

Research Article

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Effects of nutrient addition on polyphenol and nutrient concentrations in leaves of woody species of a savanna woodland in Central Brazil

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Abstract

We investigated whether changes in nutrient availability affected N, P, S and polyphenol concentrations in different leaf-development stages of three brevideciduous and three evergreen dominant woody species in a nutrient-limited savanna woodland in Central Brazil. Treatments included eight years of annual fertilization with 100 kg ha⁻¹ of N, P, N plus P and control, each replicated in four randomized 15 × 15-m plots. All species increased S concentrations (minimum 28%) in young and mature leaves in fertilized plots. *Dalbergia miscolobium* decreased total phenol concentrations with P (−34.3%, −23.7%) and NP fertilization (−28.2%, −17.1%). *Blepharocalyx salicifolius* increased total phenol (27.6%, 18.8%) and tannin (46.3%; 43.5%) in P fertilized and increased total phenol (33.9%) and tannin (27.8%, 43.5%) in NP fertilized plots. Total phenol concentration decreased with leaf age in *Ouratea hexasperma*, *Styrax ferrugineus* and *Blepharocalyx salicifolius*, which also decreased tannin concentration with leaf age. For all treatments, brevideciduous species had higher N, P, total phenols and tannin concentrations and lower S concentration than evergreens. These differences between phenological groups suggest that tropical ecosystems responses to environmental changes are more complex than anticipated by global vegetation models, with consequences for predictions in ecosystem functions and resilience.

Introduction

Polyphenols are the most widespread secondary metabolites in the plant kingdom (Hättenschwiler & Vitousek 2000) and are involved in nutrient cycling, water retention, soil temperature and P availability, and exert a dominant role in controlling plant-litter-soil interactions in a wide range of ecosystems (Hättenschwiler *et al.* 2005). They comprise about 40% of the dry weight of leaves and stems and a significant portion of the carbon in terrestrial ecosystems (Herms & Mattson 1992) and are a key component in many plant ecological processes. Polyphenols can reduce N mineralization through linking of organic N and removal of microorganisms, resulting in lower N leaching (Chapin 1995) and inhibit litter decomposition, increasing persistence of soil organic matter and cationic capacity exchange (Northup *et al.* 1995a). Soil nutrients affect polyphenol production (Hättenschwiler *et al.* 2003, Haukioja *et al.* 1998, Wright *et al.* 2010), and higher polyphenol levels are often associated with acid soils with low nutrient content (Haukioja *et al.* 1998, Northup *et al.* 1995a, 1995b, 1998; Kraus *et al.* 2004).

The savanna biome extends across Africa, Australia and South America and comprises a mosaic of plant physiognomies, from grasslands to forests (Lehmann *et al.* 2011). The savanna is the second largest biome of Brazil and the world's largest savanna (Sarmiento 1984). It is a global biodiversity hotspot due to high levels of endemism and rapid conversion of natural habitats into agriculture (Myers *et al.* 2000). Besides local and regional drivers of change, global changes also affect ecosystem functioning in this biome. Savanna woodland plants have morphological (Bieras & Sajo 2009, Sternberg *et al.* 2004) and physiological traits (Franco *et al.* 2005, Goldstein *et al.* 2008, Scholz *et al.* 2008) associated with growth under a markedly seasonal precipitation regime and low nutrient availability (Haridasan 1992). Responses to these changes may differ among functional and phenological groups. The phenological groups of woody species vary in leaf longevity, internal water balance (Lenza & Klink 2006), foliar nutrient concentrations (Franco *et al.* 2005), root architecture and temporal patterns of sap flow (Scholz *et al.* 2008). Some savanna-woodland tree species have high polyphenol leaf concentrations (Gonçalves-Alvim *et al.* 2006, 2011; Santos *et al.* 2002, Skidmore *et al.* 2010). These are positively correlated with Al soil concentration and negatively correlated with soil pH and concentrations of Ca, Mg, Mn, P and K (Jacobson *et al.* 2005).

