a*CO_2 (400 ppm) and *e*CO_2 (550 ppm). Chlorophyll fluorescence of photosystem II (PSII) was used to determine photosystem II quantum yield using a PhotosynQ MultispeQ V2.0 (PhotosynQ Inc., USA). All fluorescence measurements were made on the new, green leaves at the 4th node down from the apical bud and in the CO_2 concentration at which the plant was grown. Leaves were angled to be in full sunlight before measurements were conducted. Due to weather variability during the experiment, measurements could not always be taken at full light saturation. Leaflets and blades of grass that do not adequately cover the cuvette as a single surface were merged with neighbouring leaves and measured with a black leaf mask for the PhotosynQ MultispeQ (PhotosynQ, 2024).

Statistical Analysis:

Stomatal conductance (g_{st}) and photosynthesis (A) were compared between CO_2 treatment, encroachment status, watering, and competition stress using a mixed effects model comparison with CO_2 treatment, encroachment status, watering and competition treatments, and leaf water potential (Ψ) as fixed effects and chamber number as a random factor. In the comparison between A and Ψ, both variables were log-transformed. To test for species effects within each encroachment status for g_{st} and A, mixed effects model comparisons with species, CO_2 treatment, drought treatment, grass competition and Ψ as fixed effects and chamber number as a random factor were run for each encroachment status. Photosystem II quantum yield (ΦPSII) was compared between encroachers and non-encroachers for treatments of drought, competition, and [CO_2] using an ANOVA. ΦPSII was also compared between CO_2 treatment, encroachment status, watering, and competition stress at different levels of light intensity using a mixed effects model comparison with CO_2 treatment, encroachment status, watering, competition stress, and photosynthetically active radiation (PAR) as fixed effects and chamber number as a random factor. To test for species effects within each encroachment status for ΦPSII, a mixed effects model comparison with species, CO_2 treatment, drought treatment, grass competition and PAR as fixed effects and chamber number as a random factor was run for each encroachment status. In order to investigate photosynthetic downregulation, A measured at 550 ppm (A_{550}) was compared to A measured at 400 ppm (A_{400}) using a mixed effects linear model comparison with encroacher status and CO_2 concentration as fixed effects and chamber number as a random factor. To test assumptions of normality in the data, quantile-quantile (Q-Q) plots were employed, and the data was log transformed where necessary. Due to the number of interacting factors and the limited sample size, no *post-hoc* tested were performed. The statistical analysis was conducted in R version 4.0.5 (R Development Core Team, 2023). All mixed effects model comparisons were made using lme4 R package (Bates, 2010).

Results:

The statistical analysis of stomatal conductance (g_{st}) showed that g_{st} was significantly affected by the leaf water potential (Ψ) (χ^2 = 17.84, *df* = 1, *P* = <0.005), the water treatment (χ^2 = 13.34, *df* = 2, *P* = <0.005), and the competition treatment (χ^2 = 8.22, *df* = 1, *P* = <0.005) (Figure 6). There was no significant effect by the CO_2 concentration (χ^2 = 1.08, *df* = 1, *P* = 0.299) or the encroachment status (χ^2 = 0.0005, *df* = 1, *P* = 0.983) on g_{st}. There was no significant difference between the g_{st} of species classified as encroachers (χ^2 = 3.44, *df* = 2, *P* = 0.179) or non-encroachers (χ^2 = 3.81, *df* = 1, *P* = 0.051). It was found that photosynthesis (*A*) was significantly affected by Ψ (χ^2 = 14.98, *df* = 1, *P* = <0.005) and competition treatments (χ^2 = 5.19, *df* = 1, *P* = 0.023), but not by CO_2 concentration (χ^2 = 0.67, *df* = 1, *P* = 0.414), water treatment (χ^2 = 4.07, *df* = 2, *P* = 0.131), or encroachment status (χ^2 = 0.76, *df* = 1, *P* = 0.383) (Figure 7). All treatments saw a decrease in *A* as Ψ declined, except for the encroachers under grass competition treatments, which had a positive slope (Figure 7). There was no significant difference between the *A* of species classified as encroachers (χ^2 = 1.22, *df* = 2, *P* = 0.544) or non-encroachers (χ^2 = 1.02, *df* = 1, *P* = 0.311).

When directly comparing *A* to g_{st}, there was a significant relationship between the two variables (χ^2 = 235.24, *df* = 1, *P* = <0.005). The encroachment status (χ^2 = 16.23, *df* = 1, *P* = <0.005) and CO_2 treatment (χ^2 = 5.83, *df* = 1, *P* = 0.016) were significant predictors in how *A* responded to g_{st}. Both encroachers and non-encroachers had an increased slope of the A to g_{st} with *e*CO_2, and under both [CO_2] encroachers had a larger slope than non-encroachers (Figure 8). This implies that encroaching species can assimilate more carbon for a given stomatal conductance than non-encroaching species and that *e*CO_2 enhances this effect.

In order to investigate photosynthetic downregulation, *A* measured at 550 ppm (A_{550}) was compared to *A* measured at 400 ppm (A_{400}). There was a significant relationship between A_{550} and A_{400} (χ^2 = 2004,63, *df* = 1, *P* = <0.005) and that CO_2 concentration had a significant effect on this relationship (χ^2 = 4.11, *df* = 1, *P* = 0.043). The encroachment status had no significant effect on A_{550} (χ^2 = 1.30, *df* = 1, *P* = 0.254), however, the interaction term between A_{400}, CO_2 concentration, and encroachment status was significant (χ^2 = 4.34, *df* = 1, *P* = 0.037). The slopes of the graphs for non-encroaching species (Figure 9) show a change from y = -1.2x + 1.8 at *a*CO_2 (R^2 = 0.87, *P* = <0.005) to y = -1.1x + 2 at *e*CO_2 (R^2 = 0.75, *P* = <0.005), implying photosynthetic downregulation. The slopes for encroaching species, however, show a change from y = -1.2x + 2.3 at *a*CO_2 (R^2 = 0.82, *P* = <0.005) to y = -1.3x + 0.81 at *e*CO_2 (R^2 = 0.86, *P* = <0.005), implying photosynthetic upregulation.

The range of photosystem II quantum yield (ΦPSII) was non-significant for encroaching and non-encroaching species under all treatments (Figure 10). ΦPSII was significantly affected by photosynthetically active radiation (PAR) (χ^2 = 315.30, *df* = 1, *P* = <0.005), the interaction of PAR and encroachment status (χ^2 = 13.03, *df* = 1, *P* = <0.005) and the interaction between encroachment status and CO_2 concentration (χ^2 = 4.58, *df* = 1, *P* = 0.032) (Figure 11). The main effects of CO_2 concentration, encroachment status, watering and competition treatments were not found to be significant predictors of ΦPSII (all *P* = >0.05). It is important to note that these results were obtained from log-transformed ΦPSII and PAR values to normalise the data distribution.

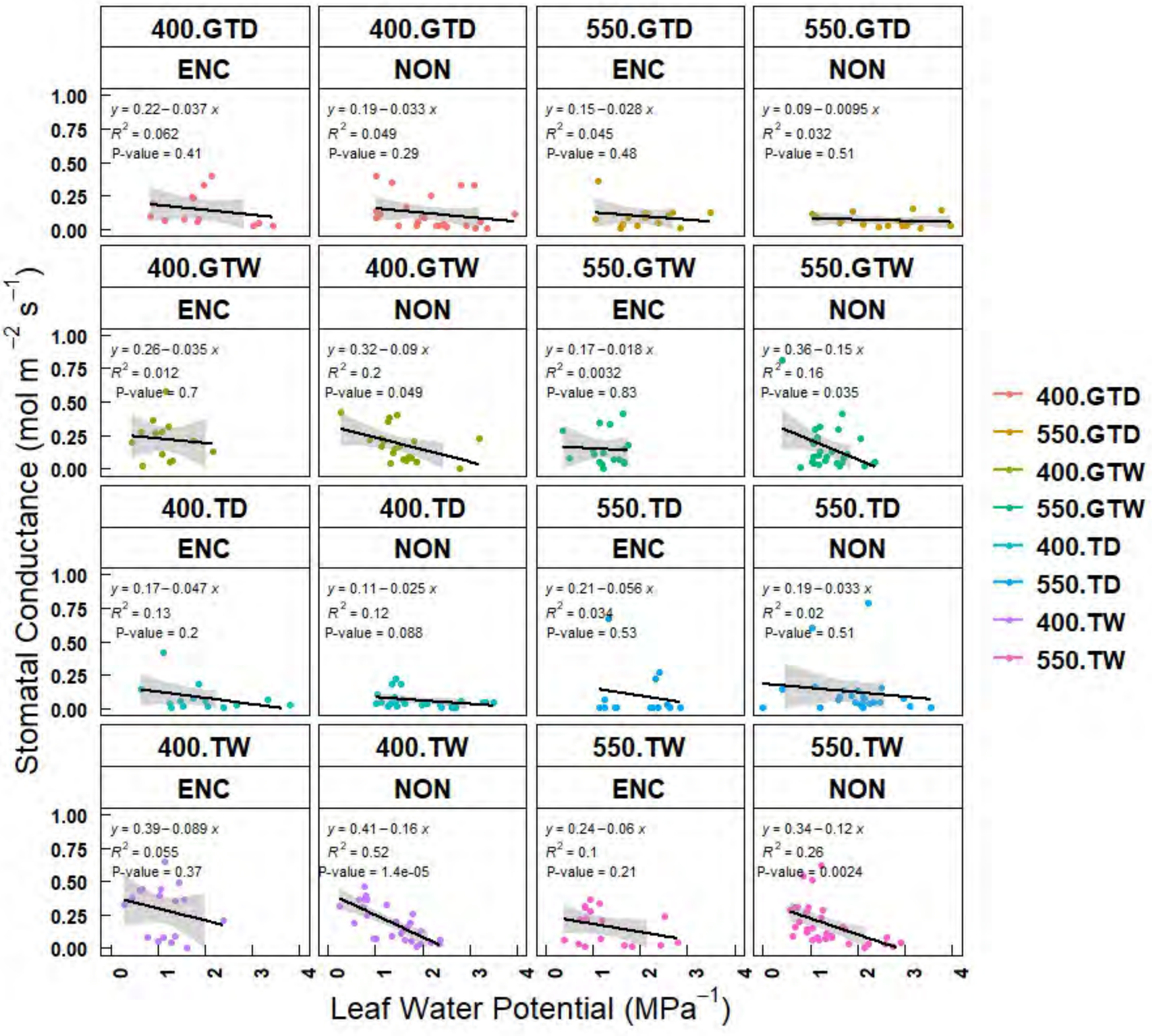

Figure 6: The relationship between stomatal conductance (g_{st}) and leaf water potential (LWP) for encroaching (ENC) and non-encroaching (NON) species under different drought and competition treatments at ambient and elevated CO_2 concentrations. Treatments are ambient (400) and elevated (550) CO_2 concentrations, grass (GT) and no-grass (T) competition, as well as drought (D) and well-watered (W) watering schedules. N = 10.

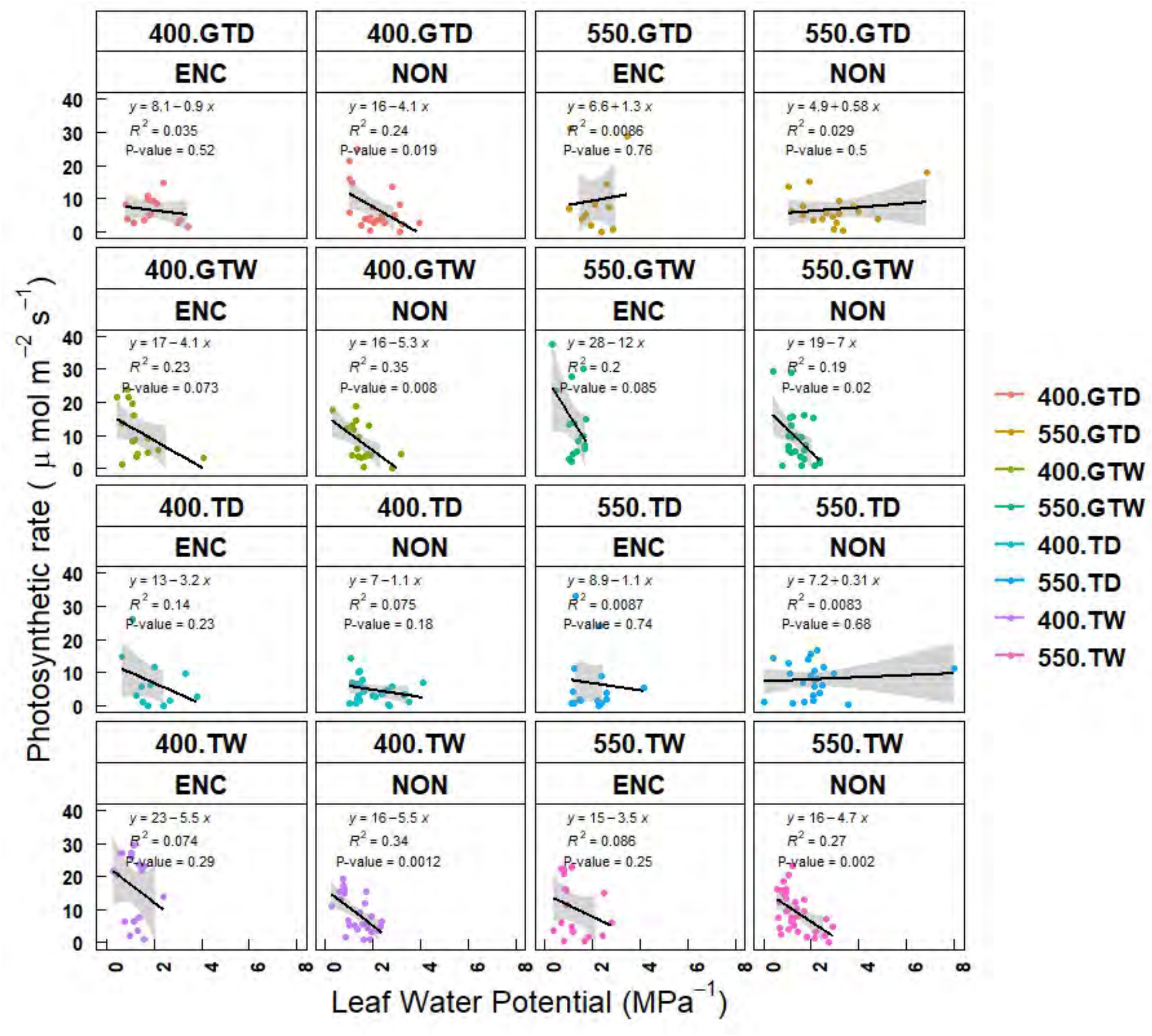

Figure 7: The relationship between photosynthesis (*A*) and leaf water potential (LWP) for encroaching (ENC) and non-encroaching (NON) species under different drought and competition treatments at ambient and elevated CO_2 concentrations. Treatments are ambient (400) and elevated (550) CO_2 concentrations, grass (GT) and no-grass (T) competition, as well as drought (D) and well-watered (W) watering schedules. N = 10.

