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Lucas Silva Costa¹ , Clapton Olimpio De Moura² , Sandra Janet Bucci³ , Julia Sonsin-Oliveira⁴ ,
Sueli Maria Gomes⁴ and Mercedes Maria Da Cunha Bustamante¹ ¹ Laboratório de Ecossistemas, Departamento de Ecologia (ECL), Instituto de Ciências Biológicas (IB), Universidade de Brasília (UnB), Brasília, DF, Brasil² Laboratório de Fanerógamas, Departamento de Botânica (BOT), Instituto de Ciências Biológicas (IB), Universidade de Brasília (UnB), Brasília, DF, Brasil³ Grupo de Estudios Biofísicos y Ecofisiológicos (GEBEF), Instituto de Bionciencias de la Patagonia (INBIOP), Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET) and Universidad Nacional de la Patagonia San Juan Bosco (UNPSJB), Comodoro Rivadavia, Argentina⁴ Laboratório de Anatomia Vegetal, Departamento de Botânica (BOT), Instituto de Ciências Biológicas (IB), Universidade de Brasília (UnB), Brasília, DF, BrasilE-mail: lscosta@yahoo.com**Keywords:** fertilization effects, sapwood hydraulic conductivity, water use, plant anatomical adjustment, water relations, vulnerability to cavitationSupplementary material for this article is available [online](#)**Abstract**

Brazilian savanna is a seasonally dry biome, highly diverse and distributed mainly on nutrient-limited soils. Interactions between water stress and nutrient availability are important evolutionary filters in these environments. Previous evidence indicated that reducing the nutritional limitation increases growth rate, optimizes water transport and decreases stomatal conductance in woody plants. However, the anatomical mechanisms that explain such responses are not well-understood. We studied the effects of long-term (20 years) nutrient addition (N, NP and P) on soil chemistry and hydraulic morphological and anatomical traits in six dominant woody savanna species. Nutrient addition and decrease in soil moisture, probably related to changes in grass cover, influenced the plant hydraulic traits at the anatomical level, namely increasing the xylem vessels' diameter. Consequently, the specific theoretical xylem conductivity (K_{tx}) increased in five species under NP and P addition. Additionally, the stomatal pore index (SPI) decreased with species-specific responses regarding the intrinsic water use efficiency (iWUE). Four species had higher vulnerability to cavitation (l_{vul}) under NP and P addition. Using community-weighted mean and structural equation modeling approaches, we observed that nutrient enrichment at the community level did not affect iWUE, while the vulnerability to cavitation (l_{vul}) strongly increased. The K_{tx} and SPI were positively and negatively affected by nutrient addition, respectively, but the effects were not as strong as expected due to contrasting species responses. These changes optimized water transport with a hydraulic safety cost and reduced water loss. In comparison with responses to N addition, the greater P-limitation in Cerrado vegetation explains the inter-specific convergence in the responses of P-fertilized individuals. We showed that long-term responses to increased nutrient availability in dystrophic soils include anatomical changes in savanna woody vegetation with relevant interactions with soil-plant-atmosphere water relations.

1. Introduction

Soil nutrient limitation is prevalent in tropical regions, including the Brazilian savannas, known as the Cerrado. Its soils are mainly dystrophic (e.g. soil of medium or low fertility), acidic and

with low cation exchange capacity (Haridasan 2000) being one of the leading environmental filters in the evolution of Cerrado (Haridasan 2008). Its highly weathered soils are mainly limited by phosphorus (P) availability (Nardoto *et al* 2006).

Soil nutrient enrichment could alter mycorrhizal association (Treseder 2004), and consequently reducing rhizomorphs, fungi structures that would transport water and nutrients in return for carbohydrates (Wallander and Nylund 1992, Gessler *et al* 2017). Besides increasing above/belowground biomass ratio, these changes in the rhizosphere are likely to promote woody plant hydraulic failure under extreme drought events (Gessler *et al* 2017). Some root traits influence nutrient addition responses, especially in plants highly adapted to the low nutrient concentration. For instance, proteoid roots in the Proteaceae family enhance nutrient uptake, allowing their growth in phosphorus-deficient soils (Lambers *et al* 2015).

The Cerrado provides and distributes water to eight of the twelve main hydrographic basins in Brazil. In particular, the biome comprises a significant part of the recharge area of the main groundwater reserve in the world, the Guarani Aquifer (Machado *et al* 2016). The Cerrado's integrity is, therefore, essential for the national and continental eco-hydrological functioning. The high biodiversity of this ancient biome is associated with its functioning and resilience. Changes in the above to belowground biomass ratio and the woody community, resulting from alterations in fire regimes, land use, soil eutrophication, and invasive species, compromise the Cerrado integrity. The biome has already lost more than 50% of its native vegetation cover and is considered one of the world's largest agricultural frontiers.

Cerrado climate is defined by a pronounced dry season with high evaporative demand and 90% of the precipitation occurs during the wet season. Thus, in addition to the nutrient limitation, Cerrado woody plants face seasonal water shortage. High atmospheric water demand during drought imposes structural (Abdala *et al* 1998), phenological (Lenza and Klink 2006) and physiological (Meinzer *et al* 1999, Bucci *et al* 2005) adaptations to Cerrado woody plants. Additionally, processes such as hydraulic lift (HL) (Moreira *et al* 2003, Scholz *et al* 2008) and stem water storage (Bucci *et al* 2004, Scholz *et al* 2008) play an important role in Cerrado vegetation water usage. Quijano *et al* (2013) stated that HL increases the soil nutrients mineralization and nutrient uptake by the woody plants, mainly N.

Responses of plant communities in seasonally dry ecosystems to increasing nutrient deposition demand more investigation considering the strong interactions between nutrient and water balances. Previous studies have investigated how Cerrado woody plants control water uptake, transport and transpiration under soil nutrient addition (Bucci *et al* 2006, Scholz *et al* 2007a, Goldstein *et al* 2013). Changes in soil nutrient availability had impacted growth rates, carbon uptake and plant–water relations (Goldstein *et al* 2013). Cerrado woody plants present high

