Plant Science 274 (2018) 181–192

Contents lists available at ScienceDirect

Plant Science

journal homepage: www.elsevier.com/locate/plantsci

Stomatal and non-stomatal limitations in savanna trees and C_4 grasses grown at low, ambient and high atmospheric CO_2

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ARTICLE INFO

Keywords:
Photosynthesis
Elevated CO_2
Global change
Poaceae
Acacia
Vachellia
Celtis
Combretum
Non-stomatal limitations
Sub-ambient CO_2

ABSTRACT

By the end of the century, atmospheric CO_2 concentration ($[CO_2]_a$) could reach 800 ppm, having risen from ~200 ppm ~24 Myr ago. Carbon dioxide enters plant leaves through stomata that limit CO_2 diffusion and assimilation, imposing stomatal limitation (L_S). Other factors limiting assimilation are collectively called non-stomatal limitations (L_{NS}). C_4 photosynthesis concentrates CO_2 around Rubisco, typically reducing L_S. C_4-dominated savanna grasslands expanded under low $[CO_2]_a$ and are metastable ecosystems where the response of trees and C_4 grasses to rising $[CO_2]_a$ will determine shifting vegetation patterns. How L_S and L_{NS} differ between savanna trees and C_4 grasses under different $[CO_2]_a$ will govern the responses of CO_2 fixation and plant cover to $[CO_2]_a$ – but quantitative comparisons are lacking. We measured assimilation, within soil wetting–drying cycles, of three C_3 trees and three C_4 grasses grown at 200, 400 or 800 ppm $[CO_2]_a$. Using assimilation–response curves, we resolved L_S and L_{NS} and show that rising $[CO_2]_a$ alleviated L_S, particularly for the C_3 trees, but L_{NS} was unaffected and remained substantially higher for the grasses across all $[CO_2]_a$ treatments. Because L_{NS} incurs higher metabolic costs and recovery compared with L_S, our findings indicate that C_4 grasses will be comparatively disadvantaged as $[CO_2]_a$ rises.

1. Introduction

All photosynthetic organisms use the same ancestral C_3 biochemical machinery in which CO_2 is fixed by ribulose 1,5-bisphosphate carboxylase/oxygenase (Rubisco) and the products are processed into sugars by dark reactions. In C_3 plants, CO_2 reaches Rubisco along a CO_2 diffusion gradient from higher atmospheric, to lower chloroplastic concentrations [1]. CO_2 diffuses into leaves through stomata – the same pathway as water vapour out – and plants regulate the rate of gas exchange by adjusting stomatal conductance (g_S) through changes in stomatal density, dimensions and aperture, which regulate evapotranspiration (E) [2]. Stomata therefore limit CO_2 diffusion into leaves and the $[CO_2]$ in sub-stomatal cavities (C_i) [3], and the extent of this limitation is called stomatal limitation (L_S). Stomata respond, not exclusively, to temperature, atmospheric humidity and CO_2 concentration ($[CO_2]_a$), and the amount of water within and supplied to leaves from the soil [4]. Limitations to A caused by other leaf-level constraints are called non-stomatal limitation, L_{NS}, and include intercellular and intracellular CO_2 diffusion, light, metabolic and biochemical constraints (Rubisco capacity, adenosine triphosphate [ATP] availability, ribulose 1,5-bisphosphate [RuBP] synthesis, and leaf nitrogen), source–sink dynamics, and leaf ultrastructure [5,6].

Rubisco can either carboxylate or oxygenate RuBP in competing photosynthetic and photorespiratory reactions. Photorespiration metabolises already fixed carbon, evolving CO_2 and offsetting net CO_2 uptake [7–9], and is largely determined by the ratio of O_2 : CO_2 concentration at the Rubisco catalytic sites [8,10]. C_4 photosynthesis reduces photorespiration by decreasing O_2 : CO_2 with a CO_2-concentrating mechanism (CCM) [11]. The C_4 pathway evolved independently ~60 times in > 18 families [12,13], many of which appeared in the Neogene (beginning ~23 Myr ago) after a reduction in $[CO_2]_a$ from ~1000 ppm towards 180 ppm [14,15]. Subsequently, savanna ecosystems expanded at the expense of closed forests under low $[CO_2]_a$ on all continents over the last 10–25 Myr [14] as monsoon-driven seasonal aridity increased [16,17]; and C_4-dominated grasslands generally expanded from mixed C_3 and C_4 grasslands ~9 Myr ago [14,18,19]. Chronic disturbance from herbivory and fires, fuelled by productive and flammable C_4 grasses, supress tree recruitment and