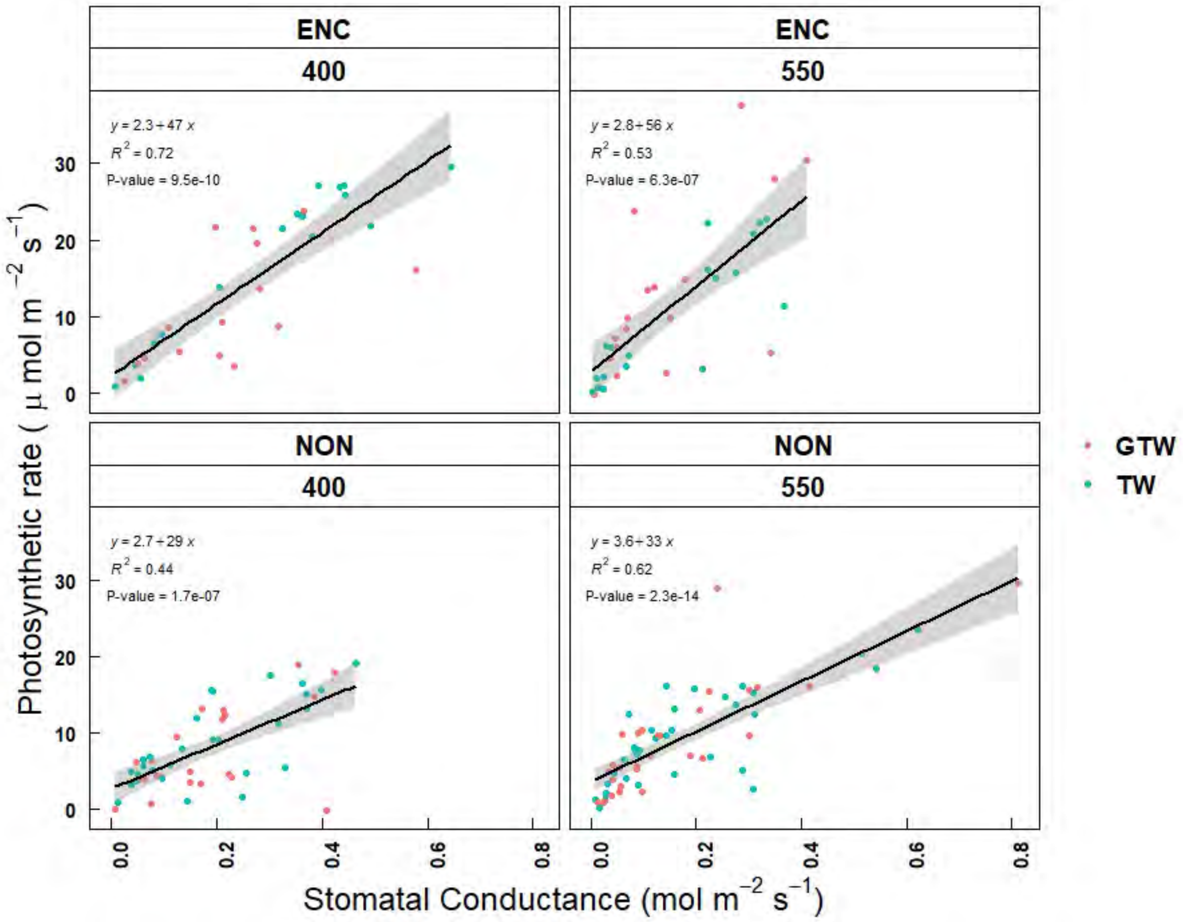

Figure 8: The relationship between photosynthesis (*A*) and stomatal conductance (g_{st}) for encroaching (ENC) and non-encroaching (NON) species under well-watered treatments at ambient (400) and elevated (550) CO_2 concentrations. Competition treatments are grass (GT) and no-grass (T) competition. Measurements of *A* and g_{st} were made at the [CO_2] under which the plant was grown. N = 10.

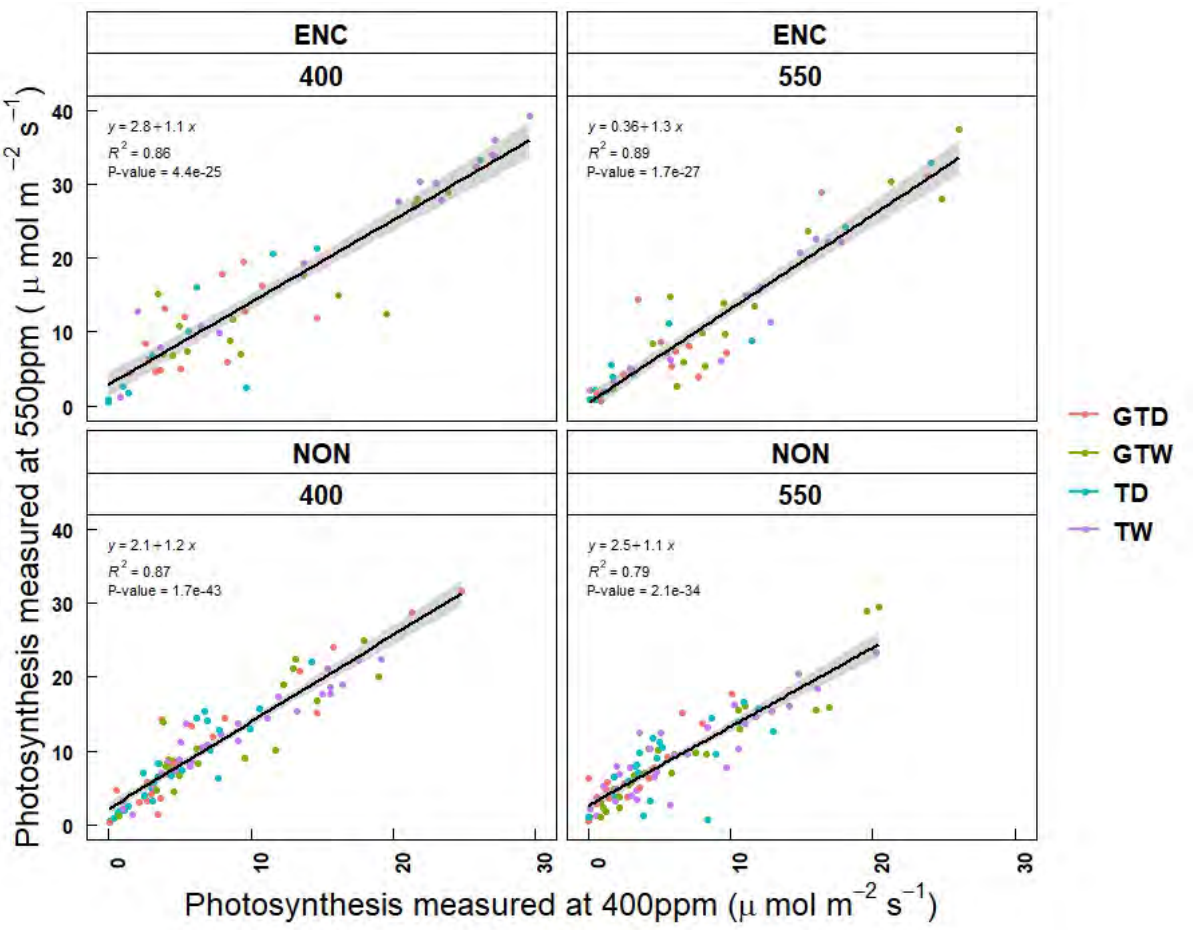

Figure 9: The relationship between photosynthesis measured at 550 ppm (A_{550}) and photosynthesis measured at 400 ppm (A_{400}) for encroaching (ENC) and non-encroaching (NON) species under treatments of grass (GT) and no-grass (T) competition, as well as drought (D) and well-watered (W) watering schedules at ambient (400) and elevated (550) CO_2 concentrations. N = 10.

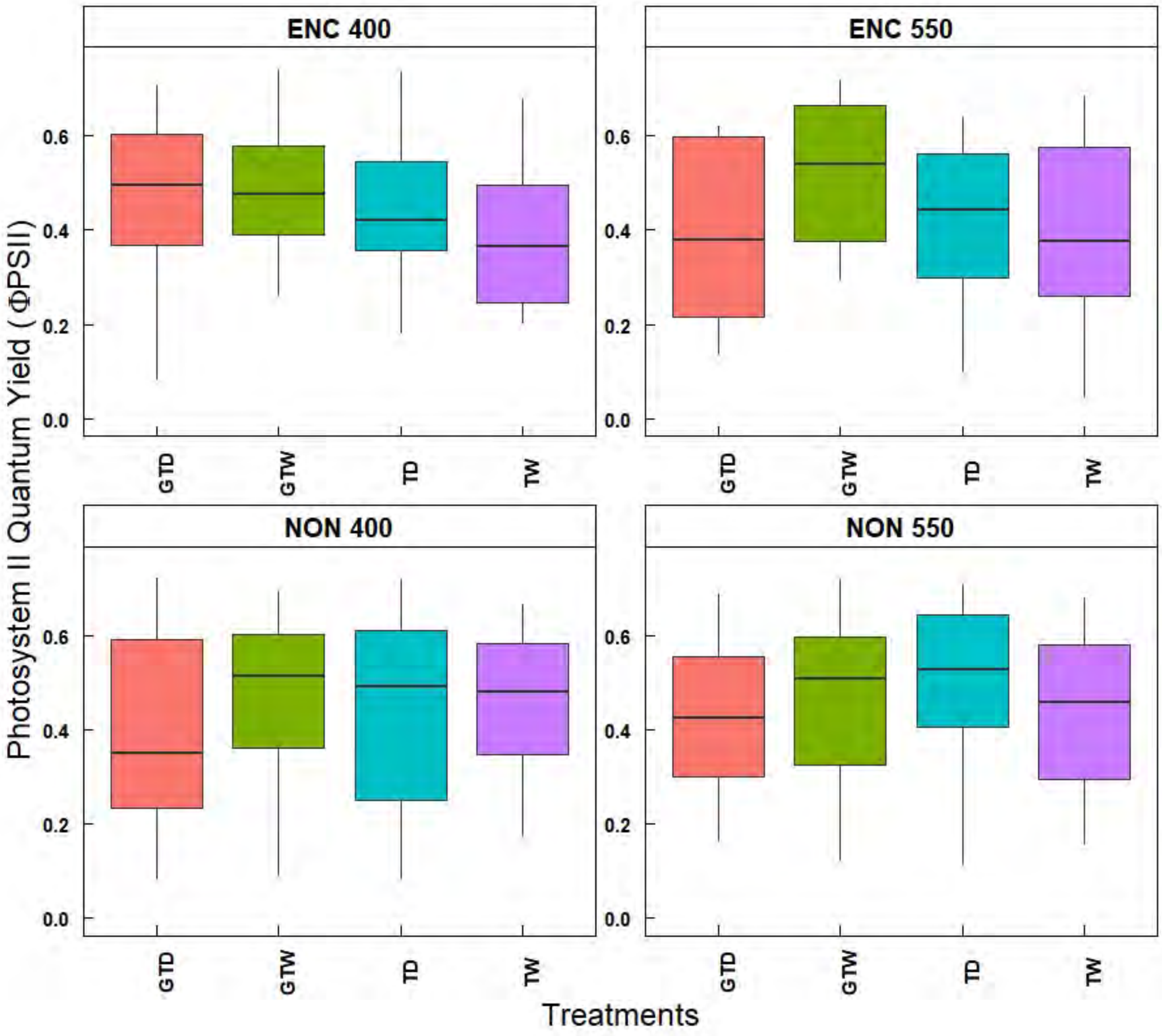

Figure 10: Boxplots showing photosystem II quantum yield (ΦPSII) for encroaching (ENC) and non-encroaching (NON) species under treatments of grass (GT) and no-grass (T) competition, as well as drought (D) and well-watered (W) watering schedules at ambient (400) and elevated (550) CO_2 concentrations. N = 10.

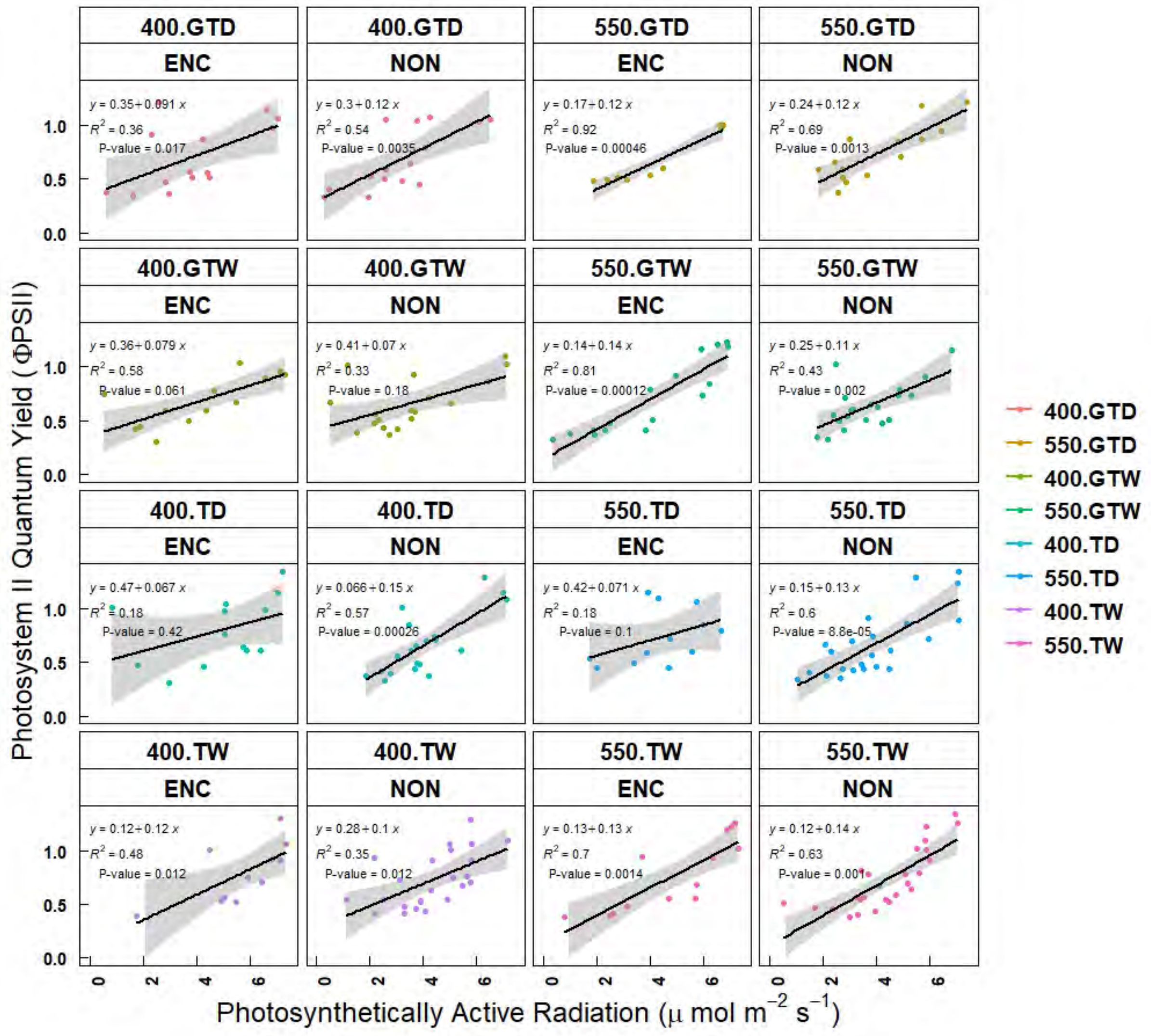

Figure 11: The relationship between photosystem II quantum yield (ΦPSII) and photosynthetically active radiation (PAR) for encroaching (ENC) and non-encroaching (NON) species under different drought and competition treatments at ambient and elevated CO_2 concentrations. All data has been log transformed. Treatments are ambient (400) and elevated (550) CO_2 concentrations, grass (GT) and no-grass (T) competition, as well as drought (D) and well-watered (W) watering schedules. N = 10.