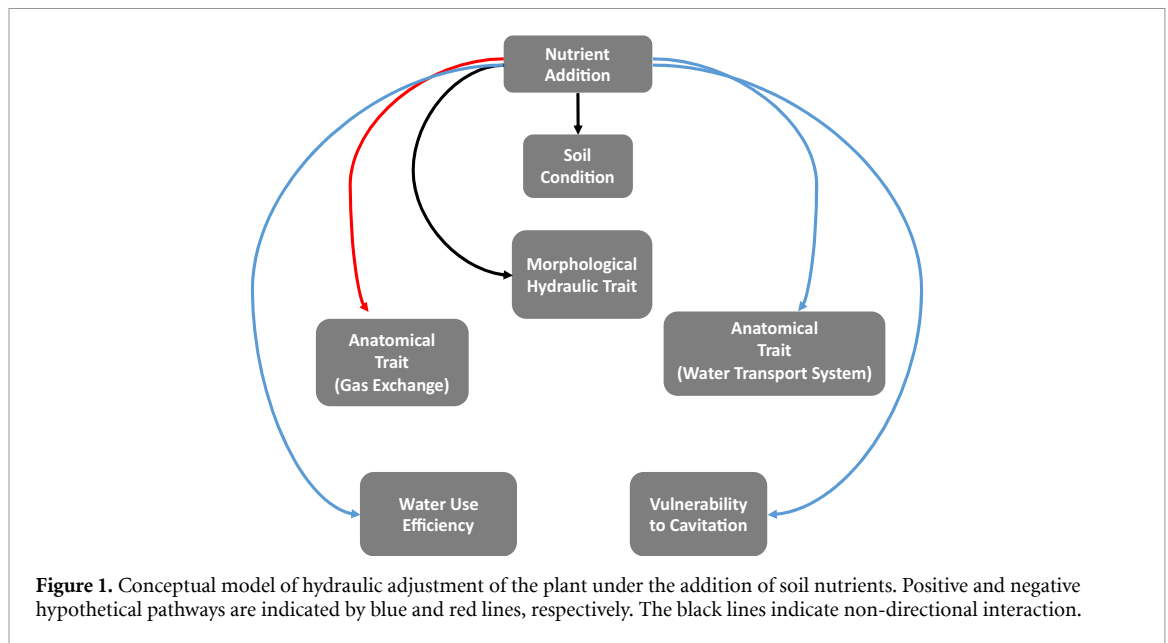
hydraulic plasticity to resource availability (Bucci *et al* 2006, Scholz *et al* 2007b). However, the anatomical mechanisms involved are still unknown. They might represent relevant plant traits to cope with long-term environmental changes. Structural changes at the cellular and whole-plant levels may increase the water use efficiency, optimize carbon assimilation and affect nutrient absorption, which are interconnected processes.

As a cumulative ecosystem response, it is expected that community composition changes under long-term soil nutrient enrichment (Farrer and Suding 2016), as observed for the Cerrado vegetation (Bustamante *et al* 2012). Plant community shifts resulting from species fitness impacts under environmental changes also result from competition asymmetry (Hautier *et al* 2009, Farrer and Suding 2016). Studies bridging environmental impacts at different scales or organizational levels are still scarce. For example, it is not well understood how changes at the cellular level influence a species' fitness and, consequently the community or whether interspecific competition may promote morphological and anatomical changes in the individual under environmental changes.

In this context, the present work raised the following question: how do long-term nitrogen and phosphorus enrichment change hydraulic architecture at the cellular, whole-plant and community levels of Cerrado woody species? We evaluated leaf and sapwood morphological and anatomical traits in six dominant woody species under the experimental addition of nitrogen (N), phosphorus (P) and both elements combined N + P (NP).

Given the evidence of increased Cerrado woody plant growth rate under fertilization (Bucci *et al* 2006, Goldstein *et al* 2013), we hypothesized higher efficiency in water use—more assimilation than water loss under fertilization—because of hydraulic adjustments. Besides morphological (leaf and wood) responses of woody plants under soil fertilization, we assessed anatomical structures responsible for hydraulic conductivity that are affected by ecosystem eutrophication.

A suggested trade-off is that increased hydraulic transport efficiency can reduce hydraulic safety (i.e. the ability of the hydraulic system to resist embolism formation and spread, and consequently loss of conductivity) (Hacke and Sperry 2001, Sperry 2015, Gleason *et al* 2016). A potential hydraulic failure due to faster-growing rate when more nutrients are available. A schematic model (figure 1) illustrates the hypotheses and suggests how fertilization could affect soil conditions, morphological and anatomical hydraulic traits, plant water use and safety.



2. Material and methods

2.1. Study site and species selection

We conducted this study in a Brazilian savanna area classified as cerrado *stricto sensu*, characterized by a continuous grass layer and a woody layer of trees and shrubs varying in cover from 10% to 60% (Ribeiro and Walter 2008). Cerrado *stricto sensu* represents 60% of the second largest South American biome. The site is located at the RECOR ecological reserve (15° 56' 41" S and 47° 53' 07" W, altitude 1100 m). The climate is Aw, according to Köppen classification. The average annual rainfall is 1500 mm and rainy season occurs from September to May. The daily maximum and minimum temperatures are 28.5 °C and 12 °C, respectively. Soil is a deep Oxisol containing about 70% clay, acidic with high Al levels, low cation exchange capacity and well-drained.

The field design consisted of 15 × 15 m plots with a buffer zone of 10 m between them and four plots per treatment. We randomly assigned the treatments to each plot. The four fertilization treatments were: control (no added fertilizer), N (100 kg of N ha⁻¹ yr⁻¹ as (NH₄)₂SO₄), P (100 kg of P as Ca(H₂PO₄)₂ + CaSO₄·2H₂O) and NP (100 kg of N + 100 kg of P in the forms previously described). The granular fertilizers were manually applied on the litter layer twice a year for 8 years (1998–2006) and resumed in September 2017. At the beginning of the experiment, the plots did not differ significantly in terms of soil nutrient concentrations and vegetation structures (Kozovits et al 2007).

Among the 20 most abundant species in the experimental area, we selected six species distributed in all plots: three evergreen with seasonal growth species—*Ouratea hexasperma* (A.St.-Hil.) Baill. (Ochnaceae), *Roupala montana* Aubl. (Proteaceae), *Styrax ferrugineus* Nees & Mart.

(Styracaceae)—and three brevideciduous/deciduous species—*Blepharocalyx salicifolius* (Kunth) O. Berg (Myrtaceae), *Caryocar brasiliense* Cambess. (Caryocaraceae) and *Qualea parviflora* Mart. (Vochysiaceae). We sampled five individuals per species per treatment (table S1 (available online at stacks.iop.org/ERL/16/055021/mmedia)).

2.2. Soil sampling and analyses

We estimated the volumetric water content (VWC) at three points below the crown of each individual using a 10 cm soil moisture sensor (HydroSense II, Campbell Scientific, USA). We collected three soil samples per plot at depth intervals 0–5 and 5–10 cm and then we prepared a composite sample for each depth interval. The soil analyses comprised soil pH in H₂O, available P and K, exchangeable Ca, Mg and Al by the Soil Laboratory of the Federal University of Viçosa (UFV), Brazil. We used composite samples from 0 to 10 cm depth for nitrogen and carbon isotopic analyses.

2.3. Foliar nutrient and water use efficiency

The foliar concentrations of nitrogen (N), phosphorus (P), potassium (K), calcium (Ca), magnesium (Mg), sulfur (S), manganese (Mn) and aluminum (Al) were determined from a composite sample from three leaves per individual (Silva 1999). The determinations were performed by Soil Laboratory of the UFV, Brazil.

Isotopic ratios of carbon (¹³C/¹²C) and nitrogen (¹⁵N/¹⁴N) of each sample were determined by a Thermo Quest-Finnigan Delta Plus isotope ratio mass spectrometer (Finnigan-MAT; CA) interfaced with an Elemental Analyzer (Carla Erba model 1110; Milan, Italy) at the Ecological Isotopic Laboratory, CENA-USP, Brazil (formula 1, table S2).