Brazilian savanna is limited by N and P (Kozovits *et al.* 2007) and nutrient cycling (especially N and P) is very conservative (Bustamante *et al.* 2006). Thus, changes in nutrient dynamics can

lead to an improved environment for some species, increasing their competitiveness (Bobbink *et al.* 2010). A long-term study of nutrient addition in a savanna woodland demonstrated that increased nutrient availability led to changes in density, dominance and richness of herbaceous and shrub-tree layer, invasion of exotic grasses and increased rates of leaf litter decomposition, altering species composition and nutrient cycling (Bustamante *et al.* 2012, Jacobson *et al.* 2011, Kozovits *et al.* 2007).

Nutrient-rich ecosystems and fertilized environments tend to have lower polyphenolic production (Haukioja *et al.* 1998, Northup *et al.* 1995a, 1995b, 1998). Nonetheless, responses to changes in nutrient cycling rates due to fertilization may vary among phenological groups. Considering that the savanna woodland is a dystrophic environment, strategies to prevent nutrient leaching are important. Thus, because leaf turnover is higher in brevideciduous species than in evergreens, an increase in nutrient concentration in brevideciduous ephemeral leaves may require greater investment in polyphenol production to slow down decomposition and prevent rapid nutrient loss after senescence (Villar & Merino 2001). Therefore, greater nutrient availability may favour species with higher leaf turnover and shift the composition of phenological groups in ecosystems. Based on these considerations and considering the relevance of polyphenols for plant and ecosystem functions, we evaluated the responses of two savanna-woodland plant functional types (based on foliar phenology) to the changes in soil nutrient availability. We hypothesized that (1) N, P and S (present in the formulation of N and P fertilizers – ammonium sulphate and superphosphate) foliar concentrations will increase in response to N, P and N plus P fertilization but responses will differ according to phenology and leaf age; (2) foliar concentrations of total phenols and tannins will differ between phenological group (being higher in brevideciduous species than in evergreen species) and will decrease in response fertilization; and (3) responses will vary according to leaf age (i.e. polyphenol concentrations decreasing with leaf age).

Methods

Study area and fertilization treatments

This study was performed in the Roncador Ecological Reserve, which belongs to the Brazilian Institute of Geography and Statistics (RECOR/IBGE), near Brasília – Federal District, Brazil (15°56'S, 47°53'N, average altitude = 1100 m asl). The soil type is Oxisol (Haplustox), an acidic soil with high Al levels and low cation-exchange capacity (Haridasan 1994). Annual precipitation during the 2-y study period was 1667 and 1184 mm. Air temperature ranged from 10.1°C (July) to 31.9°C (October). The vegetation is classified as cerrado *sensu stricto* (savanna woodland) and is the most common savanna vegetation type in Brazil. It consists of a continuous grassy layer and a tree and shrub layer with 20% to 60% canopy cover (Eiten 1983).

The fertilization experiment began in 1998 with a completely randomized experimental design, with four nutrient-addition treatments and four replicates randomly divided into 16 plots of 225 m² (15 × 15 m) at least 10 m apart from each other. The following treatments were applied to the litter layer without incorporation into the soil: control (C; without fertilization), +N (addition of ammonium sulphate, (NH₄)₂SO₄), +P (addition of 20% superphosphate, Ca(H₂PO₄)₂ + CaSO₄·2H₂O), and +NP (simultaneous addition of ammonium sulphate plus 20% superphosphate). Between 1998 and 2006, 100 kg ha⁻¹ of N, P and +NP were applied

twice a year (at the beginning and end of the rainy season). The study was conducted after 8 y of fertilization, and in 2005, all the plots in the study area were accidentally burned but the plots were reinstalled immediately after the fire event (Bustamante *et al.* 2012, Jacobson *et al.* 2011, Kozovits *et al.* 2007). The burning duration was short due to the rapid consumption of fine fuel (mainly dry mass of grasses) thus reducing the impacts on the woody layer.