Discussion:

Over the last 100 years, there has been a directional shift towards the increasing establishment of woody species in savannas alongside the increases in atmospheric CO_2 (Wiegand et al., 2006; Bond, 2008). The seedling stage is crucial for the establishment of savanna tree species (Skarpe, 1992; Bond, 2008), hence species that are better able to take advantage of changing climatic conditions in this stage can be expected to increasingly escape this demographic bottleneck as CO_2 levels rise. Using a selection of widespread savanna species (*V. karroo, V. robusta, V. seiberiana,* and *S. cafra*), the way photosynthetic parameters are affected by projected future [CO_2] and relative levels of water stress was investigated. The results from Chapter 2 (Figures 2 & 5) show that there is a difference in water use strategies between encroaching and non-encroaching species, with encroachers displaying stronger anisohydric characteristics than non-encroachers (Cory et al., 2022). The results here mirror that, with encroachers grown in well-watered conditions being able to assimilate more carbon for a given stomatal conductance than non-encroaching species, even at *a*CO_2. It was also found that encroaching species grown under well-watered conditions had slightly increased rates of A and g_{st} as Ψ decreased. Encroachers were also able to absorb more light energy in photosystem II for a given PAR The presence of grass competition suppressed A and g_{st} for both encroachers and non-encroachers. This result is consistent with the work done by Ketter & Holdo (2018), which demonstrated strong support for competitive effects of grasses on two common savanna species (*Senegalia nigrescens* and *Colophospermum mopane*) and the work done by Campbell & Holdo (2017), which found that C assimilation was negatively related to interspecific variation in tree response to grass competition. Well-watered plants in the *e*CO_2 treatment saw the sharpest declines in A with Ψ, indicating that the presence of eCO_2 increases the competition for water and resources when other factors are not limiting.

Non-encroaching species showed evidence of potential photosynthetic downregulation with *e*CO_2, while encroaching species exhibited the opposite; evidence of photosynthetic upregulation with *e*CO_2. While photosynthetic downregulation has been observed in multiple studies (e.g., Flexas et al., 2004; Ronchi et al., 2006; Nebauer et al., 2011), photosynthetic upregulation is much less frequent and generally only occurs when other nutrients, particularly N, are abundant (Bloom et al., 2010; Vicente et al., 2017). While the data implies evidence of photosynthetic acclimation to growth [CO_2], this response cannot be fully confirmed without measurements of J_{max} and V_{cmax} (Ainsworth & Rogers, 2007; Zhu et al., 2012). Nevertheless, these findings suggest that encroaching species may have increased carbon sink capacities over non-encroaching species (Ainsworth & Long, 2005), which could explain the observed differences in photosynthetic rate. More research needs to be done to confirm this, however.

While photosynthetic and stomatal parameters are largely subject to the prevailing environmental conditions and biochemical pathways, it is the structure and function of the water transport systems that ultimately constrain these metabolic processes (Tyree & Sperry, 1989; Brodribb, 2009). In the well-watered treatments, where environmental conditions should not have been constraining, there were significant improvements to both the photosynthetic rates and ratios of photochemical to non-photochemical energy dissipation for encroachers over non-encroachers. This result is just as predicted by the hydraulic response observed in Chapter 2 (Figure 5), which shows that encroachers have higher hydraulic conductance than non-encroachers when grown in well-watered conditions.

These data suggest that the encroaching species investigated are able to assimilate more carbon than the non-encroaching species by increasing their rates of photosynthesis and stomatal conductance when conditions are favourable. The presence of $e\text{CO}_2$ enhances this effect by increasing the carbon available to the plant, which increases net photosynthesis more for encroachers than non-encroachers. The presence of drought and grass competition vastly reduces the benefits of $e\text{CO}_2$, meaning increased rates of carbon assimilation might allow encroaching seedlings to escape the demographic bottlenecks more rapidly only when the availability of resources such as water, light and nutrients are not limited or under competitive pressure (Reich et al., 2014; Raubenheimer & Ripley, 2022). Thus, encroachers in the seedling stage may begin to increase their competition with non-encroachers in ecosystems with favourable environmental conditions.

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Chapter 4: Evidence for Plant Water Source Partitioning in *V. karroo* in response to drought

Abstract:

Rain events and water availability in semiarid and arid ecosystems, such as savannas, have significant implications for effective plant water use strategies. For example, the recruitment of bush encroaching species is often highly episodic and may require two consecutive favourable rainfall seasons. This is largely due to competition with C_4 grass in the upper layers of soil. C_4 species in general are more water use effective than C_3 species, and multiple studies have demonstrated that elevated CO_2 (*e*CO_2) amplifies this. Once fully established, C_3 savanna species generally deal with drought by accessing deeper groundwater layers, allowing for stable co-existence with the C_4 grass layer. Encroaching seedlings are also more vulnerable to water stress, as they have higher hydraulic conductance and xylem vulnerability when compared to non-encroachers, making them more dependent on rain. This makes identifying the stage at which these woody species can fully establish and access groundwater to escape competition important for understanding savanna woody species recruitment. The relative abundance of hydrogen (δ^2H) and oxygen (δ^{18}O) stable isotopes in xylem water provides a means to determine the plant's water source. Gradients in δ^2H and δ^{18}O isotopic signatures form across soil layers of different depths, making it possible to determine which water source(s) is currently being exploited by the plant. This study examined the relationship between tree size and water source for *Vachellia karroo*, one of the most significant encroaching species in South African savannas, across size classes (small, medium, and large trees) in a semi-arid savanna site in the Eastern Cape of South Africa. It was found that large trees were able to maintain lower leaf water potentials into drought (-3.20 ± 0.25 MPa) and were therefore much less reliant on rainwater than small trees (-3.84 ± 0.26 MPa). Isotopic analyses revealed that large trees consistently access groundwater, even when rainwater is available, while small trees only begin accessing significant amounts of groundwater when water stress increases. These results indicate that small savanna trees are significantly more susceptible to drought stress than large plants. This further highlights the importance of understanding how plants in this stage respond to changing climate when investigating mechanisms behind woody bush encroachment.

Keywords: stable isotopes, estimation of source water, woody bush encroachment, elevated CO_2, drought

Introduction:

In semiarid systems, such as savannas, rain often falls in large but rare events. These episodic events lead to percolation that refills the groundwater table, with extended periods of drought in between (Scanlon et al., 2006). This results in the deep-rooted soil-plant systems being highly efficient at rain use (Seyfried et al., 2005). These rain events have significant implications for effective plant water use strategies. For example, the recruitment of bush encroaching species is often highly episodic and requires two consecutive favourable rainfall seasons (Joubert et al., 2013). This is largely due to the competition with C_4 grasses in the upper layers of soil, before the plant can access groundwater (Scholes & Archer, 1997; Ward et al., 2013). It is well documented that once established, woody savanna species tend to access water from the upper layers of soil when soil moisture is abundant, switching to ground water at greater depths when water is limited (Dawson & Pate, 1996; Xu et al., 2011; Geißler et al., 2019). Many of the trees that can access groundwater are deeply reliant on it to get through drought periods and allow more stable coexistence with the C_4 grass layer (Shadwell & February, 2017). This makes identifying the stage at which these woody species can fully establish and access groundwater to escape competition important for understanding savanna woody species recruitment. It also has important implications for neighbouring plants due to the effects of hydraulic lift, a process where deep-rooted plants take up water from the lower, wetter layers of soil and exude it in the upper, drier layers (Horton & Hart, 1998).

Stable isotope values have been developed as a method of generating a better understanding of plant water use. Plant water source can be estimated by comparisons of the stable isotopic composition of water (δ^2H and δ^{18}O) extracted from xylem tissue and the potential water source(s). This is possible as root water uptake is generally considered a non-fractionating process, thus the isotopic signature of water found in plant xylem is considered to be a mixture of signatures from all the water sources that the plant has absorbed (Dawson & Ehleringer, 1991; Lubis et al., 2014). Gradients in δ^2H and δ^{18}O across soil layers form through seasonal differences in the input of moisture into the soil (Friedman et al., 1964; Rozanski et al., 1982), evaporation in the uppermost layers of soil (Allison et al., 1983; Barnes & Allison, 1983), or to differences in isotopic composition between the bulk soil water and the ground water (Fritz & Fontes, 1980; Gat, 1980). The isotopic signature of precipitation is variable as well, meaning that groundwater recharge from rain can vary slightly in isotopic composition across rainfall events (Rozanski et al., 2013). Isotopic signatures from precipitation, stream, and groundwater often have very distinct signals, allowing for plant water sources to be determined by testing the isotopic signatures of the xylem water (Ehleringer & Dawson, 1992; Geißler et al., 2019).

Changing climate is causing significant alterations to precipitation patterns and vegetation structure in many of these semiarid ecological systems (Reynolds et al., 2007; Intergovernmental Panel on Climate Change, 2014). The observed increase in woody species in these ecosystems indicates that the changes in climate have caused a release from the demographic bottlenecks that maintained the C_4 grass dominance (Bond & Midgley, 2012). C_4 species show a markedly different response to rising atmospheric [CO_2] than C_3 species due to vast differences in metabolism (Wand et al., 1999; Ward et al., 1999; Reich et al., 2018). C_3 trees, particularly fast-growing species with a large carbon sink capacity, generally see a much greater stimulation of carbon assimilation under long-term *e*CO_2 than C_4 grasses (Ainsworth & Long, 2005). Given that C_3 metabolism is carbon-limited under current atmospheric CO_2 conditions (Long et al., 2004), their rate of growth is expected to keep increasing with changing climate (Ainsworth & Long, 2005). Encroachers are better at taking advantage of water pulses, particularly under *e*CO_2 conditions (Chapter 2; Figures 2 & 5), indicating that an increasing number of encroaching seedlings might be able to escape their demographic bottlenecks with changing climates. This increase will not be uniform across landscapes, as local environment stress can hinder a seedling's ability to realise the benefits of *e*CO_2 (Raubenheimer & Ripley, 2022). The establishment of *V. karroo* also allows for the formation of bush clumps, which facilitate the establishment of other encroaching species and subsequently expand in size, further increasing the rates of woody bush encroachment (Jamison-Daniels et al., 2021).

This study aims to quantify the relationship between tree size and water source for *Vachellia karroo*, one of the most significant encroaching species in South African savannas. This relationship will be investigated during periods of high and low precipitation. Investigations will be made to determine whether access to groundwater significantly changes the hydraulic pressure on the plant by looking at both pre-dawn and midday water potentials (Ψ). In particular, the following questions will be addressed:

(1) Do small, medium, and large *V. karroo* trees have distinct hydrogen (δ^2H) and oxygen ($\delta^{18}O$) isotopic signatures that can be linked to differences in source water?

(2) Does water source change for small, medium, and large *V. karroo* between periods of high and low precipitation?

(3) Do small, medium, and large *V. karroo* trees have different water potentials that reflect their access to different water sources?

Methods:

Field experiment:

This study was conducted at a field site known as Endwell Safaris, located in the Smaldeel region of the Eastern Cape province of South Africa (32°44'15.7"S 26°29'06.6"E). The land is used predominantly for livestock farming, particularly cattle and goats (Martens et al., 1996; Smit, 2004). The dominant tree species in this area is *Vachellia karroo* (Acocks, 1988). The site has a mean annual precipitation of 700 – 800 mm (Cole et al., 2018) and rainfall is generally higher in the summer months (Martens et al., 1996). The vegetation in Smaldeel is classified as a semi-arid savanna, with mixture of trees and a discontinuous grass layer. More specifically, it is a combination of Bedford Dry Grassland and Bisho Thornveld (Mucina & Rutherford, 2007). This field site was monitored for roughly four weeks during a period of high precipitation (April 2023) and a period of low precipitation (September 2023). The two data sampling periods were defined based on the total amount of precipitation in the 3 months leading up to the start of the experiment. During the high precipitation period, the 3 months leading up to the field experiment saw 389.11 mm of rain. During the low precipitation period, the 3 months leading up to the field experiment saw 117.12 mm of rain with 17 days of no rain before the first measurement.

Vachellia karroo individuals were randomly selected from areas of similar soil and topography, to represent a range of size classes. The trees were classified into three size classes based on tree height: small (<1 m), medium (1-3 m), and large (>3 m). Since fire is absent from this system, tree height is an approximate proxy for tree age, and one would expect that larger trees have more developed root systems (Persson, 2002). The distance between each selected individual and the nearest neighbour was ~3 m, to minimise any inter- or intraspecific competitive effects. Trees were marked with GPS coordinates and metal tags to allow them to be reidentified for repeated measurements. For the isotope analyses, however, it was not always possible to harvest samples from the same individual, particularly in the small size class. For these cases, the nearest individual of approximately the same size was collected. Each individual had its water potential measured pre-dawn and midday to test the hydration status of the plants during dry downs.

Water potentials (Ψ):

In order to assess the water status of the plants in the field, both predawn water potential (Ψ) and midday water potential (Ψ) were taken. Predawn Ψ measurements were made between 5:00 am and 7:00 am and midday Ψ measurements were made between 12:00 pm and 2:00 pm. All Ψ measurements were made using a PMS pressure chamber (Model 1505D). Leaves chosen for Ψ measurements were excised from the plant using a fresh razor blade and then immediately sealed in a ziplock bag. Each bag had a small piece of moist tissue that was exhaled into before inserting the leaf and sealing. The time between collecting the leaf and running the measurement was generally 5 minutes. Leaves selected for midday Ψ were fully expanded mature leaves, with all leaflets still present, sitting in direct sunlight.

Isotopic analysis:

To determine the plant water sources, stable isotope ratios of hydrogen (δ^2H) and oxygen (δ^{18}O) were analysed. Comparing the signatures of δ^2H and δ^{18}O stable isotopes found in xylem water to potential water sources is a strong indicator of which water source the plant is currently exploiting. This test is possible due to gradients in hydrogen and oxygen isotopic signatures forming across soil layers of different depths (Ehleringer & Dawson, 1992) and root water uptake being considered a non-fractionating process (Dawson & Ehleringer, 1991). Rainwater was captured in a rain gauge and immediately sealed into glass scintillation vials. Rainwater samples were collected from multiple different rain events. Each rain event where water was collected had at least 10mm of rain. Groundwater samples were collected from a borehole on the farm by pumping out the water at the borehole site. Samples collected to undergo isotope analyses were mature, suberized stem sections of roughly 50 cm in length (Dawson & Ehleringer, 1993). The stem sections were immediately defoliated, cut into shorter segments, and sealed in a glass scintillation vial. The glass vial was then sealed with parafilm on the inside of the lid and insulation tape on the outside, to prevent any evaporation. Samples were then taken back to the lab and stored in a dark, cool cupboard until cryogenic vacuum distillation (CVD) could be performed. The cupboard was maintained at 23-27 °C.

Cryogenic Vacuum Distillation (CVD) was used to extract the xylem water from collected samples at iThemba Labs, Johannesburg for the high precipitation period samples and at Rhodes University, Makhanda for the low precipitation period samples. Prior to CVD, all samples had their bark and phloem removed to prevent the heavy isotope-depleted phloem water from interfering with the xylem water isotopic signature (Nehemy et al., 2022). Samples were cut into sections of ~5 cm and placed into small glass beakers. These beakers were then connected to cold fingers on a central manifold that connected to a vacuum pump. Once connected, the beakers with samples were submerged in water heated to ~80-90 °C and the cold fingers were submerged for roughly half their length in a dry ice slurry at ~-80 °C or in liquid nitrogen at ~-196 °C. The dry ice slurry was used at iThemba Labs and liquid nitrogen was used at Rhodes University due to availability issues. A vacuum of ~10 kPa below atmospheric was then pulled on the samples through the central manifold. All samples were left for 4 hours extraction time. After the water had been quantitatively transferred from the sample beakers to the cold fingers, the cold fingers were removed, sealed with parafilm, and left to sit at room temperature to defrost. Once defrosted, the contents of the cold fingers were carefully decanted into Eppendorf tubes that were sealed with parafilm. The cold fingers were then placed into a dry oven for a minimum of 18 hours between measurements.