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https://doi.org/10.1016/j.plantsci.2018.05.028
Received 7 March 2018; Received in revised form 24 May 2018; Accepted 25 May 2018
Available online 29 May 2018

promote open habitats, meaning savanna vegetation patterns are closely linked to the productivity of C_4 grasslands [20–23]. Changes in disturbance drivers can induce rapid transition between open, C_4-dominated grasslands with scattered trees, and closed forest [24,25], and savanna vegetation responses to disturbance are likely to be modified by changing $[CO_2]_a$.

Today, savannas experience $[CO_2]_a$ levels that are higher than in any point during their evolutionary history, but the effect of rising $[CO_2]_a$ on savanna vegetation patterns is difficult to predict, in part because potential differences in the relative roles of stomatal and non-stomatal limitations in the photosynthetic responses of C_3 and C_4 plants to $[CO_2]_a$ are not well understood [22,26–29]. When stomatal factors limit photosynthesis during a drought, for example, A is restored by increasing C_i through stomatal opening upon restoration of soil water availability; consequently, L_S does not impair or reduce metabolic function [6,30,31]. Conversely, metabolic constraints imposed by L_{NS} are generally not immediately relieved with increases in soil water and g_S, necessitating metabolic repair and prolonging recovery of A to pre-drought levels [32]. Under mild water limitation – that might be experienced daily or weekly in open, semiarid savannas – L_S is thought to predominate limitations to A in C_4 leaves, with L_{NS} becoming more important as leaf water status continues to decline [6,33,34]. However, compared with C_3, C_4 leaves are more susceptible to L_{NS} [32,35] and the speed of leaf dehydration may govern the mode of limitation to A [35]. Although the severity of water limitation affects the relative influence of L_S and L_{NS}, few studies have assessed stomatal and metabolic contributions to C_3 and C_4 photosynthetic inhibition under moderate soil drying. Consequently, the extent and proportionality of stomatal and metabolic inhibition of A with moderate reductions in leaf water status are largely unknown for either C_3 or C_4 plants. Moreover, absolute declines in g_S with increasing growth $[CO_2]_a$ are generally larger for C_3 than C_4 leaves [10,36]. If, however, C_4 plants suffer from increased L_{NS} relative to C_3 under moderate fluctuations in water availability this will impinge on their performance even under future rises in $[CO_2]_a$. Quantifying these processes will be important for predicting shifts in savanna vegetation patterns.

Here we aim to resolve how the relative contributions of L_S and L_{NS} respond to $[CO_2]_a$ and affect CO_2 fixation in C_3 forest and savanna trees and C_4 savanna grasses. We measured photosynthesis in three tree species (*Vachellia karroo*, *Celtis africana* and *Combretum apiculatum*) and three C_4 grass species (*Eragrostis curvula*, *Heteropogon contortus* and *Themeda triandra*) grown at either low (200 ppm), ambient (400 ppm) or elevated (800 ppm) $[CO_2]_a$. We grew the plants in replicated controlled-environment growth chambers and measured photosynthetic potential over typical wetting–drying cycles by watering plants to 80% of pot capacity and allowing soil moisture to decline over 2–3 days during which measurements were taken. We characterised photosynthetic potential with A–response measurements to parameterise empirical models for direct comparison between the trees and grasses, quantify L_S and L_{NS}, and assess differences in the $[CO_2]_a$-acclimation responses of the trees and grasses.