The intrinsic water use efficiency (iWUE), is it the ratio of net photosynthesis rate to transpiration (formula 2, table S2), it was calculated following Farquhar *et al* (1989) and Pérez-Harguindeguy *et al* (2013) protocol. WUE changes rapidly and the isotopic methodology represents longer-term conditions, especially during favorable periods.

We used the foliar δN^{15} to assess if the species are using different N sources by comparing differences among δN^{15} values in samples from control and treatment plots.

2.4. Morphological traits

We measured stem diameter at 30 cm of tree base and total height for each individual. We estimated the total leaf area (TLA) by counting all leaves and multiplying by the average leaf area. We stored terminal branches in a thermal box and fresh materials were analyzed within 6 h after collection. The specific leaf area (SLA), leaf area to sapwood area (LA/SA) and stem specific wood density (SSD) were determined according to Pérez-Harguindeguy *et al* (2013). All parameters from three samples of each individual and for SSD we removed the bark and pith. The LA/SA_{PLANT} is the TLA per basal area, which represents the sapwood transversal area.

2.5. Leaf and sapwood anatomy

We used the same terminal branches used in the previous procedures for the sapwood anatomy analysis. We made transverse sections in a sliding microtome with a 25 μm thickness and selected between 30 and 40 sections. We then mounted the slides following Johansen's (1940) protocol and quantified vessel diameter (VDia) and vessel density (VD) with Image-Pro Plus software version 6.0.

Based on the Hagen–Poiseuille law, we calculated the specific theoretical xylem conductivity (K_{tx}) from the VDia and normalized by sapwood cross-section area (formula 3, table S2).

We calculated the vulnerability to the cavitation index (I_{vul}) by the ratio of mean VDia to the VD as Carlquist *et al* (2001). High I_{vul} values indicate high xylem functional vulnerability to water stress.

We collected three leaves fully expanded and sun-exposed of each individual and fixed them in FAA 50° GL and stored in 50% alcohol following (Johansen 1940). We prepared the foliar transversal sections using histological resin following the protocol suggested by the manufacturer (Leica) and using the rotating microtome (Leica RM 2145, Wetzlar, Germany). We used a light microscope (Olympus BX40) to obtain the anatomical images from leaf sections of 0.08 μm thickness. The stomatal density (StoD) and length (StoL) were obtained from paradermic sections using Franklin's solution. We did the mesophyll tissues and stomatal characterization for five of six species. We chose to exclude *C. brasiliense* from

mesophyll analysis and give priority to its stomatal-related traits. The stomatal pore index, SPI (formula 4, table S2), was calculated for all species, except to *S. ferrugineus* that has stomata hidden in crypts.

2.6. Statistics

We compared obtained data from control and fertilized individuals using a pairwise *t*-test after verifying data normality. We used a permutation test to explore differences among data distribution, considering the effect size and the 95% confidence interval. We performed a two-way PERMANOVA test to compare the treatments and inherent species traits influences. We made a principal components analysis (PCA) to identify possible patterns among selected species and nutrient enrichment effects.

To understand how community composition changes due to soil nutrient enrichment affect hydraulic traits, we calculated the community-weighted mean (CWM, formula 5, table S2) for selected traits. We used the relative abundance of each species (i.e. the number of individuals of one species as a percentage of all species in each plot). Subsequently, the CWM traits were used as inputs for the structural equation modeling (SEM). SEM is a multivariate statistical framework and allows a rigorous estimation of direct effects and tests for the overall fit of a complex network of variables (traits) (Grace *et al* 2010). We considered nutrient addition treatments (N, P, and NP) as an exogenous predictor. We chose the best-fitting model which satisfies the valid model premises, e.g. comparative fit index higher than 0.96 and standardized root mean square residual less than 0.09 (Hooper *et al* 2008). The best-fitting SEM was interpreted as a proxy of the Cerrado woody community under soil nutrient enrichment, mentioned hereafter as an aggregated approach. We conducted analyses in R (R Core Team 2018). The CWM and SEM analysis scripts are available in the supplementary material.

3. Results

3.1. Soil physical and chemical properties

Control and treatment plots differed significantly regarding soil variables (table S3). The soil pH in all treatments was lower than in the control plots. The VWC was also reduced in NP and P plots. The available soil P concentration was lower in the N plots and higher in NP and P plots. There were no significant differences in total N concentration between plots (table S3).

3.2. Foliar nutrients

The nutrient addition treatments altered the foliar nutrient concentrations in comparison to control (table S4). P foliar was higher in all species except in *R. montana*, whereas the N concentrations were significantly higher in *C. brasiliense* and *Q. parviflora*.

The foliar N/P ratio of *B. salicifolius*, *O. hexasperma* and *S. ferrugineus* decreased significantly in NP and P treatments. In five of the six studied species, one of the elements (N or P) showed a decrease when the other element was manipulated (e.g. significantly lower N foliar values in P-fertilized plots). Our results point to a stronger P-limitation and a balancing of foliar N and P.

The leaf δN^{15} natural abundance ranged from -3.50 (*B. salicifolius*) to $+4.89$ (*R. montana*) in control plots. The addition of N alone or in combination with P increased leaf δN^{15} . *B. salicifolius* presented significantly higher δN^{15} in N and NP plots, *C. brasiliense* in N plots and *S. ferrugineus* in NP plots (table S4).

The iWUE also increased in *B. salicifolius* and *S. ferrugineus*, under P and NP addition, respectively. On the other hand, *Q. parviflora* and *R. montana* presented a decrease in P-fertilized individuals (figure S1).

3.3. Plant morphological traits

Under N addition, three species increased LA/SA_{PLANT}, a tree architectural index of potential constraints on water supply to transpiration demand: *B. salicifolius* (60% in N and 20% in NP), *C. brasiliense* (50% in N) and *S. ferrugineus* (130% in N and also in P). LA/SA_{PLANT} showed a considerable intra-specific variation, 57% (table S5).

Branch SSD did not differ significantly between control and fertilized individuals (table S4). Regarding SLA, *B. salicifolius* increased by 12% (NP), while *C. brasiliense* decreased by 14% in N. *O. hexasperma* decreased by 12% (P), while *Q. parviflora* increased by 41% in NP. *R. montana* decreased approximately 30% in all treatments and finally, *S. ferrugineus* did not change more than 10% in any treatment.

3.4. Sapwood anatomy

The most striking changes under nutrient enrichment were observed in the sapwood anatomy. The vessel diameter (VDia) increased in *S. ferrugineus* (30% (N), 34% (NP), 36% (P)); *O. hexasperma* (10% (N), 19% (NP), 30% (P)); *C. brasiliense* (16% (N), 35% (NP), 12% (P)) and *B. salicifolius* (12% (NP), 11% (P)). Only *R. montana* presented a significant decrease in VDia ($-13%$ (N), $-17%$ (NP), $-23%$ (P)) (table 1, figure S2).

Five species (*C. brasiliense*, *O. hexasperma*, *B. salicifolius*, *S. ferrugineus* and *Q. parviflora*) presented smaller VD (from 4% to 41% reduction) in at least one of three treatments respect to the control. Once again, *R. montana* showed a different pattern, with an increase of VD of 27% and 38% in NP and P plots, respectively (table 1, figure S3).