Once the water was extracted from each sample, it was stored in an Eppendorf tube until it could undergo isotope analysis at iThemba Labs. All isotopic analyses were conducted on a Trace Gas Spectrometer (LGR ICOS V09.09.11.H2O_4ISO.LWIA, Los Gatos Research Inc., USA). Each sample was injected 8 times, with the first 3 measurements being discarded as a stabilisation process. The mean and standard deviation is then calculated for the retained measurements. Using 5 LGR standards, measurements were made each standard, with increments of 6 unknown samples in between. Once the all the standards have been measured, the cycle repeats until 4 measurements are made for each standard. The mean and standard deviation was then calculated for the measurements on the standards and regressed against their known values. This linear regression was then used to correct the measured isotope values to the final numbers used in this study.

Statistical analysis:

The midday and pre-dawn water potential (Ψ) was compared between size classes, during both low and high precipitation periods, using a linear mixed effects model with size class and length of drought as fixed effects and plant ID as a random factor. For data from the low precipitation period, proximity to rain events was used instead of days without rain. The relative abundance of oxygen ($\delta^{18}O$) and hydrogen ($\delta^{2}H$) stable isotopes were compared separately between different size classes against drought stress, during both low and high precipitation periods, using a linear mixed effects model with size class and days without rain as fixed effects and plant ID as a random factor. The relative abundance of $\delta^{18}O$ and $\delta^{2}H$ stable isotopes were plotted for each size class using a regression model and compared with Global and Local Meteoric Water Lines (GMWL and LMWL). LMWL was calculated from the regression line of the isotopic ratios of all the rain samples collected at the field site. Intercepts of the regression lines of each size class and the GMWL were calculated. Due to the number of interacting factors and the limited sample size, no *post-hoc* tested were performed. The statistical analysis was conducted in R version 4.0.5 (R Development Core Team, 2023). All mixed effects model comparisons were made using lme4 R package (Bates, 2010).

Results:

High precipitation period data:

Water potential

Length of drought had a significant effect (χ^2 = 286.33, *df* = 1, *P* = <0.005) on predawn water potential, but size class did not (χ^2 = 0.69, *df* = 2, *P* = 0.708). Predawn water potentials see a fairly uniform decrease in Ψ across size classes as the drought progresses (Figure 12). Both size class (χ^2 = 6.22, *df* = 2, *P* = 0.045) and length of drought (χ^2 = 376.89, *df* = 1, *P* = <0.005) had significant effects on the midday water potential of *V. karroo*. With midday water potentials, Ψ showed the highest variation in the small size class from low (-0.83 ± 0.29 MPa) to high (-3.83 ± 0.28 MPa) water stress, and the large size class showed the lowest variation from low (-1.78 ± 0.33 MPa) to high (-3.21 ± 0.25 MPa) water stress (Figure 13). At the onset of drought, small trees had significantly less negative Ψ than large trees but had significantly more negative Ψ at the end of the drought (Figure 13). Results show that large trees have more negative Ψ (-1.78 ± 0.33 MPa) than small trees (-0.83 ± 0.29 MPa) at low levels of drought but, as the drought progresses, large trees have a less negative Ψ (-3.21 ± 0.25 MPa) than small trees (-3.83 ± 0.28 MPa). The medium size class was not statistically different from any other size class for either midday or predawn Ψ.

Isotope ratios

Both hydrogen (δ^2H) (χ^2 = 13.82, *df* = 2, *P* = <0.005) and oxygen (δ^{18}O) (χ^2 = 7.82, *df* = 2, *P* = 0.02) isotopic ratios were significantly affected by the size class. The length of drought was also a significant predictor of δ^2H and δ^{18}O. When compared to isotopic values of the ground and rainwater samples, small trees are much more reliant on rainwater (soil water of potential groundwater recharge) than medium or large trees (Figures 14 & 15). At the onset of drought, the medium and large size classes clustered together for both δ^2H and δ^{18}O, however as the drought progressed, medium trees clustered much more closely with small trees. Results show that large trees are better able to utilise groundwater reserves than small or medium trees, which allows them to maintain higher levels of hydration during drought.

Low precipitation period data:

Water potential

Proximity to rain event (χ^2 = 346.18, *df* = 1, *P* = <0.005) and size class (χ^2 = 8.77, *df* = 2, *P* = 0.013) both had significant effects on predawn water potential of *V. karroo*. Small trees began to separate from medium and large size classes just before the first rains for predawn Ψ. Both size class (χ^2 = 10.23, *df* = 2, *P* = 0.006) and proximity to rain event (χ^2 = 946.13, *df* = 1, *P* = <0.005) had significant effects on the midday water potential of *V. karroo*. With midday water potentials, Ψ showed the highest variation in the small size class from low (-1.11 ± 0.25 MPa) to high (-3.84 ± 0.23 MPa) water stress, and the large size class showed the lowest variation from low (-1.27 ± 0.26 MPa) to high (-3.18 ± 0.24 MPa) water stress (Figure 17), mirroring the results from the high precipitation period (Figure 13). Small trees had significantly more negative Ψ (-3.84 ± 0.23 MPa) than large trees (-3.18 ± 0.24 MPa) leading up to the first rains but had no significant difference from the other size classes after the rain (Figure 17). Results here complement those of the high precipitation period, with large trees being better able to maintain moisture as drought progresses when compared to small trees. After the rain events, there was little statistical difference between the size classes.

Isotope ratios

Neither hydrogen (δ^2H) (χ^2 = 3.41, *df* = 2, *P* = 0.18) or oxygen (δ^{18}O) (χ^2 = 1.85, *df* = 2, *P* = 0.40) isotopic ratios were significantly affected by the size class. Proximity to rain event was a significant predictor of both δ^2H (χ^2 = 17.93, *df* = 1, *P* = <0.005) and δ^{18}O (χ^2 = 7.18, *df* = 1, *P* = 0.007). As the drought progressed, a significant split between the size classes began to develop, however all size classes clustered together again after the rain event. Results here show that reliance on pulses of rainwater has some seasonal dependence, with all size classes using predominantly groundwater during periods of low precipitation.

Estimation of source water:

Figures 20 and 21 show stable isotope values for different size classes of *V. karroo* plotted against the GMWL. Regression lines for all stable isotope data are compiled in Table 1. The slope of regression lines ranged from 1.90 to 2.74 during the high precipitation period and from 4.29 to 4.66 during the low precipitation period. Small trees have the steepest slope in both sets of data (Table 1). Most data points from the low precipitation period sit above the GMWL in the deuterium enriched zone. Intercepts between the species-specific regression lines and the GMWL are compiled in Table 2. Intercepts are similar between small (-4.70 : -28.05) and large (-4.94 : -29.97) size classes in the high precipitation period and between medium (-1.09 : 1.48) and large (-1.07 : 1.63) size classes in the low precipitation period. The LMWL was plotted against the GMWL (Figure 22), and regression lines were compared (Table 1). The regression line for LMWL (δ^2H = 5.21 δ^{18}O – 30.16) fell outside of the usual range expected for LMWL (Putman et al., 2019).

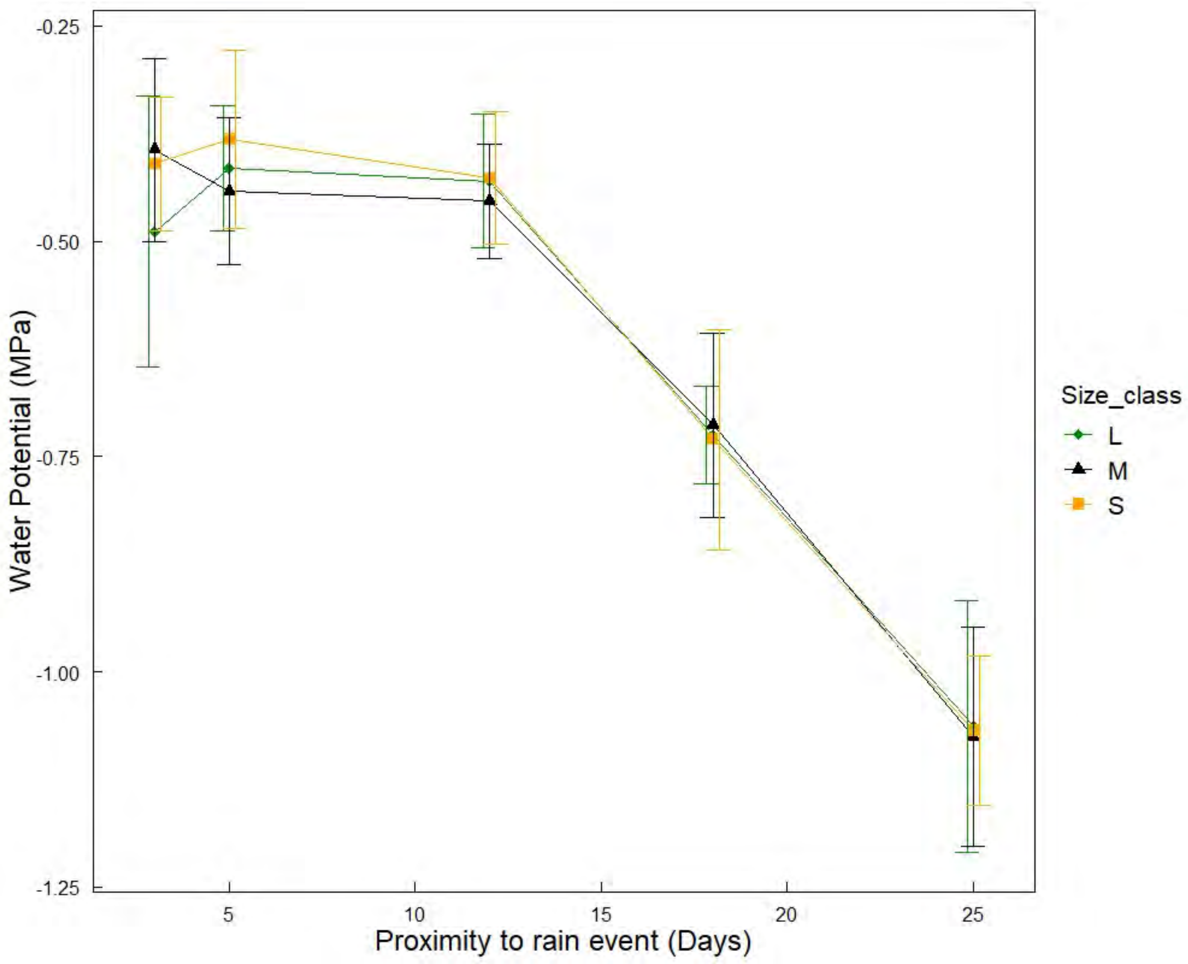

Figure 12: Pre-dawn Water Potentials (Ψ) over time during the high precipitation period. Size classes are small (<1 m) (S), medium (1-3 m) (M), and large (>3 m) (L). N = 6.

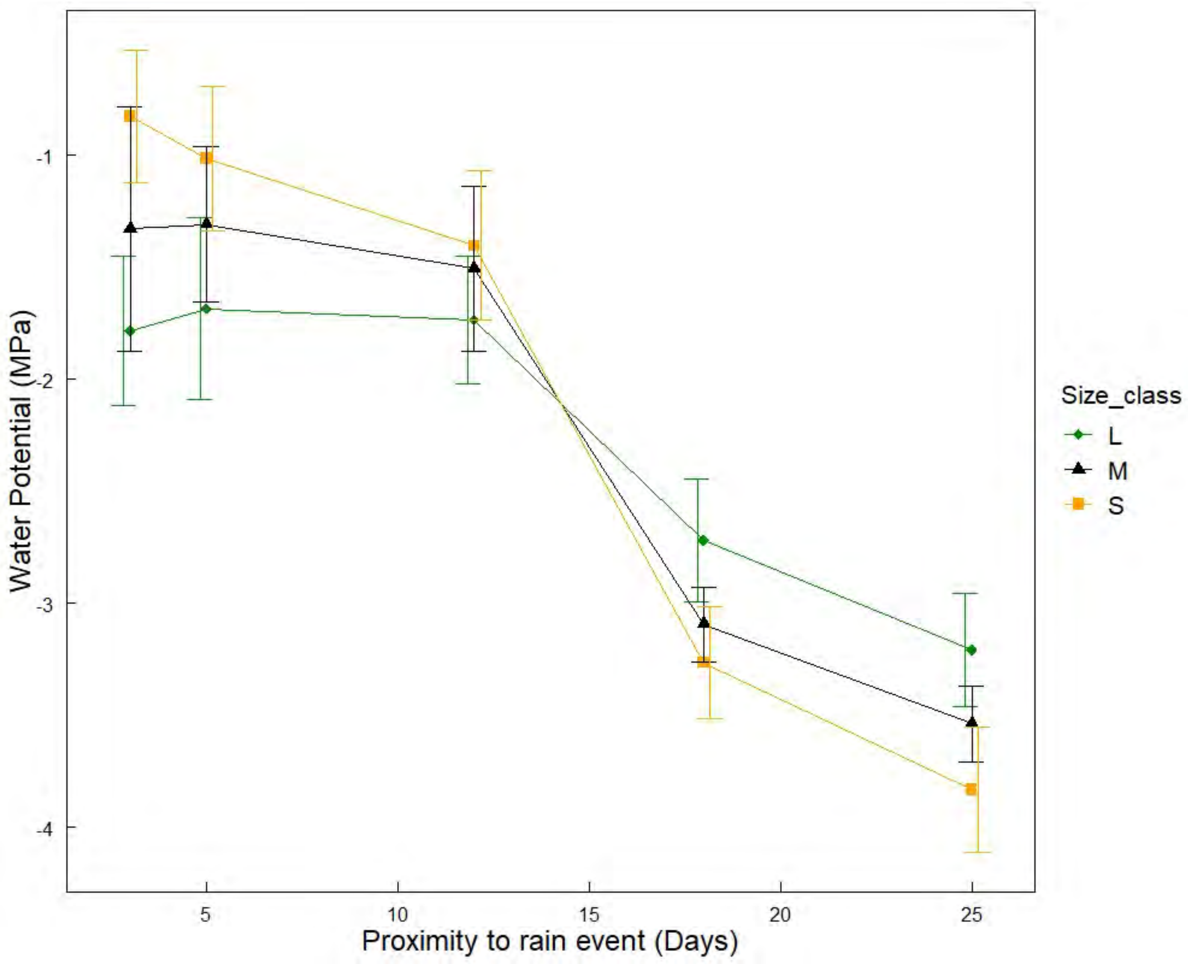

Figure 13: Midday Water Potentials (Ψ) over time during the high precipitation period. Size classes are small (<1 m) (S), medium (1-3 m) (M), and large (>3 m) (L). N = 6.