Soil sampling and analysis

Composite soil samples were collected at the beginning of the rainy season (October), consisting of two samples from each plot at five depths (0–10, 10–20, 20–30, 30–40 and 40–50 cm). We determined soil pH in water, total N (micro-Kjeldahl method), available P, K, and exchangeable Ca, Mg and Al. Soil P and K were extracted with Mehlich 1 solution, and Al was extracted with 1 M KCl solution. The available P concentration was estimated by colorimetry with ammonium molybdate. Exchangeable Al was quantified by titration with NaOH. Exchangeable Ca, Mg, and Fe concentrations were determined by atomic absorption spectrophotometry, and K concentration by flame emission (EMBRAPA 1999). Soil pH values were lower in the +N, +P and +NP plots than in the control plots at 0–30 cm depth. Available P concentration was higher in the +P and +NP plots at 0–40 cm depth. Soil Ca concentrations were higher at 0–30 cm depth in +P plots than in control plots. Soil Al concentration was higher at all depths in +NP plots and at 0–30 cm in +N plots. Soil pH values increased with depth in all plots, whereas total N, P, K, Mg and Al concentrations showed the opposite pattern. There were no differences in soil N, K and Mg concentrations between control plots and fertilizing treatments. For more details about soil results, see Jacobson *et al.* (2011).

Woody species

Brazilian savanna plant biodiversity comprises a large number of rare species and few oligarchic species (Ratter *et al.* 2012). In this study, we selected six woody plant species in the experimental plots from two phenological groups (three brevideciduous and three evergreen). *Caryocar brasiliense* A. St.-Hil. (Caryocaraceae), *Blepharocalyx salicifolius* (HBK) O.Berg. (Myrtaceae) and *Dalbergia miscolobium* Benth. (Leguminosae-Papilionoideae) are brevideciduous (with complete canopy replacement during the dry season, and a short period with few or no senescent leaves). The mean leaf lifespan of deciduous and brevideciduous species is 279 days (Souza 2012). *Oouratea hexasperma* (A. St.-Hil.) Baill. (Ochnaceae), *Roupala montana* Aubl. (Proteaceae) and *Styrax ferrugineus* Nees & Mart. (Styracaceae) are evergreens. They produce a cohort of new leaves that precedes or is concomitant with the loss of the older leaf cohort (Lenza & Klink 2006). The mean leaf lifespan of evergreens is 400 days (Souza 2012). These species, representing ~38% of community tree density, are among the 10 with the largest importance value index (IVI) (Jacobson *et al.* 2011). They are common savanna species in Brazil, as demonstrated by the analysis of 625 Brazilian savanna areas, occurring in 10.7% (*B. salicifolius*) to 62.2% (*R. montana*) of the sampled areas (Françoso 2014, Françoso *et al.* 2016).

Leaf sampling

Twenty individuals of each species were selected and marked (five individuals per treatment, totalling 120 individuals), considering health status and accessibility to foliage. Samples were collected between October and January of 2007 (i.e. beginning to middle

of the rainy season). Leaf samples for each individual were collected, at the same time, in three distinct growth stages: newly sprouted leaves (that had emerged less than 2 wk before), young leaves (younger than 2 mo, fully expanded, relatively thin with lighter pigmentation), and mature leaves (older than 4 mo, fully expanded, thicker with darker pigmentation). The leaves were dried at 60°C for 48 h and ground in a Wiley mill (40-mesh). For nutrient analysis, only young and mature leaves were used.