The specific theoretical xylem conductivity (K_{tx}) increased in all studied species under nutrient addition, except in *R. montana* that showed a decrease of K_{tx} in $-47%$ (N), $-11%$ (NP) and $-28%$

(P) (figure 2). *S. ferrugineus* increased K_{tx} in the three treatments respect to the control (193% (N), 256% (NP) and 279% (P)). In *B. salicifolius* and *O. hexasperma*, K_{tx} increased only in P and NP plots (approx. 40% in NP and 90% in P plots). *Q. parviflora*, K_{tx} increased by about 40% with N and NP addition but decreased by 23% under P addition. Similarly, the K_{tx} in *C. brasiliense* increased K_{tx} in 232% in NP plots and decreased by 23% in P plots.

The vulnerability to cavitation index (l_{vul}) was higher (from 29% to 96%) in five species (figure 3). In the case of *B. salicifolius*, this index increased in (NP) and (P) but decreased in N plots. *Q. parviflora* individuals showed an increase of 11% under P addition and a decrease under N and NP treatments. *R. montana* presented a reduction of l_{vul} in all treatments comparing to control (figure 3).

3.5. Leaf anatomy

Leaf anatomy changed in response to soil nutrient enrichment, but responses were species-specific regarding mesophyll organization. *Q. parviflora* and *S. ferrugineus* showed bundle sheath extension, while *R. montana* leaves presented sclerenchyma tissue underneath the adaxial epidermis (figure 4). *S. ferrugineus* differed from the other species regarding the adaxial epidermis thickness, the abaxial epidermis roughness, presence trichomes and the lower leaf thickness (LT). This species also presented significantly thinner abaxial epidermis in all treatments compared to control (figure 4).

There was a noteworthy decrease in the stomatal length (StoL) for fertilized individuals of *Q. parviflora*, *B. salicifolius*, *C. brasiliense* and *R. montana*. However, StoL did not change in *O. hexasperma* under nutrient enrichment (table 1, figure S4). The stomata density (StoD) followed an inverse tendency to StoL. However, changes in StoD were lower than the differences in StoL under soil nutrient addition (table 1, figure S5).

The SPI decreased under nutrient enrichment in four of the five species. *Q. parviflora* showed lower (40%–50%) SPI in all treatments respect to control plots. The SPI of *C. brasiliense* decreased by 30% in P plots and *B. salicifolius* presented a decrease of 20% in N plots compared to control. *R. montana* responded to both treatments with lower SPI in N and P treatments (18% and 23%, respectively) (figure S6).

3.6. Anatomical structures of gas exchange and water transport systems under soil nutrient enrichment

Soil nutrient enrichment has changed anatomical structures involved in gas exchange and water transport ($p < 0.0003$, PERMANOVA). However, despite the converging changes among species due to nutrient enrichment, these were more explained by species-specific traits than by nutrient treatments ($p < 0.0001$, pseudo- $F_{Species} = 29\ 000$; Pseudo- $F_{Treatment} = 8000$).

Table 1. Leaf and sapwood anatomy traits under nutrient addition. Values are average \pm SD ($n = 4-6$). Significant effects of fertilization with respect to the control are indicated as * $p < 0.05$ and ** $p < 0.01$ (pairwise t -test). Nitrogen (N), nitrogen + phosphorus (NP) and phosphorus (P) treatments. The methodology adopted by our study, regarding vessel density, limited the analysis within each species. Due to the automated procedure, the program may have considered the axial parenchyma along with the vessel lumen. This methodology is proposed for experiments in which it can be compared only between the studied groups: control and under nutrient addition individuals.