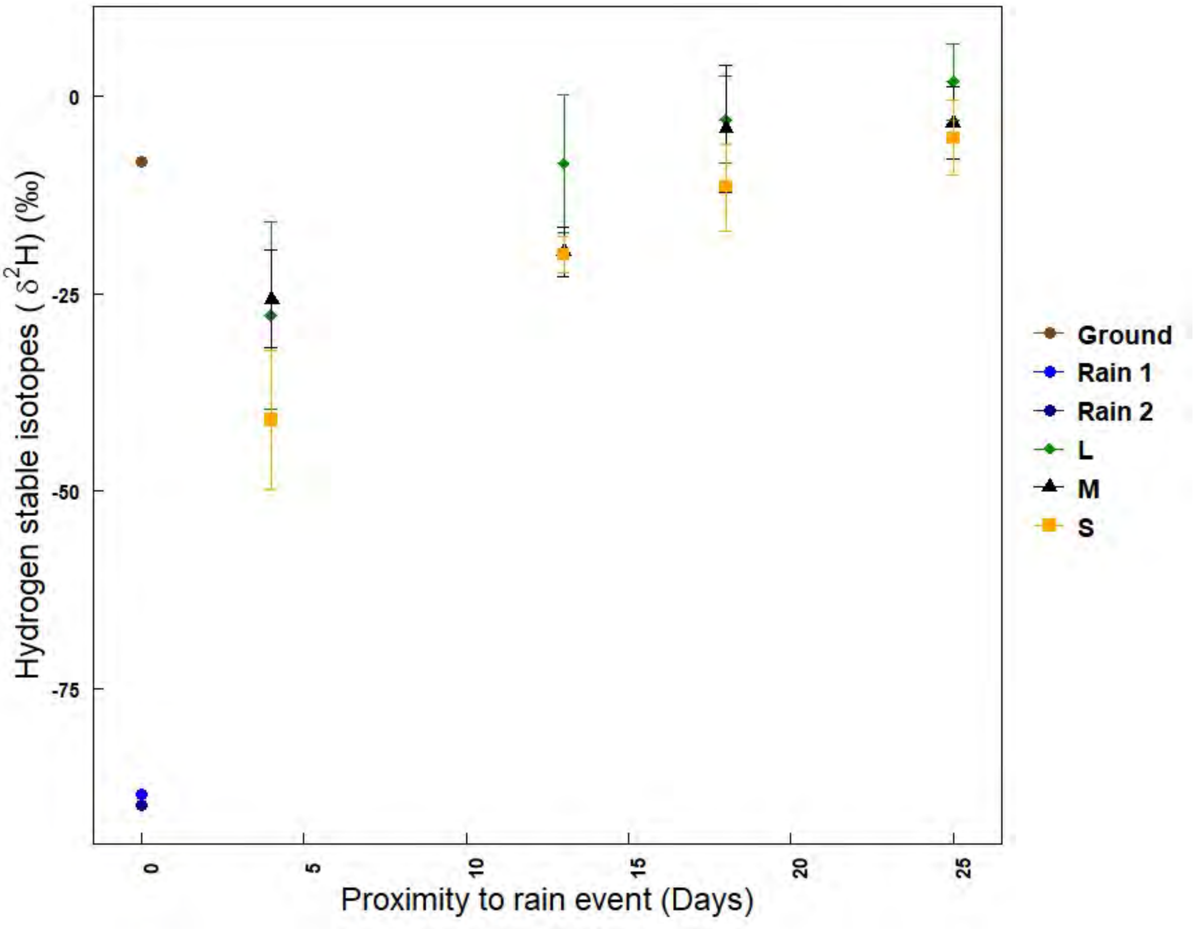

Figure 14: Change in δ^2H over time during the high precipitation period. Small size classes cluster much closer to the isotopic signature of rain when compared to large size classes. Size classes are small (<1 m) (S), medium (1-3 m) (M), and large (>3 m) (L). N = 5.

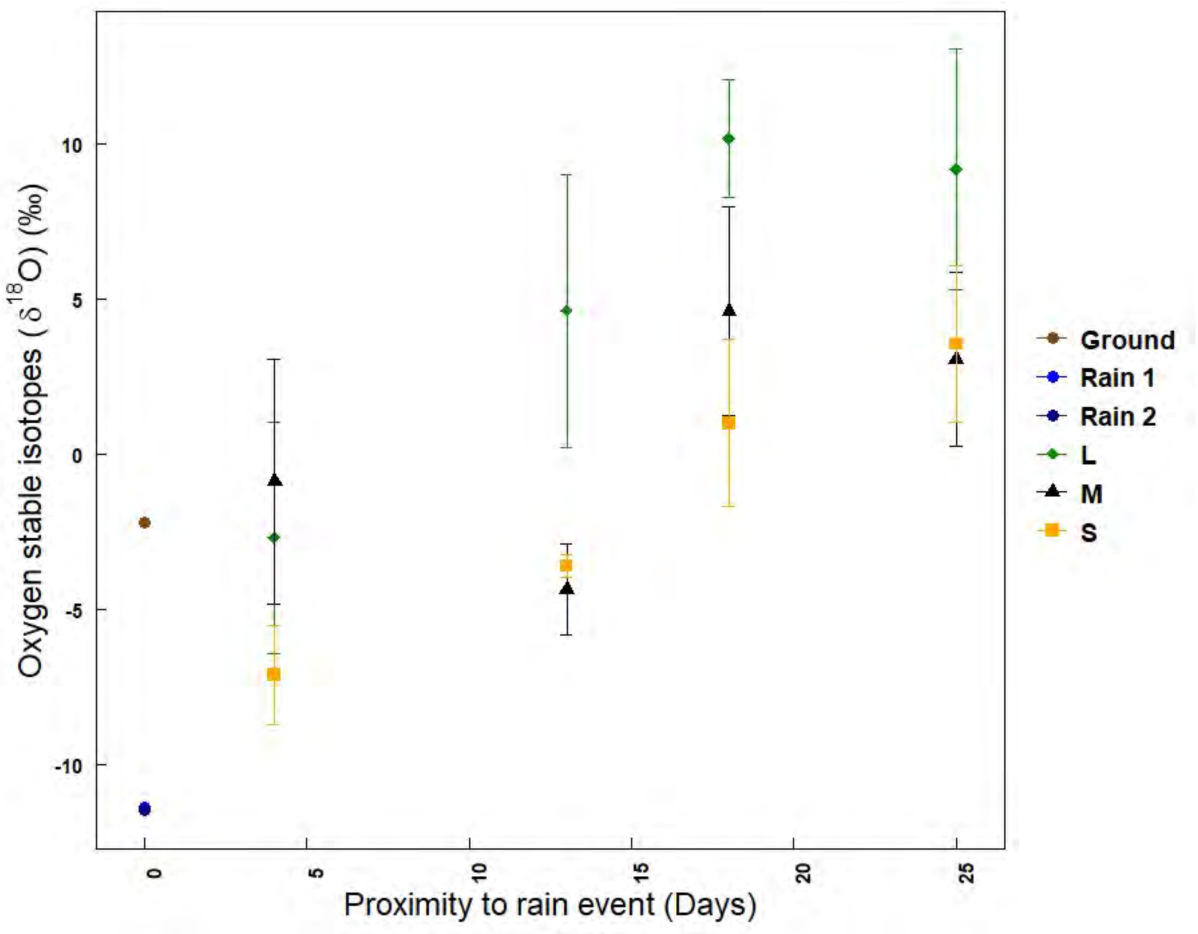

Figure 15: Change in $\delta^{18}O$ over time during the high precipitation period. Small size classes cluster much closer to the isotopic signature of rain when compared to large size classes. Size classes are small (<1 m) (S), medium (1-3 m) (M), and large (>3 m) (L). N = 5.

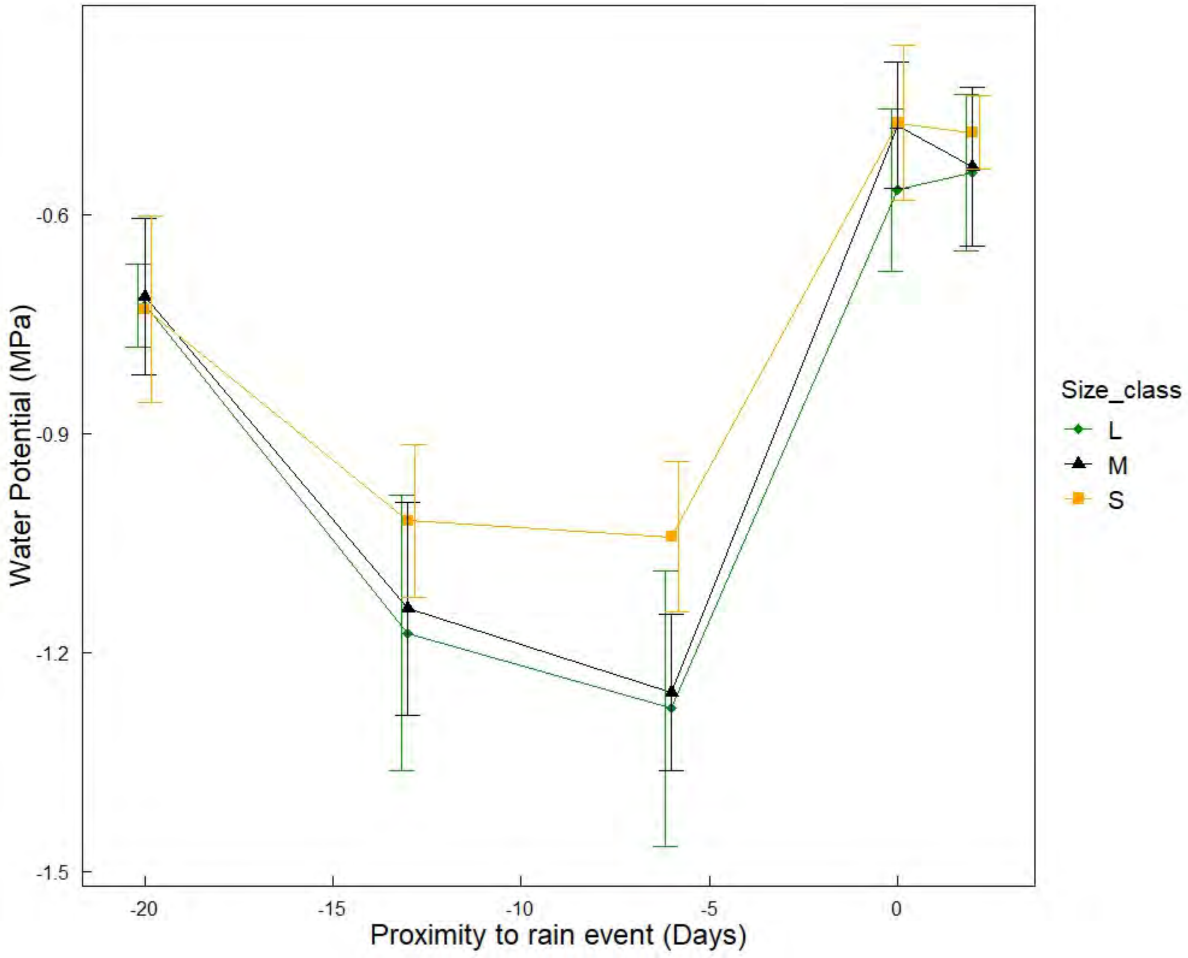

Figure 16: Pre-dawn Water Potentials (Ψ) over time during the low precipitation period. Size classes are small (<1 m) (S), medium (1-3 m) (M), and large (>3 m) (L). The first three points are at 18, 25, and 32 days without rain respectively. N = 6.

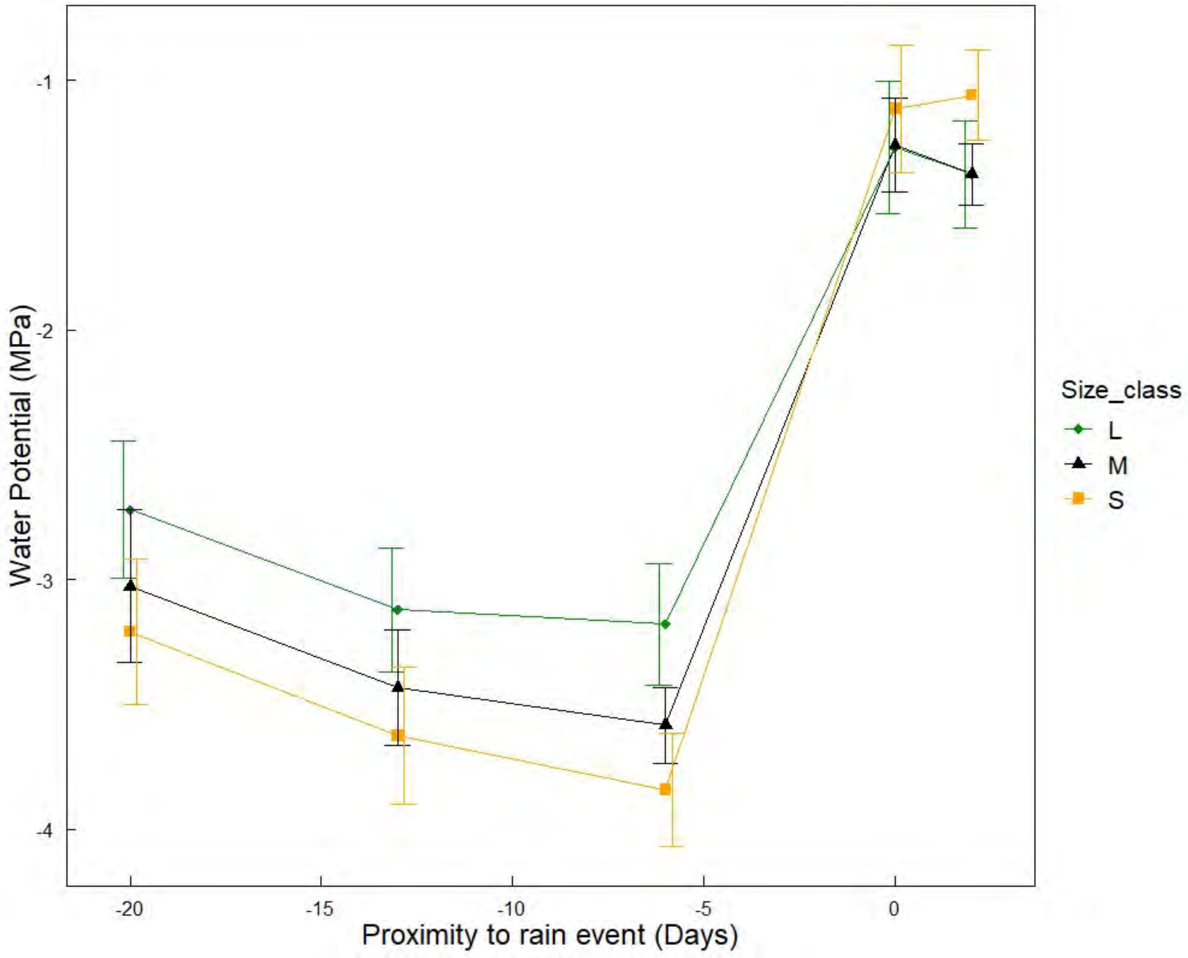

Figure 17: Midday Water Potentials (Ψ) over time during the low precipitation period. Size classes are small (<1 m) (S), medium (1-3 m) (M), and large (>3 m) (L). The first three points are at 18, 25, and 32 days without rain respectively. N = 6.