Total phenol extraction and analysis

Leaf extraction was performed at room temperature using 1 g of ground leaf for every 15 ml methanol, 50% (v/v). The samples were extracted over a 24-h period in a dark room and mixed with solvent renewal (+15 ml) (twice for 30 min). The extracts were filtered through 25- μ m filter paper (Whatman International, England) and stored at -2°C (Hagerman 2002). The Folin–Ciocalteu method was used to determine total phenol concentration (Forrest & Bendall 1969), by adding 2.5 ml of 10% (v/v) Folin–Ciocalteu reagent (Sigma-Aldrich) to 0.5 ml extract and 2 ml sodium carbonate solution (0.75 mg g⁻¹). The solution was left at room temperature for 1 h and analysed by spectrophotometry at 760 nm (Shimadzu UV-1203; Shimadzu Corporation, Japan). A standard curve was created using tannic acid (Sigma-Aldrich), and concentrations were expressed as percentage (dry mass) of tannic acid equivalents. All measurements were performed in triplicate.

Tannin extraction and analysis

Leaf tannin was analysed using the precipitation method (Hagerman & Butler 1978). Briefly, 1 ml extract was added to 2 ml bovine serum albumin fraction V (1 mg ml⁻¹; Sigma-Aldrich). The solution was left at room temperature for 15 min and centrifuged (3000 rpm) for 15 min. After removing the supernatant, the precipitate was dissolved in 4 ml solution of sodium dodecyl sulphate (Sigma-Aldrich) and triethanolamine (Sigma-Aldrich), and 1 ml ferric chloride (0.01 M) was added. After 30 min, absorption was measured at 510 nm using a Shimadzu UV-1203 spectrophotometer (Shimadzu Corporation). A standard curve was created using tannic acid (Sigma-Aldrich), and concentrations expressed as percentage (dry mass) of tannic acid equivalents. All measurements were performed in triplicate.

Foliar nutrients

Leaf samples were digested in a solution of nitric, perchloric and sulphuric acid (10:2:1). We determined the concentrations of sulphur with turbidimetry. Phosphorus foliar concentrations were determined with colorimetric analysis with ammonium molybdate and ascorbic acid, and nitrogen foliar concentrations were determined with the micro-Kjeldahl distillation method (EMBRAPA 1999).

Statistical analysis

Normal distribution of the variables was analysed with Kolmogorov–Smirnov test ($\alpha < 0.05$). Since data were not normally distributed, even after transformation, they were compared using the Mann–Whitney non-parametric test ($\alpha < 0.05$). Nutrient concentrations at each soil depth were compared with the F and Student's t-test ($\alpha < 0.05$). Comparisons with the control were made for each depth ($n = 4$). Arcsine square-root transformation was used for total phenols (dry mass %), tannins (dry mass %), and N (mg g⁻¹). Logarithm transformation was used for concentrations of P (mg g⁻¹) and S (mg g⁻¹) (Zar 1999). Differences in polyphenol

and nutrient concentration between treatments (control, +N, +P, +NP), phenological groups (brevideciduous, evergreens), species and growth stages (newly sprouted leaf, young leaf and mature leaf) were tested by repeated-measures ANOVA using analysis of mixed linear models, followed by the Bonferroni adjustment ($\alpha < 0.05$) and Dunnett test ($\alpha < 0.05$) for multiple comparisons. In the mixed linear model, errors and random effects allow the display of correlations between non-constant variables. Factors are assumed to have a linear relationship with the dependent variable. This model can be used to conduct repeated-measures tests, identifying factors that contribute significantly to the model (McCulloch *et al.* 2008). These analyses were performed using SPSS 15.0 for Windows (SPSS Inc., USA).