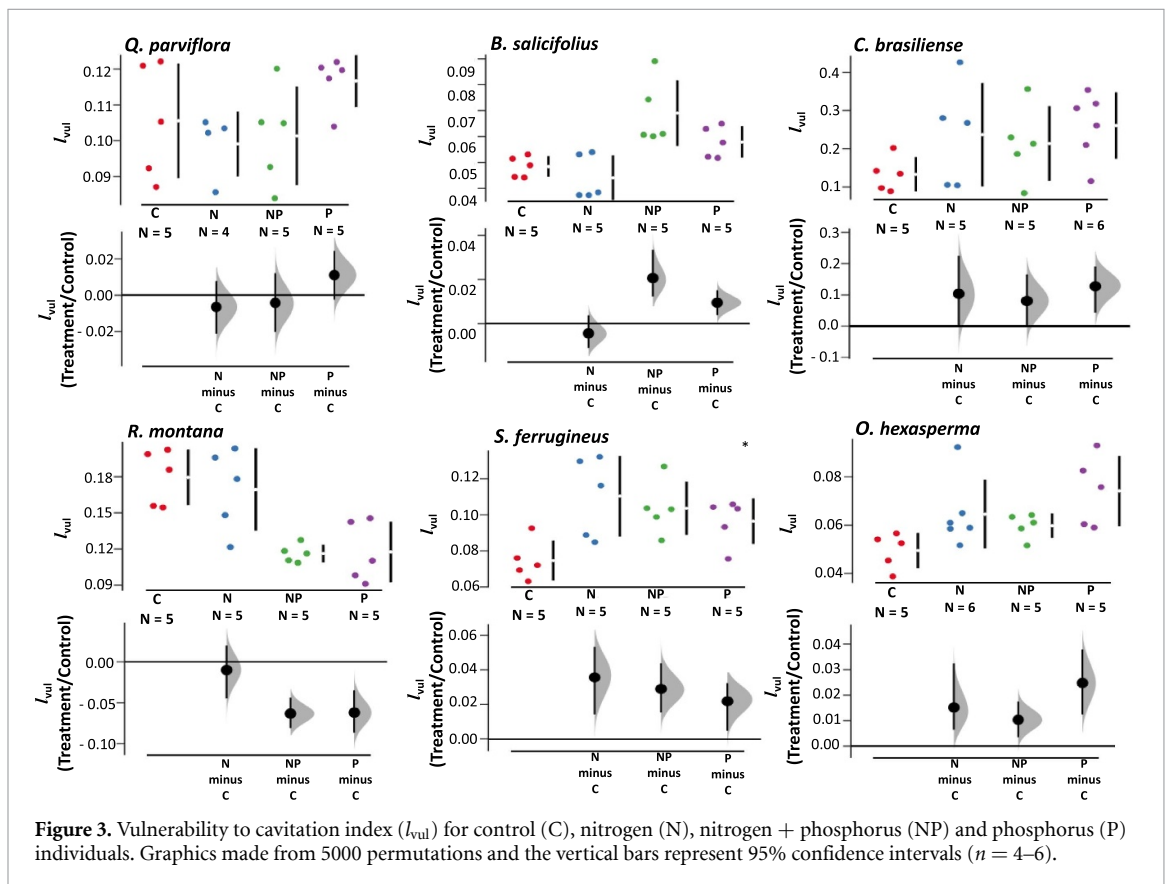
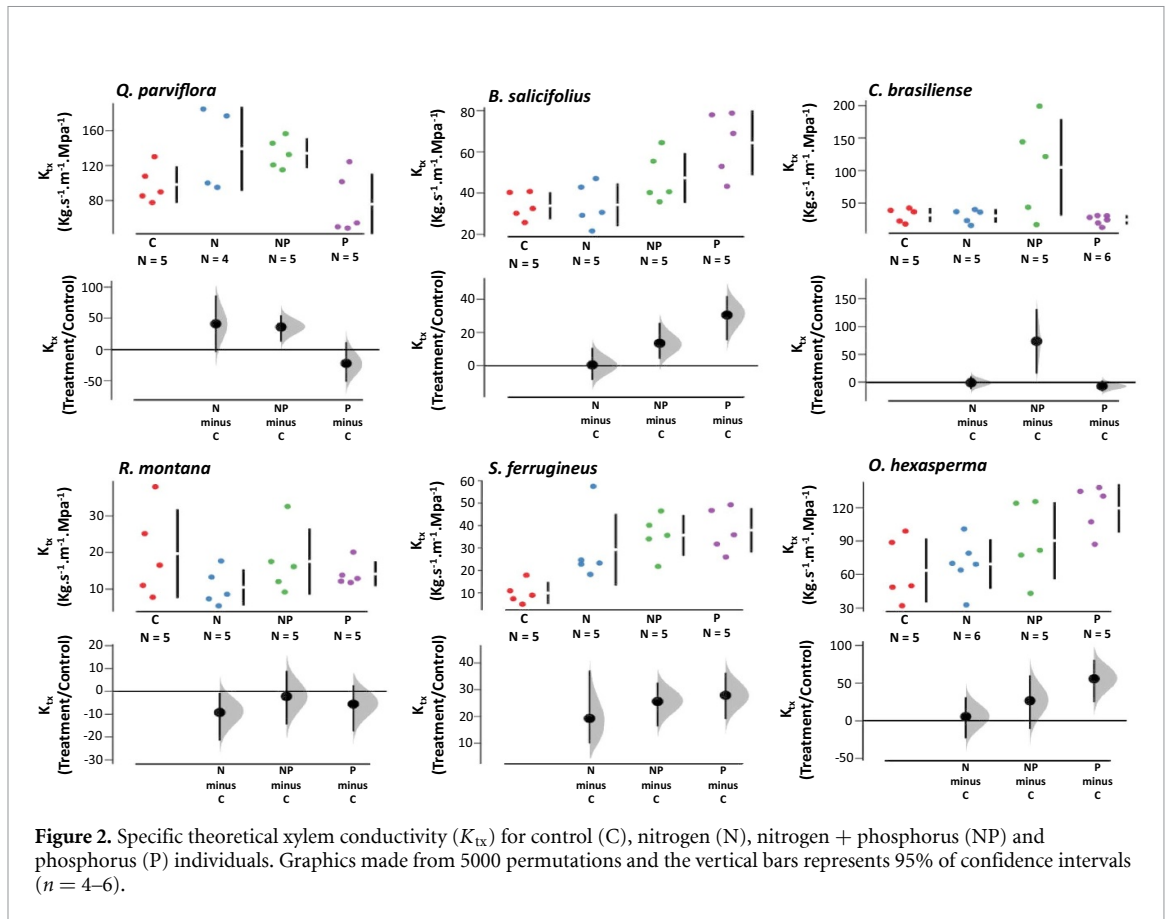
Species and treatment	Stomatal density (stomata: mm^{-2})	Stomatal length (μm)	Vessels density (vessel: mm^{-2})	Vessel diameter (μm)
<i>B. salicifolius</i>		Leaf		Sapwood
Control	356.2 \pm 25.6	16.7 \pm 2.8	551.9 \pm 38.7	26.6 \pm 1.3
N	378.2 \pm 19.7	15.5 \pm 2.0	587.8 \pm 72.6	25.5 \pm 3.1
NP	467.0 \pm 122.7	14.6 \pm 0.9	436.7 \pm 54.3	29.7 \pm 2.7
P	399.7 \pm 89.0	15.2 \pm 1.2	513.3 \pm 51.1	29.6 \pm 3.1
<i>Caryocar brasiliense</i>				
Control	263.6 \pm 26.5	21.1 \pm 2.0	395.2 \pm 112.4	48.6 \pm 2.2
N	253.2 \pm 25.1	23.4 \pm 4.5	308.9 \pm 162.8	56.2 \pm 5.2*
NP	205.5 \pm 44.9	22.9 \pm 1.7	352.6 \pm 121.2	64.0 \pm 17.8
P	243.8 \pm 41.5	18.7 \pm 1.6	231.7 \pm 77.5*	54.6 \pm 8.4
<i>O. hexasperma</i>				
Control	328.7 \pm 49.1	28.0 \pm 1.4	626.6 \pm 101.5	30.4 \pm 2.3
N	315.8 \pm 64.3	28.1 \pm 3.6	528.0 \pm 95.9	33.5 \pm 4.6
NP	338.2 \pm 47.8	28.0 \pm 1.5	610.4 \pm 53.8	36.3 \pm 1.9**
P	344.4 \pm 69.6	27.0 \pm 2.6	541.9 \pm 86.0	39.2 \pm 1.6**
<i>Q. parviflora</i>				
Control	382.7 \pm 87.2	22.9 \pm 2.1	387.7 \pm 57.1	40.2 \pm 1.2
N	340.2 \pm 77.6	17.8 \pm 3.0	423.6 \pm 24.0	41.9 \pm 4.0
NP	351.3 \pm 130.5	19.0 \pm 3.9	404.6 \pm 60.6	40.3 \pm 1.4
P	346.7 \pm 14.5	18.7 \pm 2.0	352.9 \pm 25.6	42.6 \pm 6.7
<i>R. montana</i>				
Control	284.6 \pm 77.2	33.4 \pm 4.5	219.8 \pm 34.1	38.9 \pm 2.1
N	279.2 \pm 43.7	29.9 \pm 1.9	204.8 \pm 40.0	33.7 \pm 1.9**
NP	277.0 \pm 33.8	34.0 \pm 4.5	279.0 \pm 43.6	32.3 \pm 3.6*
P	283.2 \pm 45.7	28.5 \pm 1.9	281.8 \pm 47.6	32.2 \pm 1.4**
<i>Styrax ferrugineus</i>				
Control	NA	NA	350.7 \pm 34.4	25.9 \pm 2.3
N	NA	NA	312.7 \pm 41.5	33.8 \pm 3.7*
NP	NA	NA	333.7 \pm 15.1	34.4 \pm 4.1*
P	NA	NA	369.5 \pm 43.9	35.2 \pm 2.0**