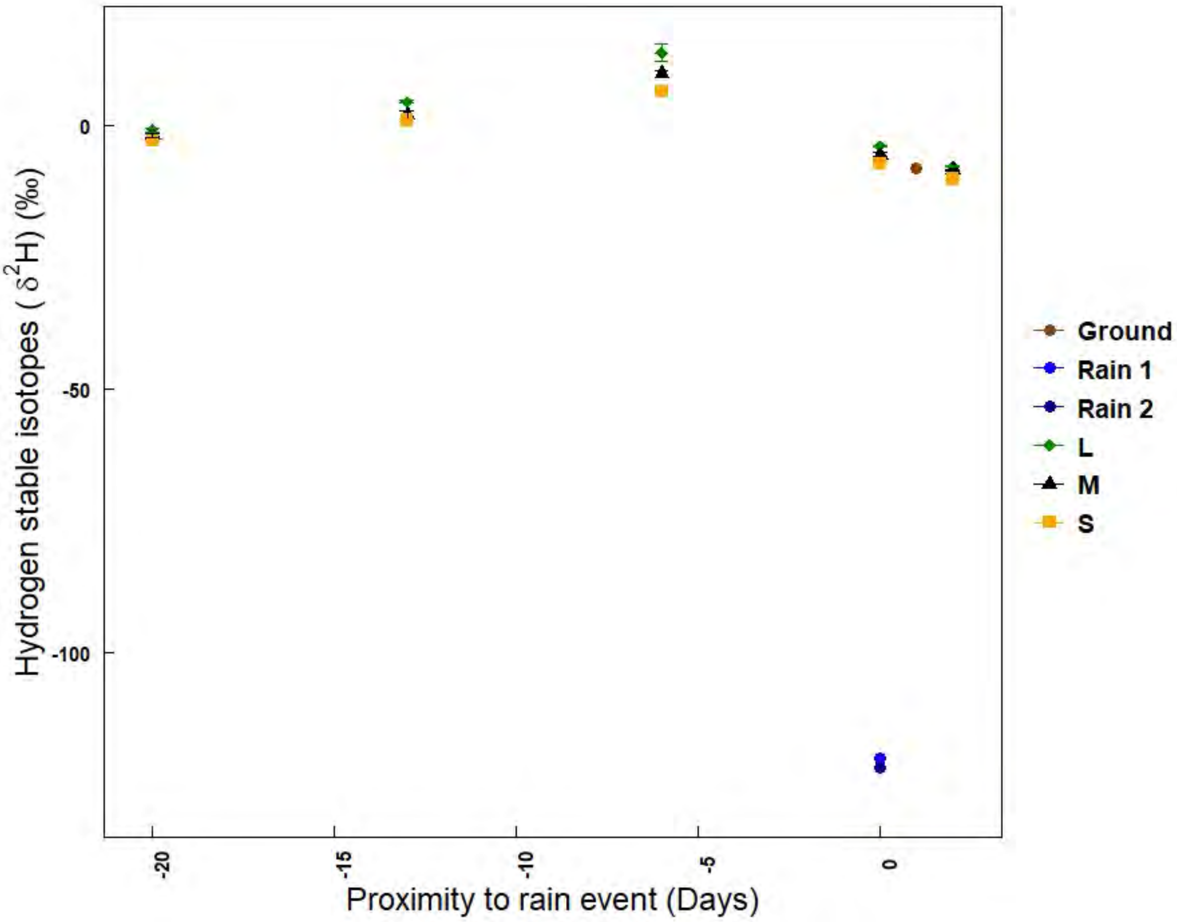

Figure 18: Change in δ^2H over time during the low precipitation period. Size classes are small (<1 m) (S), medium (1-3 m) (M), and large (>3 m) (L). N = 6.

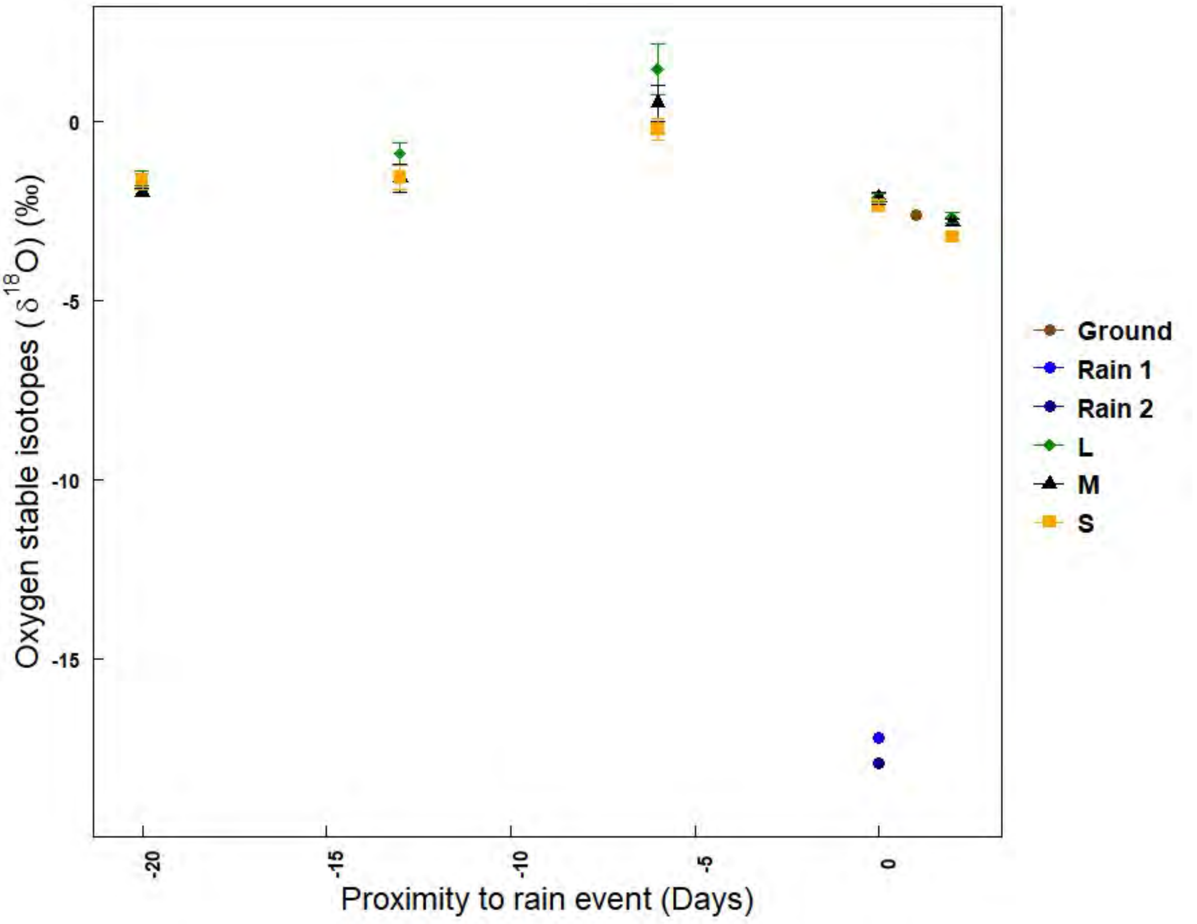

Figure 19: Change in δ^{18}O over time during the low precipitation period. Size classes are small (<1 m) (S), medium (1-3 m) (M), and large (>3 m) (L). N = 6.

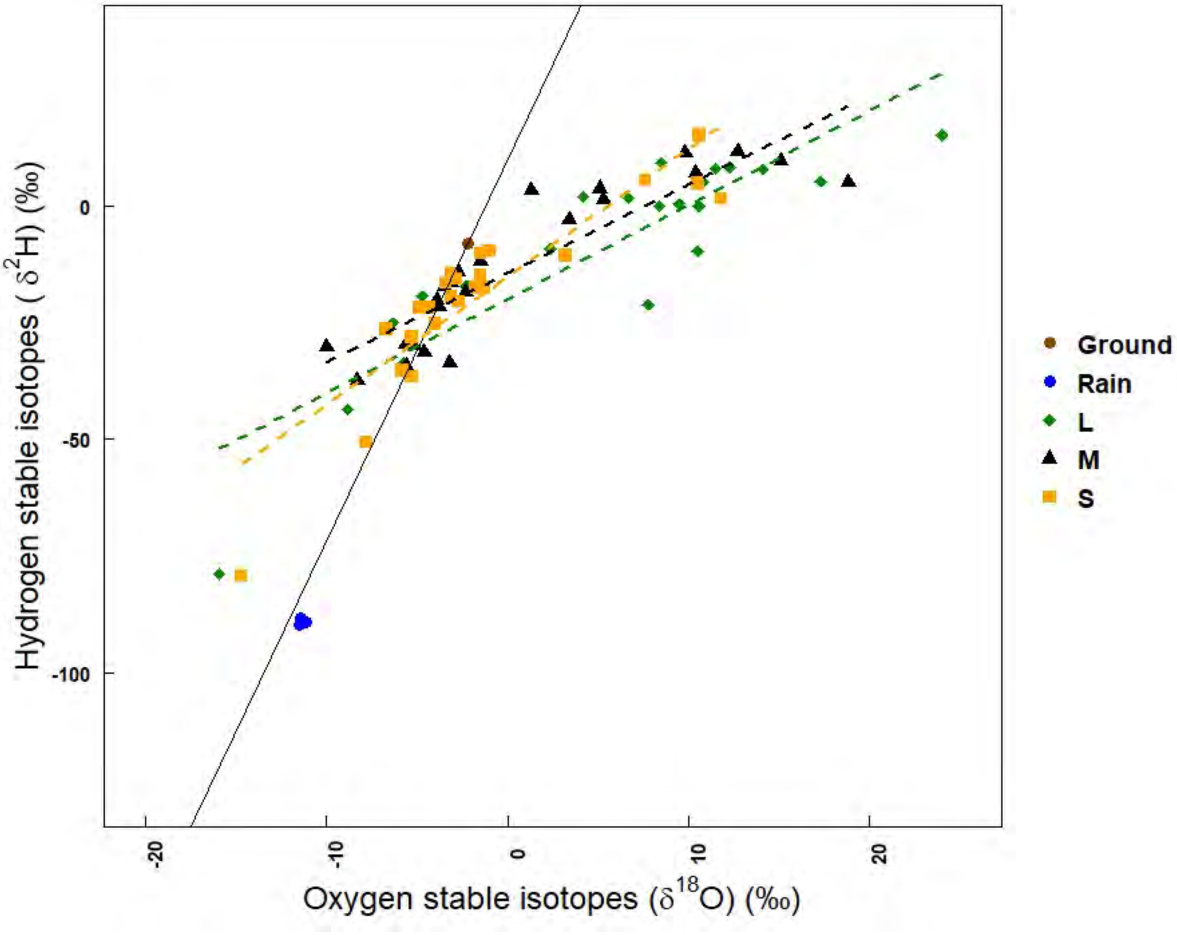

Figure 20: Estimation of water source for small (<1m) (S), medium (1-3 m) (M), and large (>3 m) (L) size classes of *V. karroo* during the high precipitation period. Trendline is the Global Meteoric Water Line (GMWL). N = 22.

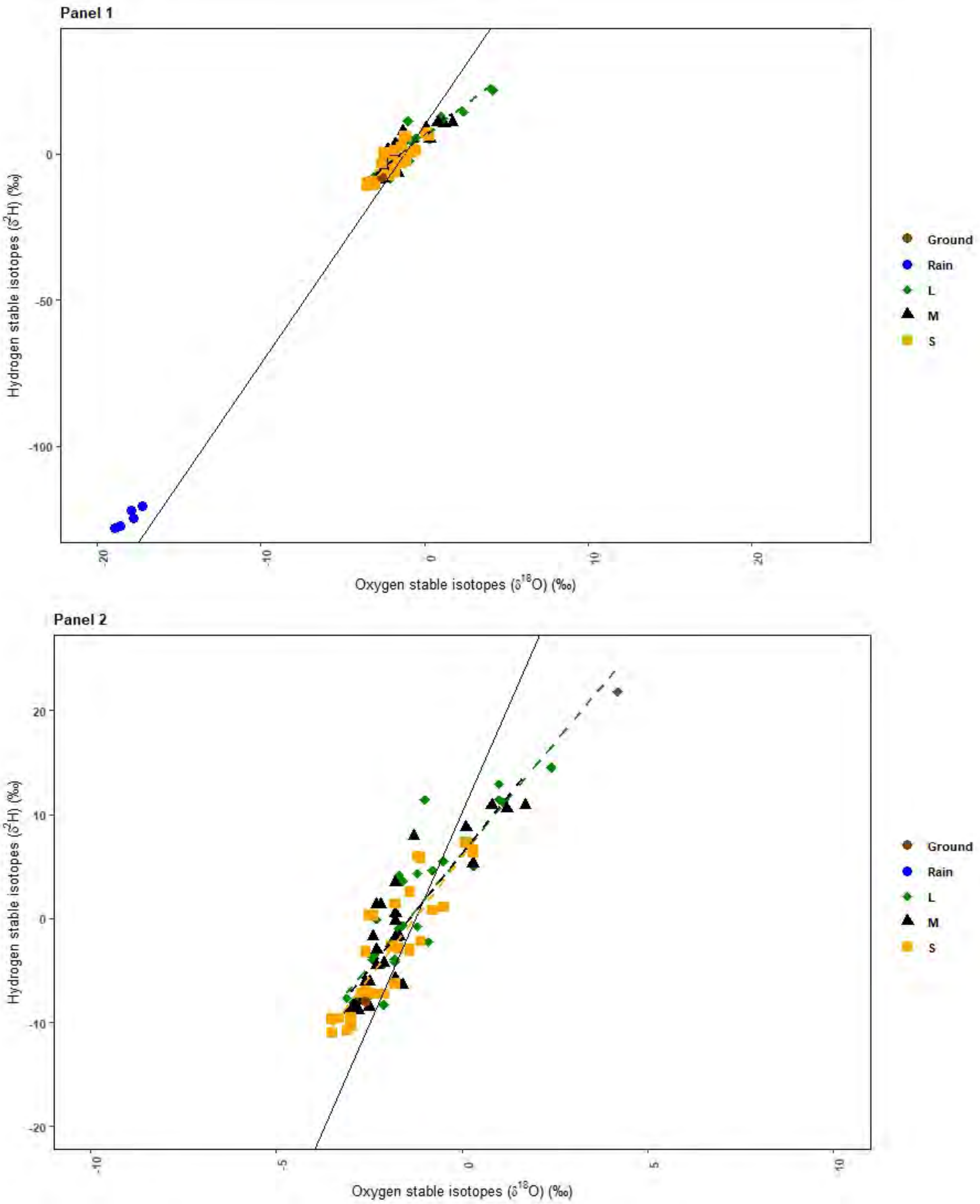

Figure 21: Estimation of water source for small (<1m) (S), medium (1-3 m) (M), and large (>3 m) (L) size classes of *V. karroo* during the low precipitation period. Panel 2 is the same data as Panel 1, but with reduced axes to improve resolution. Trendline is the Global Meteoric Water Line (GMWL). N = 30.