Results

Nutrient, total phenols and tannin foliar concentration in control plots

N mean foliar concentration was 14.1 \pm 4.1 mg g⁻¹, and ranged from 10.0 to 22.3 mg g⁻¹. Mean N concentration was 16.5 \pm 4.4 mg g⁻¹ in brevideciduous and 11.6 \pm 1.7 mg g⁻¹ in evergreens. Foliar P concentration was 0.67 \pm 0.2 mg g⁻¹, and ranged from 0.5 to 1.3 mg g⁻¹ (0.77 \pm 0.3 mg g⁻¹ in brevideciduous and 0.57 \pm 0.1 mg g⁻¹ in evergreens). S foliar concentration was 0.41 \pm 0.1 mg g⁻¹ (0.38 \pm 0.1 mg g⁻¹ in brevideciduous and 0.43 \pm 0.1 mg g⁻¹ in evergreens). Total phenol concentration (% dry leaf mass) was 13.5 \pm 6.3%, and ranged from 3.5 to 26.6% (18.9 \pm 3.3% in brevideciduous and 8.1 \pm 2.9% in evergreens). Tannin concentration was 5.2 \pm 3.2%, and ranged from 1.8–10.5% (6.1 \pm 3.3% in brevideciduous and 4.4 \pm 3.0% in evergreens) (Supplementary material table 1).

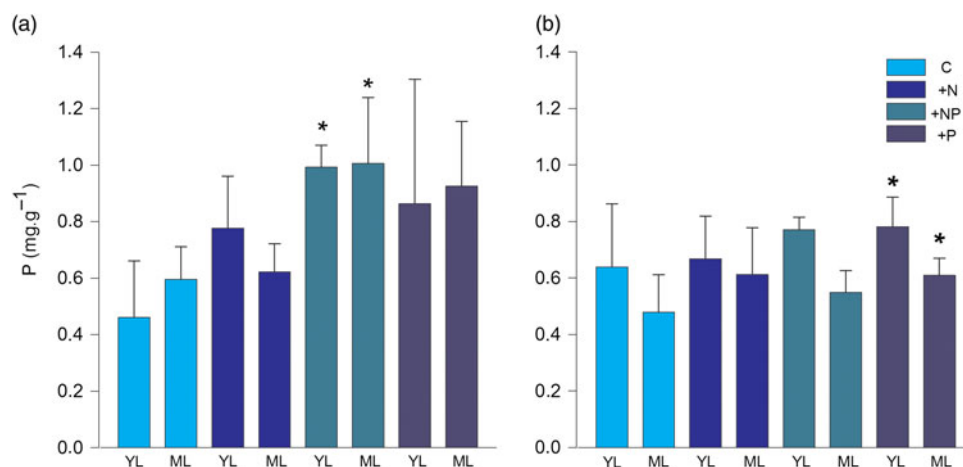
Nutrient, total phenol and tannin foliar concentrations in response to fertilization treatments

N, P, S, total phenols and tannin foliar concentration and differences between leaf growth stages in brevideciduous and evergreen species, in control and fertilized plots are presented in supplementary materials tables 1 and 2. Significant differences between N, P, S, total phenols and tannin foliar concentrations between control and fertilized plots, in six savanna woodland species are presented in Table 1.

Table 1. Significant differences (Dunnett, $P < 0.05$) in N, P, S (mg g⁻¹), total phenol and tannin foliar concentrations (% dry leaf) between treatments (C – control, N – nitrogen addition, P – phosphorus addition, NP – nitrogen plus phosphorus addition) in brevideciduous (*Caryocar brasiliense* (CB), *Blepharocalix salicifolius* (BS), *Dalbergia miscolobium* (DM)) and evergreen species (*Oureatea hexasperma* (OH), *Roupala montana* (RM), *Styrax ferrugineus* (SF)) in a savanna woodland in Central Brazil.