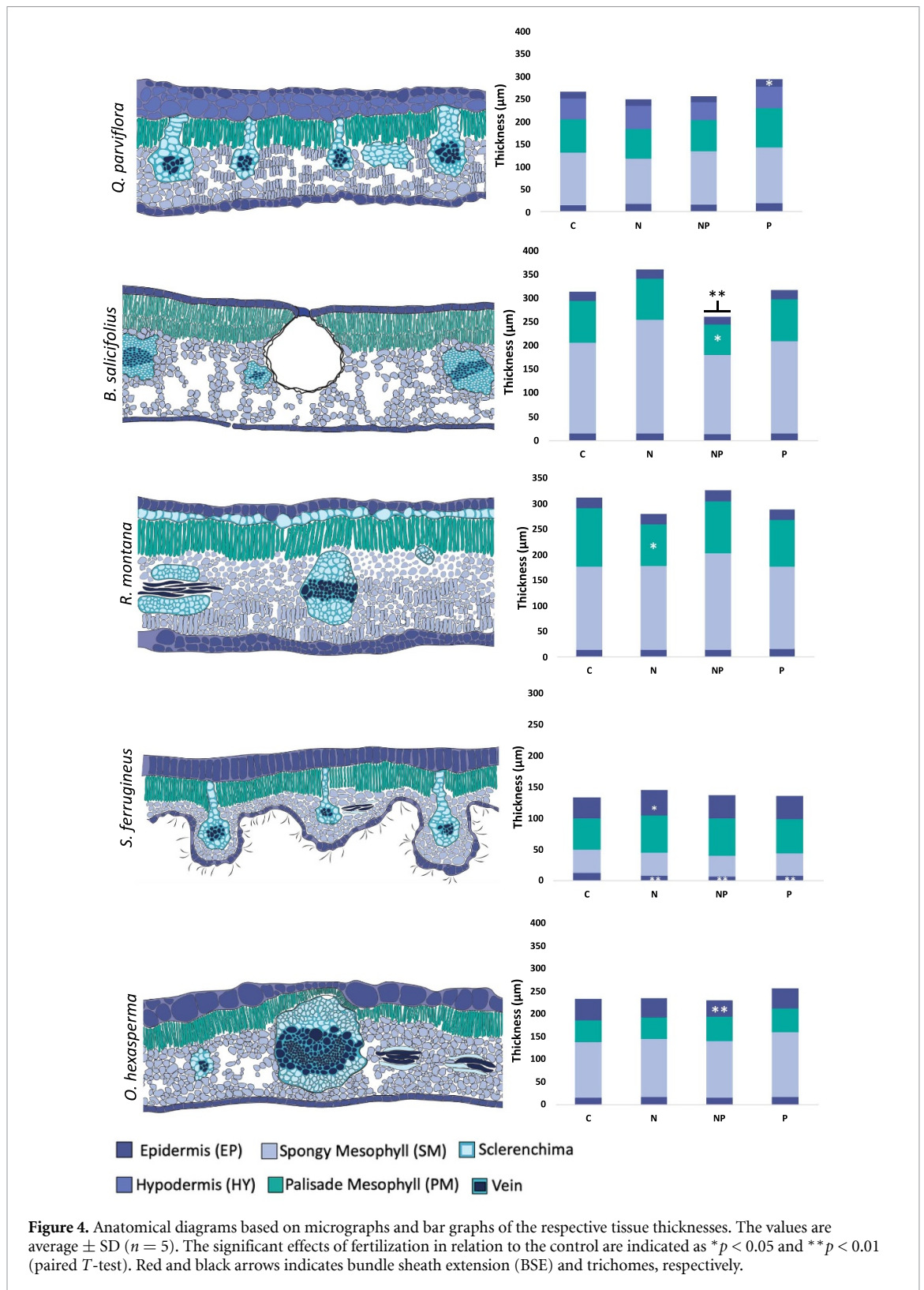
Foliar anatomical structures were analyzed based on two aspects, resistance and regulation of gas exchange. We accessed the leaf resistance to gas exchange measuring total LT and the contribution of the palisade mesophyll and spongy mesophyll to total LT. The regulation of gas exchange is basically the function of stomata, evaluated in terms of size (StoL) and density (StoD). We analyzed the wood anatomical structures of water transport as vessel diameter (VDia) and density (VDen).

A PCA was made based on the anatomical structures of gas exchange and water transport systems. PC 1 was more related to the foliar mesophyll traits (figure 5). Mesophyll thickness is related to leaf gas exchange resistance, e.g. *S. ferrugineus* leaves presented more gas exchange resistance than *B. salicifolius* (figures 4 and 5). PC 2 was composed mainly of transport (vessels) and regulation (stomata) hydraulic traits. We found a negative relationship between VDen and VDia (figure 5).

3.7. The anatomical hydraulic system of savanna woody species under soil nutrient enrichment: an aggregated approach

The use of the most representative species of the study area in an aggregate manner is a proxy for woody plant community responses to soil nutrient enrichment. Using CWM traits makes it possible to address how woody plant abundance shifts over long-term soil nutrient enrichment could influence our results. The best-fitting SEM showed a positive interaction of soil nutrient addition on the water transport system (K_{tx}). Conversely, nutrient enrichment negatively affected leaves gas exchange traits (SPI). However, due to contrasting species-specific responses, the effect was not as strong as we expected, e.g. $p > 0.05$ (figure 6). On the other hand, the vulnerability to cavitation index (I_{vul}) was the strongest positive relationship found by the model, e.g. fertilized communities presented a higher vulnerability to cavitation. Nutrients enriched plots presented lower soil moisture values, represented by the