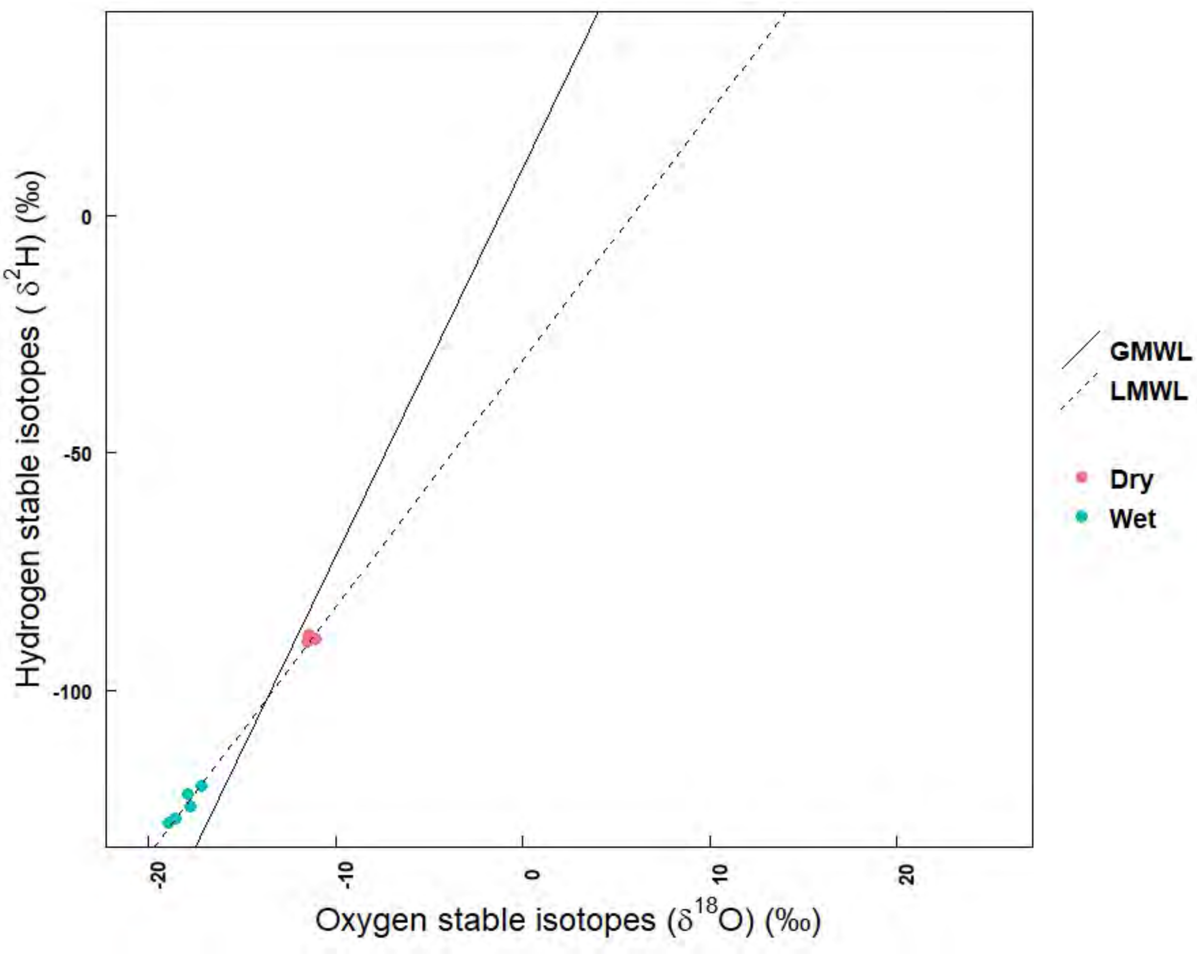

Figure 22: Local Meteoric Water Line (LMWL) for Endwell farm compared to the Global Meteoric Water Line (GMWL), based on readings taken from rain samples from high (Wet) and low (Dry) precipitation periods. N = 8.

Table 2: Equations for the regression lines; R^2 values.

Water source:	High precipitation period:	Low precipitation period:
Large trees	$\delta^2H = 2.02\ \delta^{18}O - 20.00$; $R^2 = 0.78$	$\delta^2H = 4.29\ \delta^{18}O + 6.21$; $R^2 = 0.84$
Medium trees	$\delta^2H = 1.90\ \delta^{18}O - 14.48$; $R^2 = 0.80$	$\delta^2H = 4.44\ \delta^{18}O + 6.30$; $R^2 = 0.77$
Small trees	$\delta^2H = 2.74\ \delta^{18}O - 15.17$; $R^2 = 0.80$	$\delta^2H = 4.66\ \delta^{18}O + 5.96$; $R^2 = 0.77$
GMWL	$\delta^2H = 8.17\ \delta^{18}O + 10.35$; R^2 = N/A	
LMWL	$\delta^2H = 5.21\ \delta^{18}O - 30.16$; $R^2 = 0.99$	

Table 3: Intersect of regression lines with the GMWL.

Water Source:	High precipitation period:		Low precipitation period:	
	$\delta^{18}O$	δ^2H	$\delta^{18}O$	δ^2H
Large trees	-4.94	-29.97	-1.07	1.63
Medium trees	-3.96	-22.00	-1.09	1.48
Small trees	-4.70	-28.05	-1.25	0.13

Discussion:

Using a common savanna encroacher (*V. karroo*) as a model species, the effect of plant size on the ability to access groundwater and how this affected the water availability to the plant was investigated. It was found that large trees were much less reliant on rainwater (soil water of potential groundwater recharge) than small trees during periods of high precipitation. Large trees consistently access groundwater, even when rainwater is available, while small trees only begin accessing significant amounts of groundwater when water stress increases. During the low precipitation period, this water stress was enough that all size classes were heavily reliant on groundwater. This is consistent with the results of studies conducted in the savannas of Kruger National Park in South Africa (Kulmatiski & Beard, 2013), the Kgalagadi Transfrontier Park in Botswana (Shadwell & February, 2017), and the Kalahari rangeland in Namibia (Geißler et al., 2019)(Geißler et al., 2019), which all found that the dominating large trees and shrubs do not rely on rainwater (soil water of potential groundwater recharge) to any significant degree. Medium trees have an intermediate response between the small and large size classes, with isotopic values clustering closely with large trees at the onset of drought by utilizing groundwater supplies. This is indicative of a relatively early investment in a deep taproot to exploit the more reliable water sources, as observed by Ehleringer & Dawson (1992). As the drought progresses, however, the isotopic values of medium sized trees begin to cluster more closely with small trees. This is likely due to increased competition for groundwater with the fully established rooting systems of large trees.

The results from the isotopic analysis were reflected in the midday Ψ measurements on the plants. As soil water stress increased, large trees that were able to access groundwater were better able to maintain less negative Ψ during the day in both low and high precipitation periods. Small trees only began reaching the Ψ of 50% xylem failure, as calculated in Chapter 2 (Figure 2), during the highest levels of water stress (Figures 13 & 17) and it is therefore most likely that they would only experience mild rates of embolisms. During the high precipitation period, all size classes saw a decline in predawn Ψ at the same rate. During the low precipitation period, however, small trees began to split from medium and large trees, maintaining less negative Ψ as drought progressed. Interestingly, none of the size classes displayed an increase in uptake of rainwater after the rain event recorded during the low precipitation period, despite showing a significant change to their Ψ. This may be due to reduced percolation during periods of drought (Kleine et al., 2020), but more research is required here to determine if this is the case.

None of the size classes in the low precipitation period accessed evaporatively enriched water and were most likely all accessing groundwater reserves (Figure 21). Regression lines were similar for all size classes, indicating that all size classes were getting water from the same source. All size classes plot on the enriched side of both the LMWL and the GMWL during the low precipitation period (Figure 21; Table 1), meaning that this system can be described as “excess deuterium” (Bershaw et al., 2020). It is likely that this system is driven by moisture from the same source as during the high precipitation period, but with moisture recycled within the landscape. Water vapour that has evaporated is usually deuterium rich, as it has to maintain the isotopic mass balance with the evaporatively enriched water that it evaporated from. If this water falls as rain and is taken up by the plant, it will fall in the excess deuterium zone (Araguás-Araguás et al., 2000; Bershaw et al., 2020). A more robust LMWL would need to be generated to fully explore this, however. There was more variation in the data from the period of high precipitation, with regression line slopes remaining similar but differences arising in the intercepts. This indicates that while all size classes during the high precipitation period accessed evaporatively enriched water, there was some variation in water source. This variation is seen in the isotopic data looking at the progression of drought (Figures 14, 15, 18 & 19). The source of water accessed during the high precipitation period is most likely from the upper layers of the soil. The variation in regression lines generated in the low and high precipitation periods show that all size classes of *V. karroo* utilise different water sources depending on the season and level of drought. Despite accessing groundwater reserves during periods of drought, seedlings saw significant drops in water potentials during these periods, indicating that rainfall events may be more significant for the establishment of encroaching species seedlings under the longer and more frequent droughts predicted in the future (Intergovernmental Panel on Climate Change, 2014).

For the estimation of water source data (Figures 20 & 21), the Global Meteoric Water Line (GMWL) was used in place of the Local Meteoric Water Line (LMWL) due to artifacts in the rain data from the low precipitation period used to generate the LMWL. There is no precedent for a LMWL to have this gradient and intercept (Figure 22; Table 1). An extensive meta-analysis of LMWLs from across the globe revealed that slopes can range from 4.8 to 10.9, with an average of 7.64 ± 0.64, and intercepts can range from -24‰ to 27‰ (Putman et al., 2019). The same analysis demonstrated that LMWLs from Southern Africa generally have slopes ranging from 6.0 to 8.0 and intercepts ranging from 5 to 15 (Putman et al., 2019). Our LMWL had a regression line of $\delta^2H = 5.21\ \delta^{18}O - 30.16$, which falls outside of these ranges. While it is generally best practice to use the LMWL, there have been published studies that compare isotope data to the GMWL (e.g. Uugulu et al., 2023). The fact that the high precipitation period rain plots onto the same line suggests unique synoptics in the area (such as local re-evaporation), but this would require much more data to fully explore (Treble et al., 2005; Hughes & Crawford, 2012). The rain event captured in the low precipitation period occurred during unusual weather conditions, which is likely what caused the artifacts. The synoptic charts taken from the time of sample collection (see Appendix A; Figures 24 [suppl.] – 27 [suppl.]) give insights into the conditions that created this anomaly. Figures 24 [suppl.] & 25 [suppl.] show low pressure systems in the Mozambique Channel and coastal lows off the Kwa-Zulu Natal coast with a high-pressure system in the South Indian Ocean. By the 29th (Figure 26 [suppl.]) the low-pressure system is disrupted, however it is likely that the moisture here came in from the tropics with the low-pressure systems. This low-pressure system is likely to have driven the stable isotope ratios to more negative values through a process known as the continental effect (Dansgaard, 1964; Kern et al., 2020). The continental effect refers to the gradual depletion in heavy isotopes of the marine moisture as precipitation falls as the air masses travel from the moisture source towards the centre of the continent (Dansgaard, 1964; Kern et al., 2020). To fully determine whether these data points have significantly affected the LMWL a lot more data is required, which is beyond the scope of this study.

Conclusion:

There was strong evidence that while the small size class of *V. karroo* can access groundwater supplies during periods of drought, their midday Ψ fell to the Ψ of 50% xylem embolism (P_{50}) after 25 days of water stress. This indicates that small savanna trees are indeed reliant on rainfall events to prevent significant damage to their water transport systems. This result was consistent with a study conducted by Joubert et al. (2013), which found that the recruitment of bush encroaching species is often highly episodic and requires two consecutive favourable rainfall seasons. With the results of Chapters 2 and 3 (Figures 2, 5, 6 & 7) indicating that encroaching species in the seedling stage are set to maximize gas exchange at the cost of increased susceptibility to water stress with increasing levels of CO_2, seedlings can be expected to develop even more reliance on rainfall events to escape their demographic bottlenecks in these ecosystems under project future climates.

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Chapter 5: Conclusions

This study demonstrates an important mechanism behind savanna bush encroachment in both *a*CO_2 and *e*CO_2: encroacher seedlings have more strongly anisohydric properties than non-encroachers in the presence of grass competition, allowing them to maximise gas exchange during periods of high precipitation at the cost of increased susceptibility to xylem cavitation during periods of low precipitation. While *Vachellia* and *Senegalia* trees are generally considered anisohydric, differences in the rates of water use and xylem vulnerability between species have been observed (Cory et al., 2022; Uni et al., 2023). With high spatial and temporal variability in environmental stresses and water availability across savanna ecosystems (O'Connor et al., 2014; Skowno et al., 2017; Venter et al., 2018) and strong evidence that CO_2 stimulation of savanna seedling growth can only be realised when conditions are suitable (Raubenheimer & Ripley, 2022), it makes sense that the most optimal strategy to escape demographic bottlenecks is to be dependent on high rates of carbon uptake during pulses of water availability and favourable environmental conditions. On a landscape scale, this effect has already been observed by a study conducted on *V. mellifera* in Namibian savannas, which found that the recruitment of this bush encroaching species is often highly episodic and requires two consecutive favourable rainfall seasons (Joubert et al., 2013). More extensive studies found that while fire and grazing are important factors, it is ultimately the interplay of environmental factors such as rainfall amount and frequency paired with soil nutrient levels that cause bush encroachment, further reinforcing the importance of water use strategies (Ward, 2005; O'Connor et al., 2014)

This study found that at a semi-arid field site, there was strong evidence that small individuals of *V. karroo*, a common savanna encroacher, are reliant on rainwater (soil water of potential groundwater recharge) to prevent significant damage to their water transport systems. Despite accessing groundwater reserves, small trees are unable to uptake water from this source as efficiently as medium or large trees, leading to faster rates of desiccation, as evidenced by more negative midday water potentials. While there are significant shifts predicted in xylem vulnerability under *e*CO_2 for encroaching seedlings, this effect may be offset by the reduction stomatal conductance under *e*CO^2 (Bellasio et al., 2018). There is extensive literature suggesting that the seedling stage is a crucial demographic bottleneck in savannas for the establishment and distribution of woody species (e.g., Chidumayo, 2013; Anderson et al., 2015; Morrison et al., 2019) and the results show that encroachers will be more susceptible to this bottleneck under *e*CO_2 in areas suffering drought, as the CO_2 fertilization effect falls away under these limiting conditions. Interestingly, a paper investigating the impacts of the 2014-2016 drought in South Africa found that the prolonged drought mostly caused mortality in taller woody species, with plants over 5m suffering the highest rates of mortality (Swemmer, 2020). While my results suggest that larger trees are able to maintain better levels of hydration into drought, the study by Swemmer (2020) was conducted after a much more severe and prolonged drought than my study. This indicates that additional research is needed to confirm the response at extreme levels of drought, which are predicted to occur more frequently as the climate changes (Intergovernmental Panel on Climate Change, 2014).

As hypothesized, the OTC experiments revealed that both encroaching and non-encroaching species displayed characteristics typical of anisohydric "water spenders", as has been found elsewhere for species of *Vachellia* and *Senegalia* (Cory et al., 2022; Uni et al., 2023). *e*CO_2 significantly shifted the hydraulic response of encroachers to drought towards even stronger anisohydric properties. Under *e*CO_2 conditions, encroaching species are better at transporting water in conditions of high water availability, but these systems quickly fail under heavy water stress due to increased xylem vulnerability. This response was most pronounced when seedlings were grown in the presence of grass competition and diluted without grass competition. All xylem vulnerability measurements were made using the pneumatic method on a newly developed device called the pneumatron (see Appendix A; Figure 23 [suppl.]) (Pereira et al., 2020; Trabi et al., 2021). While this device was tricky to set up and get functioning properly, it provides a low-cost and easy to use method of improving understanding of plant-water relations.