2. Materials and methods

2.1. Plants and growth conditions

Seeds of *Vachellia karroo* (Hayne) (formerly *Acacia karroo*) were obtained from the Desert Legume Program, (Tucson, AZ, US), and both *Combretum apiculatum* (Sond.) and *Celtis africana* (N.L.Burm.) from Silverhill Seeds (Cape Town, ZA). *V. karroo* is a leguminous tree typical of open savannas, *Combretum* spp. are common in miombo closed savanna woodland, and *C. africana* is a forest tree. Germinated seeds were randomly distributed between six controlled-environment growth chambers (Conviron BDR16, Conviron, Manitoba, CA) and grown for 18 months prior to measurements. C_4 grass seeds of *Eragrostis curvula* ([Schrad.] Nees) (accession number PI-155434), *Heteropogon contortus* ([L.] P.Beauv. ex Roem. & Schult.) (PI-228888) and *Themeda triandra* (Forssk.) (PI-208024) were obtained from the Germplasm Resources Information Network (GRIN, Agricultural Research Service, USDA, Washington D. C., US). These grasses span a range of adaptations to fire and drought and are broadly representative of open African savannas. Once established, a plant from each grass species was randomly selected, split into individuals at the rhizome, distributed between the growth chambers, and grown for 12 months prior to measurements. We refer to the plants by genus from here on.

Plants were grown in 2.5 dm^3 pots (n = 4–10) filled with three-parts commercial loam-free top soil (Boughton Ltd. Kettering, GB) plus one-part John Innes No.3 compost (John Innes Manufacturers Association, Reading, GB). Growth chambers (two per $[CO_2]_a$ treatment) were maintained at three $[CO_2]_a$ levels of 200, 400, or 800 ppm and otherwise constant conditions of 26 : 17 °C and 70 : 50% relative humidity (day : night). A 12-hr photoperiod with a midday peak photosynthetic photon flux density (*PPFD*) of 800 μmol m^{-2} s^{-1} was imposed at canopy level. Light was provided from a 3:1 mix of 39-W white-fluorescent tubes (Master TL5, Philips, Eindhoven, NL) and 39-W red–blue fluorescent tubes (Grolux T5, Havells-Sylvania, Newhaven, GB), augmented with six 105-W halogen light bulbs (GLS, Havells-Sylvania). Plants were rotated weekly within, and monthly between, cabinets along with environmental settings to minimise block effects. From the outset, plants were watered to gravimetrically determined 80% pot capacity three times per week after 24–32 photoperiod hours since last watering and all pots were provided with 150 ml of 3:1:2 N:P:K soluble nutrient mix (Miracle-Gro® All Purpose Plant Feed, Scotts Miracle-Gro, Marysville, OH, US, diluted to 5 g nutrient mix l^{-1} water) every two or three weeks as part of the watering volume.

2.2. Leaf gas exchange and water potential

Instantaneous mid-afternoon leaf gas exchange was measured three times over six weeks on all plants using an infrared gas analyser, IRGA (LI6400XT, LI-COR Biosciences, Lincoln, NE, US) fitted with a 6 cm^2 cuvette and a red–blue LED light source (6400-02B, LI-COR Biosciences) under operational environmental conditions (denoted by subscript '$_{op}$') within the growth chambers after ~12 photoperiod hours since watering on young, fully expanded leaves. Two to four grass blades were carefully aligned side by side and held together with insulation tape, avoiding any overlapping between blades, and clamped between the gaskets such that the area of the gas exchange cuvette was filled entirely. Where tree leaves did not fill the cuvette we made leaf area measurements using scaled, digital images of each leaf, taken while still attached to the plant using a bespoke leaf clamp and camera stand. Leaf area was calculated using ImageJ software (NIH, Bethesda, MA, US) and was used to correct gas exchange data at the time of measurement.

To minimise environmental perturbations and the time for leaf gas exchange to stabilise, the cuvette and integrated gas analyser was placed inside the growth chambers, which were opened briefly to switch plants between measurements, while air was supplied from within the closed chambers to the IRGA console outside using plastic tubing and CO_2 was supplied from cartridges (Liss–Group, Répcelak, HU). We set reference air $[CO_2]$ (C_a, 200, 400 or 800 μmol mol^{-1}), block temperature (26 °C) and light intensity (500 μmol m^{-2} s^{-1}) in the cuvette to correspond to those of the growth chambers at the time of measurement (mid-afternoon), set a flow rate of 235 μmol s^{-1} and took a 10-s average reading after readings had stabilised. Pilot studies indicated that this regime, particularly *PPFD* of the growth and measuring environment, ensured optimal growth for both trees and grasses and captured responses between fully lit and shaded leaves. During operational leaf gas exchange measurements, we sampled an adjacent, young, fully expanded leaf from each plant and immediately determined midday leaf water potential (Ψ_{leaf}) using a Scholander pressure chamber (PMS Instrument Company, Model 1000, Albany, OR,