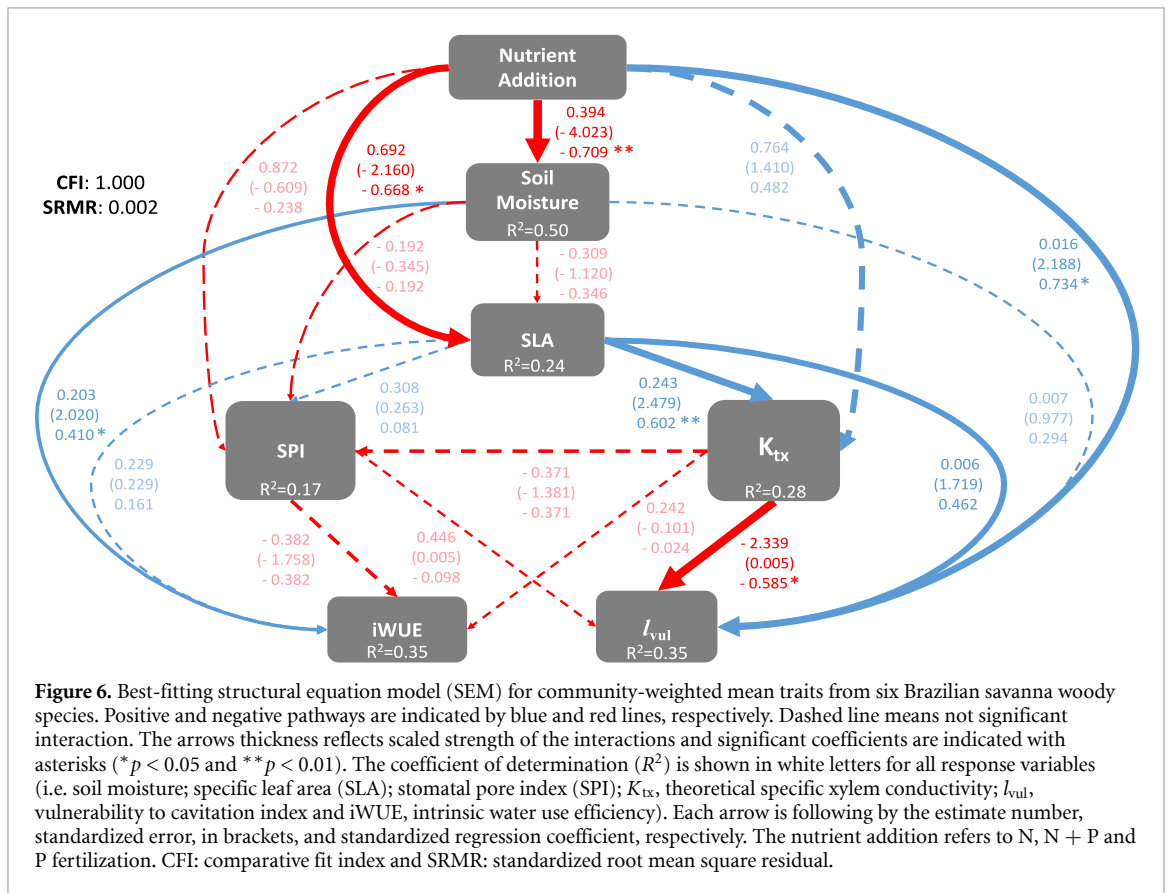
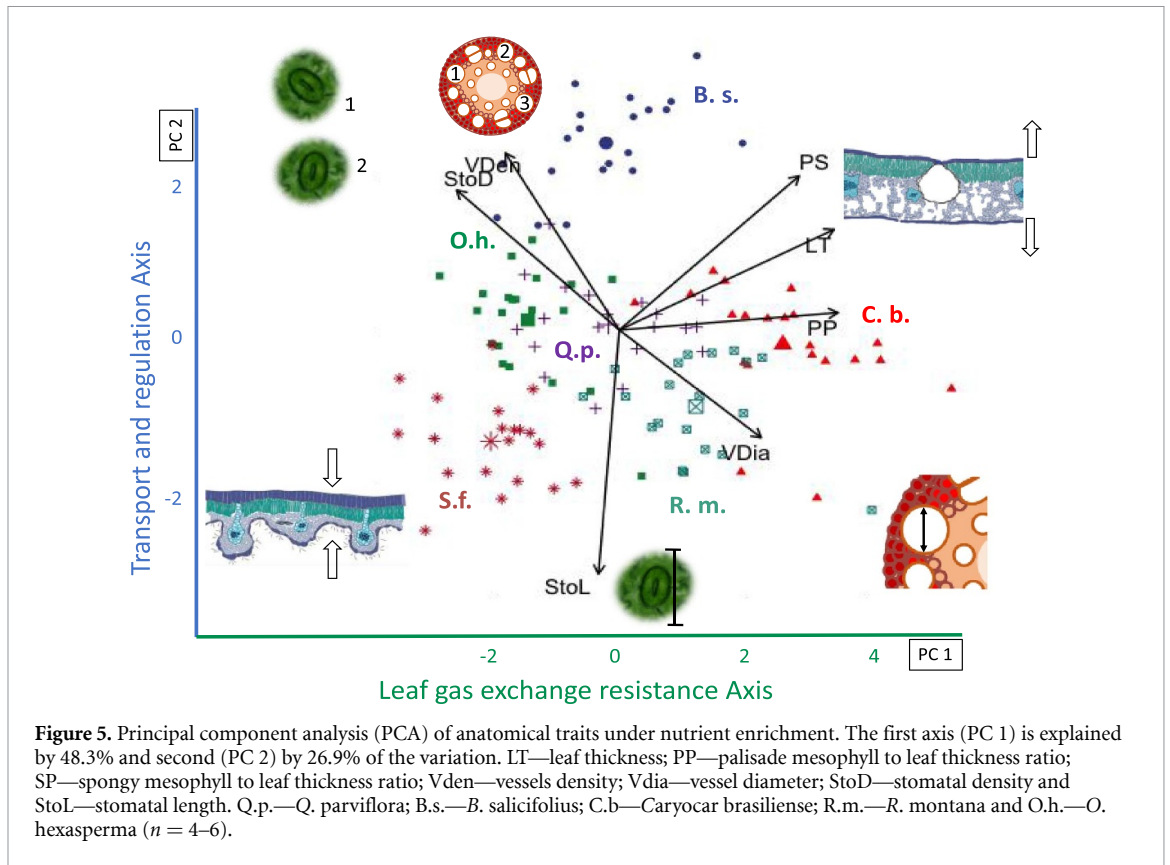


thick red arrow in figure 6. The studied woody plant communities over wetter soils presented higher water use efficiencies (iWUE). SLA appeared as an important morphological trait, decreasing under nutrient enrichment and showing a positive influence on the water transport system (K_{tx}) and vulnerability to cavitation index (I_{vul}). Finally, a negative relationship

between K_{tx} and I_{vul} might be explained by how the attributes were calculated (table S2).

4. Discussion

The nutrient enrichment changed soil characteristics directly by adding chemical elements and indirectly



by long-term changes in the plant community. The N and P fertilizers that contain sulfur resulted in soil acidification (Jacobson et al 2011). The invasion

by the African grass *Melinis minutiflora* P.Beauv. in response to P-addition (Bustamante et al 2012) may explain the decrease in the water availability observed

in the topsoil of P-fertilized plots. The VWC was negatively correlated with the coverage of the invasive grass (Mello 2019).

Most of the studied species are using additional P. The significant increase in foliar P highlights this nutrient's limitation in seasonally dry tropical ecosystems with highly weathered soils such as the Brazilian Cerrado (Nardoto *et al* 2006). The stoichiometric balance of the elements found in fertilized individuals' leaves also evidenced the co-limitation by P and N (table S4).

The wide range of foliar δN^{15} natural abundance in Cerrado plants indicates the use of different N forms or pools (Bustamante *et al* 2004). The N-addition (alone or in combination with P) changed the isotopic signature in three (*B. salicifolius*, *C. brasiliense* and *S. ferrugineus*) of the six selected species. Changes in the isotopic signature mean that these three species were using added N as a nutrient source. However, we cannot discard that the other species did not use the additional N, only that the foliar isotopic signature did not provide evidence.

The increase in TLA in four of the six selected species (table S4) may have been responsible for the rise in the evaporative demand to water supply index (LA/SA_{PLANT}). The N-addition has increased the TLA, but the sapwood, responsible for the water supply did not increase in the same magnitude. Thus, in morphological terms, the N-addition causes potential restrictions in water transport to the canopy. Spann *et al* (2016) have also reported foliage increase in woody plants under N-addition, while Zhang *et al* (2018) found a similar pattern for temperate and subtropical plants.