The rate of water and nutrient transport places a physical limit on the rates of gas exchange and metabolic function that cannot be overcome (Tyree & Sperry, 1989; Brodribb, 2009). The photosynthetic response reflected this, as in treatments where environmental conditions should not have been constraining, there were significant improvements to both the photosynthetic rates and photosystem II quantum yield for encroachers over non-encroachers. There was also evidence that implies a novel trait in encroaching species: photosynthetic upregulation. This is a novel trait as photosynthetic downregulation has been observed in multiple studies (e.g., Flexas et al., 2004; Ronchi et al., 2006; Nebauer et al., 2011), however photosynthetic upregulation is reported much less frequently and generally only occurs when other nutrients, particularly N, are abundant (Bloom et al., 2010; Vicente et al., 2017). This may suggest that encroachers have an increased carbon sink capacity over non-encroachers (Ainsworth & Long, 2005), which may explain why they can assimilate more carbon for a given stomatal conductance.

Here, this research provides valuable insights into the mechanisms behind woody encroachment in the context of rising [CO_2]. This research provides an in-depth analysis of how the eco-physiological mechanisms via which savanna plant water-use strategies affect photosynthetic responses in the seedling stage, and how this response affects the ability of a species to encroach savanna ecosystems in the context of rising [CO_2]. The outcomes of this complex response will largely depend on the extent of changes to biotic and abiotic factors across spatial and temporal zones caused by climate change. Encroachers are better at taking advantage of spatial and temporal pockets wherein growth conditions are suitable under e$CO_{2,}$ and it is therefore very likely that rates of encroachment will accelerate in most savannas, apart from those undergoing prolonged drought. Future studies can further investigate the validity of these findings by testing rates of xylem cavitation and mortality in encroachers and non-encroachers during an extended drought. The greenhouse experiment of this study was conducted exclusively on seedlings, so more research is required to test whether adult trees have the hydraulic responses to these conditions. Further research into the potential photosynthetic upregulation response to eCO_2 observed in encroachers is also required. This is of particular interest as many of the encroachers, such as *V. karroo*, are nitrogen fixers (Barnes, 2001), and photosynthetic upregulation has only been observed in plants grown in environments where nutrients such as nitrogen are not limiting (Bloom et al., 2010; Vicente et al., 2017).

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Appendix A:

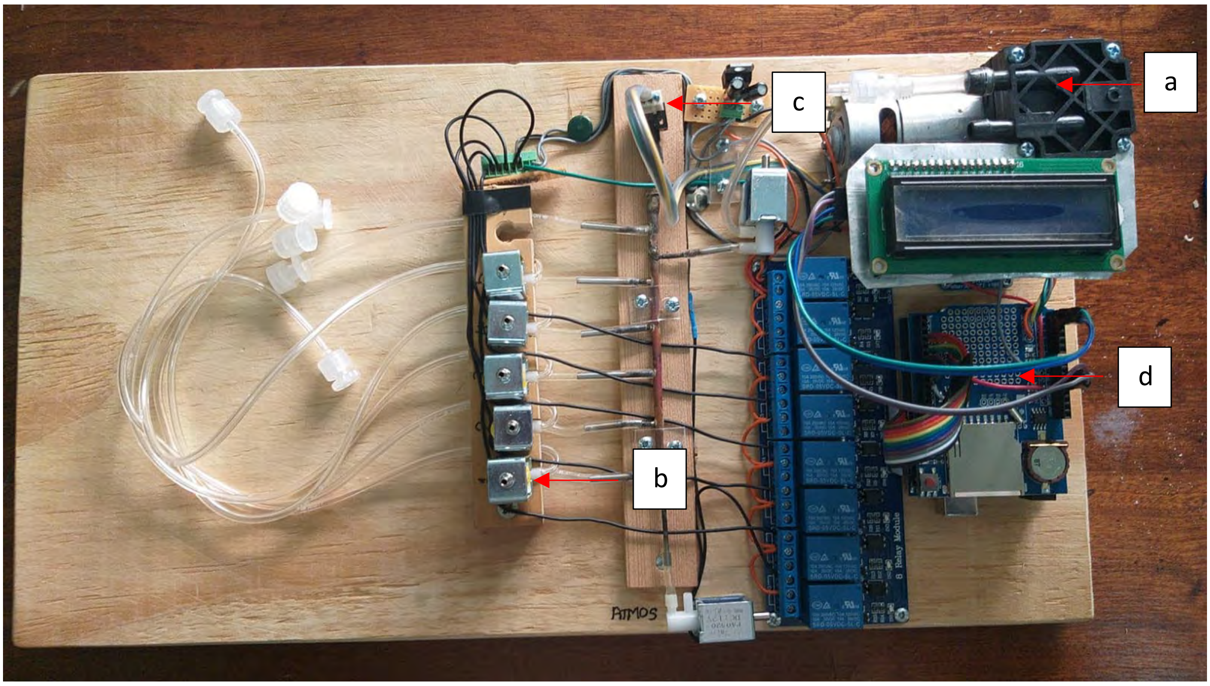

Figure 23 [suppl.]: Photograph of the pneumatron device used in this study. Labels are for (a) the vacuum pump, (b) a mini solenoid valve, (c) a pressure transducer, and (d) an Arduino Uno microcontroller.

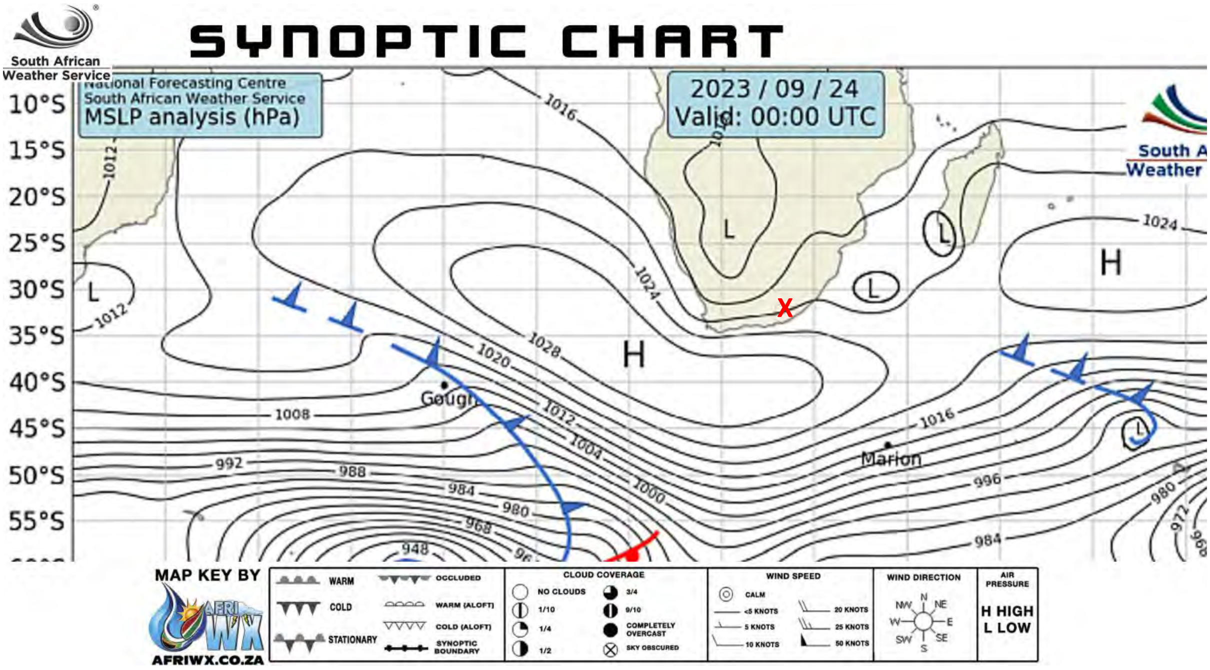

Figure 24 [suppl.]: Synoptic chart of South Africa for 24 September 2023. Red X is the study site where samples were collected.

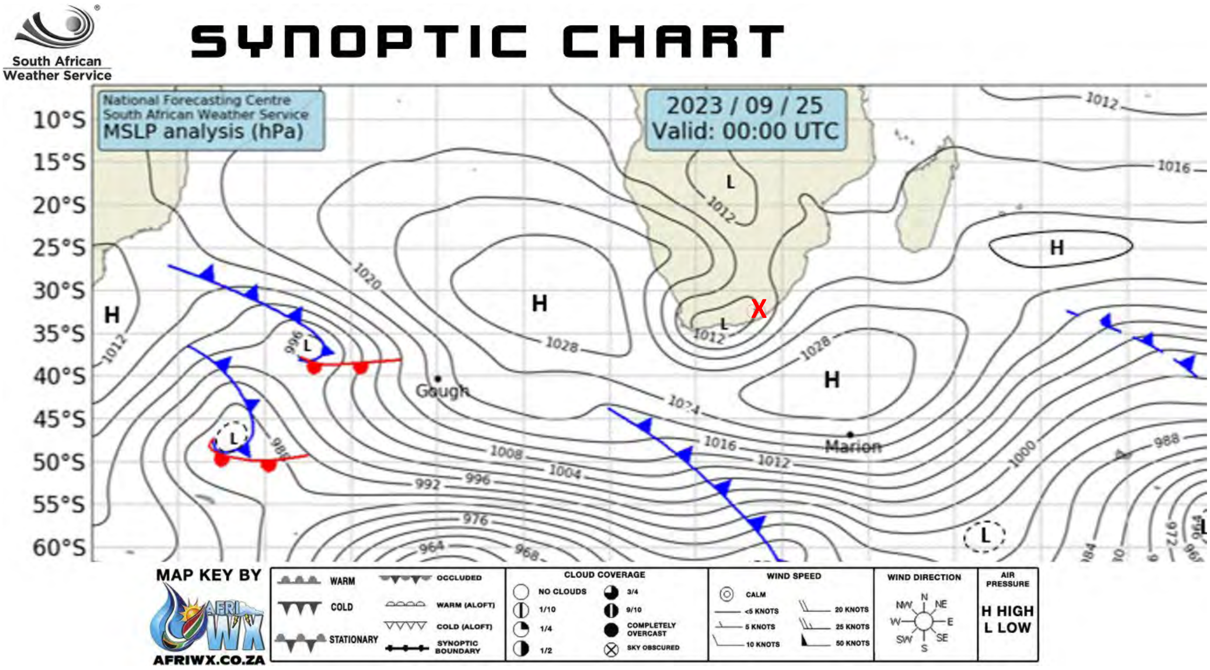

Figure 25 [suppl.]: Synoptic chart of South Africa for 25 September 2023. Red X is the study site where samples were collected.

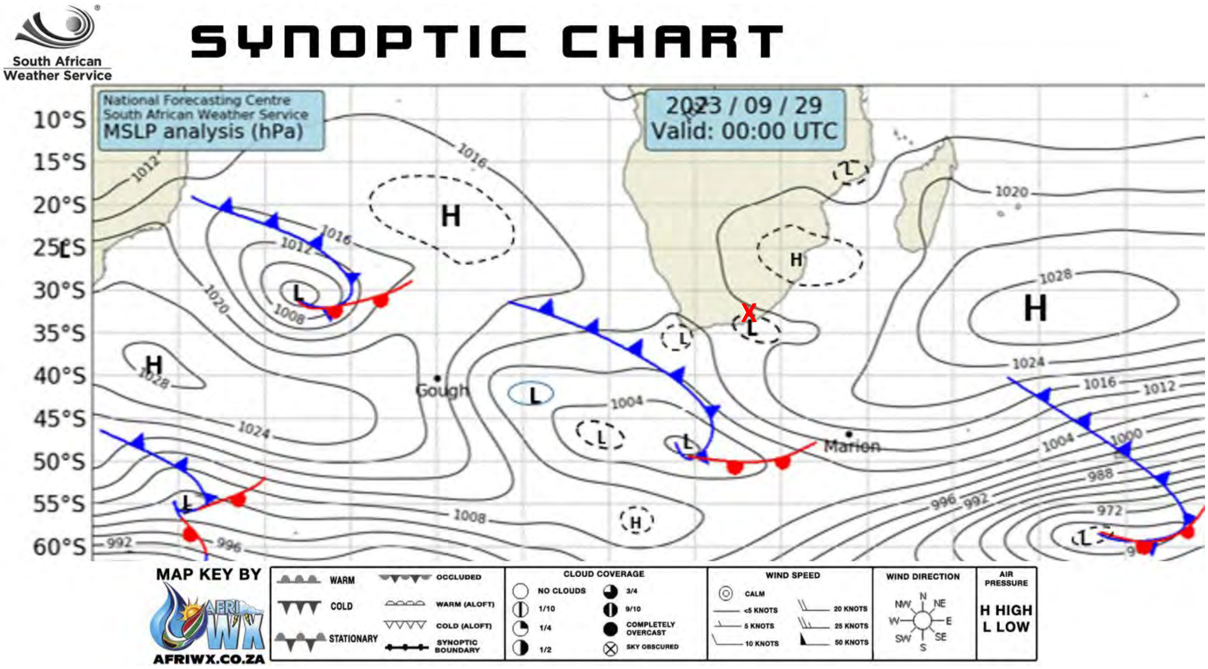

Figure 26 [suppl.]: Synoptic chart of South Africa for 29 September 2023. Red X is the study site where samples were collected.

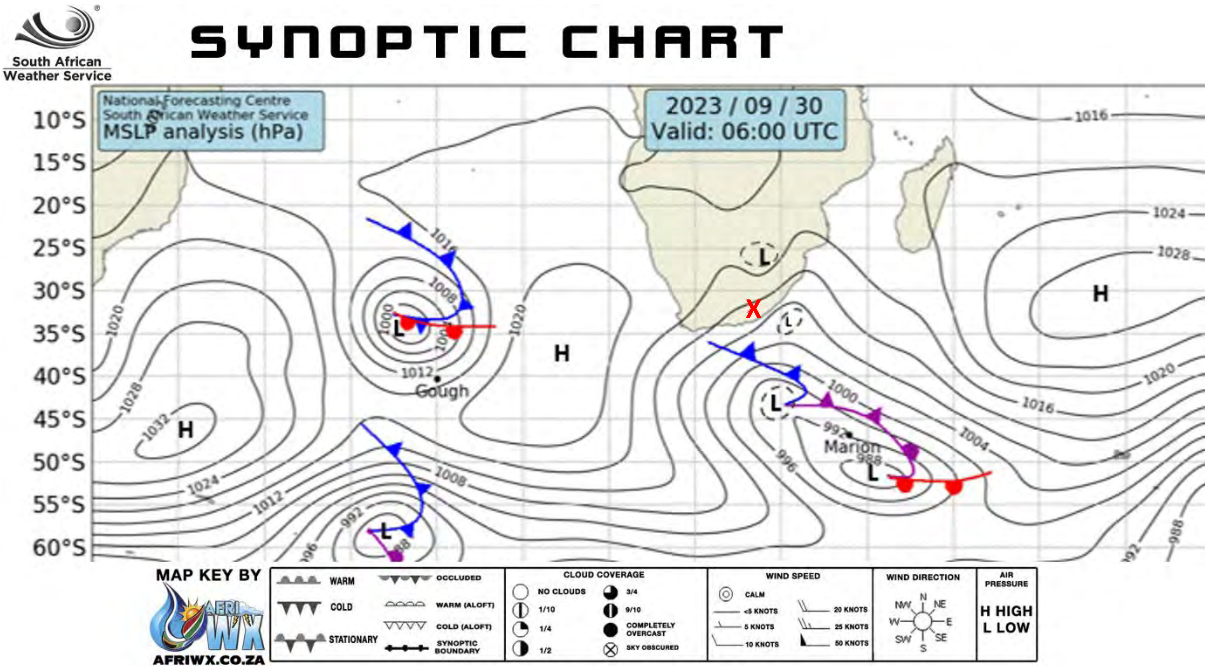

Figure 27 [suppl.]: Synoptic chart of South Africa for 30 September 2023. Red X is the study site where samples were collected.