The sapwood vessels' diameter increased with nutrient enrichment. Based on previous studies at the same experiment plots (Bucci *et al* 2006), the increased growth rate under fertilization may result in changes in xylem structures. Our findings on changes in water transport structures are consistent with studies in temperate (Zhang *et al* 2020), subtropical (Zhang *et al* 2018) and tropical ecosystems (Spann *et al* 2016). We evidenced anatomical mechanisms behind changes in the xylem hydraulic conductivity under nutrient addition. Additional sapwood traits, such as vessel arrangement and vessel length, could also be relevant as found by Spann *et al* (2016). Despite the importance of morphological traits, such as wood density, we verified anatomical features affecting hydraulic conductivity without directly reflecting on morphological characteristics. Additionally, Bucci *et al* (2004) and Scholz *et al* (2008) have shown that wood tissues play a significant role in water storage processes. The stem water recharge rates and withdrawal for *S. ferrugineus* and *O. hexasperma* indicate a high water storage capacity. While Scholz *et al* (2008) also found evidence of stem expansion and contraction for *B. salicifolius* and *C. brasiliense*,

B. salicifolius presented a higher magnitude of withdrawal and recharge of stored water. High capacities of water storage constrain the magnitude of leaf water deficits in Cerrado trees (Scholz *et al* 2008).

Despite the expected increase in xylem conductivity (K_{tx}) the highly diverse Cerrado woody plant community also presented different K_{tx} responses under nutrient addition. Contrasting wood and leaf types can lead to unlike anatomical responses to nutrient enrichment (Zhang *et al* 2020). The species *R. montana*, in particular, was less responsive in comparison to the other species. It belongs to the Proteaceae family, known by specialized roots architecture, to improve P acquisition (Lambers *et al* 2015). The increased soil P-availability may have generated some toxicity to this species, limiting its growth and, consequently, its water transport system. Although there is evidence of P-toxicity affecting the growth of Proteaceae plants (Hawkins *et al* 2008, de Campo *et al* 2013), more studies are needed on the physiology of *R. montana* under the P-addition, as it showed an increase in abundance at P plots.

Higher hydraulic efficiency under nutrient addition resulting from greater vessels is associated with a higher vulnerability to cavitation (higher l_{vul}). These results are consistent with the finding of Spann *et al* (2016) in tropical tree species and with those of Hacke *et al* (2010) in an N-addition experiment. However, they diverge from the results of Bucci *et al* (2006), Villagra *et al* (2013) and Borghetti *et al* (2017) that observed a decrease in vulnerability to drought in fertilized woody species. Different methodological approaches to infer vulnerability to cavitation can also lead to controversial results. Hydraulic safety depends on several characteristics also involving vessel arrangement, membrane traits, cell wall, fibers and tracheids (McCulloh and Sperry 2005, Sperry *et al* 2006, Pratt *et al* 2007).

The concurrent decrease in stomatal length (StoL) across different species under fertilization shows the responsiveness of stomata to environmental changes. Decreasing StoL could improve stomatal sensitivity (Drake *et al* 2013). The soil nutrient enrichment reduced the structures responsible for water loss in the most studied species. The lower SPI, in fertilized plants, suggests reducing maximum stomatal conductance (Sack *et al* 2003) in woody savanna plants. The increase in TLA, while SPI decrease, suggests an adaptive response to enhance carbon gain while reducing water loss under a changing environment (Hetherington and Woodward 2003).

Plant anatomical responses under environmental changes is an emerging issue. Anatomical hydraulic traits might be related to woody species fitness at long-term nutrient limitation alleviation in Cerrado ecosystems. In the experimental plots, the abundance of *S. ferrugineus* increased by two-fold when N

and P were added separately and four-fold in NP plots (unpublished results). *S. ferrugineus* is an evergreen species with highly adapted leaves to minimize water loss (figures 4 and 5). Its leaf traits reduce the physical influence of air, increasing the leaf surface aerodynamic resistance to gas exchange. With low water loss, this species would increase the carbon gain and outcompete other species under fertilization. The abundance of *R. montana* increased threefold under P-addition (NP and P treatments). However, this species presented very conservative hydraulic responses under nutrient addition.

On the other hand, the occurrence of *O. hexasperma* decreased in the same plots where *R. montana* increased. *O. hexasperma* showed increased hydraulic efficiency but no adaptation to reduce water loss. Its response may have harmed *O. hexasperma* that maintains its leaves during the dry period (evergreen leaf habit). The abundance (Jacobson *et al* 2011) and growth (Bucci *et al* 2006) of *B. salicifolius* were positively affected by N addition in the first decade of the experiment. After 19 years, the abundance of *B. salicifolius* only decreased in P plots (unpublished results). *B. salicifolius* had dimorphic root systems and also evidences of HL (Moreira *et al* 2003, Scholz *et al* 2008). Quijano *et al* (2013) argues that HL increases the soil nutrients mineralization and uptake by woody plants, mainly N and in higher diverse vegetation. Probably it may explain the *B. salicifolius* success on N-fertilized plots (N and NP). While in *Q. parviflora*, an aluminum accumulator species, gas exchange traits decreased in all treatments. Aluminum accumulator species are highly adapted to dystrophic Cerrado soils (Haridasan *et al* 1986) and the abundance of *Q. parviflora* decreased in NP plots. Finally, *C. brasiliense* increased its SPI and TLA in N plots, indicating more significant water loss, while its abundance decreased. The results indicate more substantial effects of nutrient addition on plant abundance of evergreen species, suggesting that nutrient enrichment in these savannas might have profound impacts on the ecosystem functioning through changes in the abundance of phenological groups.

The aggregated approach elucidates emerging patterns in woody savanna plants' anatomical mechanisms under nutrient enrichment. Although we found a high diversity of morphological and anatomical responses, the aggregated approach was robust enough to represent a proxy for the highly diverse Cerrado woody community. Besides the particular effects of each species, it was possible to observe convergent responses, mainly under P-addition. This convergence's evidence is the strong influence of nutrient enrichment in the vulnerability to cavitation index (I_{vul}) and the SLA. Changes in the fitness of the studied species may relate to changes in the anatomy of the water transport system, as well as associated with the asymmetric competition with impacts

on ecosystems. The role of nutrients in studies of drought-induced woody plant mortality is an incipient but rising topic (Gessler *et al* 2017). Our results reveal the interactions between nutrient limitation and water transport anatomical structures weighted by community species shift in seasonally dry ecosystems that might affect ecosystem functioning under eutrophication.

The Cerrado, the most biodiverse savanna globally, is threatened by the expansion of cattle raising and large scale agriculture that imply an intensive use of agrochemicals and fertilizers. Changes in land management also affect the remnants of native vegetation due to border effects and environmental pollution. Our study pointed out eutrophication impacts at different scales and organizational levels, where changes at the cellular level influence the woody plant community's structure. On the other hand, the interspecific competition and the entry of invasive grasses have altered cellular hydraulic structures. Understanding how nutrient inputs into ecosystems with low fertility soils is crucial to define environmental-friendly thresholds for fertilizers use in tropical agriculture.

Data availability statement

The data that support the findings of this study are openly available at the following URL/DOI: <https://doi.org/10.6084/m9.figshare.13252052.v1>. Data will be available from 01 February 2021.


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