	N	P	S	Total phenol	Tannin
CB	-	-	(NP>C); (P>C)	-	-
BS	-	(NP>C)	(N>C); (NP>C); (P>C)	(NP>C); (P>C)	(NP>C); (P>C)
DM	(N>NP); (N>P)	-	(NP>C)	(NP>C); (P<C)	-
OH	-	-	(NP>C); (P>C)	-	-
RM	-	-	(N>C); (NP>C); (P>C)	-	-
SF	-	(P>C)	(N>C); (NP>C); (P>C)	-	-

Figure 1. Phosphorus concentration (mg g^{-1}) in young leaves (YL) and mature leaves (ML) in the brevideciduous species *Blepharocalyx salicifolius* (a), and in the evergreen species *Styrax ferrugineus* (b) in control plots (C) and fertilized plots (+N, nitrogen, +P, phosphorus; and +NP, nitrogen plus phosphorus) in a savanna woodland in Central Brazil. * Indicates significant differences with control plots (Dunnett, $P < 0.05$). Error bars represent the standard deviation.



Foliar N ($F = 108$, $n = 120$, $P < 0.001$) and P ($F = 72.8$, $n = 120$, $P < 0.001$) concentrations were higher in brevideciduous species whereas foliar S concentrations were higher in evergreens ($F = 20.2$, $n = 120$, $P < 0.001$).

Only the brevideciduous species *Blepharocalyx salicifolius* (+NP plots) and *Styrax ferrugineus* (P plots) presented higher P concentrations in P fertilized plots compared with control plots, both in young ($F = 3.8$, $P = 0.02$) and mature leaves ($F = 4.3$, $P = 0.01$) (Figure 1).

Foliar N:P ratios varied in response to fertilization ($F = 6.6$, $P < 0.001$) (Figure 2). Among brevideciduous species, the N:P ratio of *B. salicifolius* was lower in NP plots than in control plots (12.6 ± 4.4) ($P < 0.001$) (Figure 2a). The N:P ratio of *Dalbergia miscolobium* was lower in +P plots (27.9 ± 2.7) than in control plots (32.5 ± 2.0) ($F = 7.9$, $P < 0.001$) (Figure 2b). Among the evergreen species, the N:P ratio in leaves of *Ouratea hexasperma* was higher in +N plots than in control plots (21.4 ± 7.6) ($F = 3.3$, $P = 0.03$) (Figure 2c) while it was lower in *Roupala montana* (17.2 ± 6.6) ($F = 4.0$, $P = 0.01$) (Figure 2d) and *Styrax ferrugineus* (16.3 ± 3.1) ($F = 2.2$, $P = 0.02$) (Figure 2e) in +P plots than in control plots (24.6 ± 6.3 in *Roupala montana* and 21.5 ± 6.0 in *S. ferrugineus*).

Both young and mature leaves of *Caryocar brasiliense* ($F = 22.2$, $P < 0.001$, Figure 3a), *Blepharocalyx salicifolius* ($F = 8.3$, $P < 0.001$, Figure 3b), *Roupala montana* ($F = 11.7$, $P < 0.001$, Figure 3e) and *Styrax ferrugineus* ($F = 32.3$, $P < 0.001$) (Figure 3f), showed higher S concentrations in all fertilized plots compared with control plots. The smallest increase in S concentration (28%) was observed in young leaves of *Dalbergia miscolobium* in +NP plots. The highest increase (6.5 times) was observed in both young and mature leaves of *Styrax ferrugineus* in +P plots.

In all treatments, mean foliar total phenol concentration was 55.5% higher in brevideciduous species, whereas the difference in tannin concentration between phenological groups was half that amount. Brevideciduous species had higher foliar total phenol ($F = 578$, $n = 154$ and 170 , $P < 0.001$) and tannin ($F = 58.6$, $n = 154$ and 170 , $P > 0.001$) concentrations than evergreen species.

Only the brevideciduous species *Blepharocalyx salicifolius* (total phenol, $F = 17.2$, $P < 0.001$) (Figure 4a) and tannin, $F = 178.7$, $P < 0.001$) (Figure 4c)) and *Dalbergia miscolobium* (total phenol $F = 25.6$, $P < 0.001$) (Figure 4b) had significant differences in concentrations between fertilized and control plots but responses differed between species and primarily in immature leaves. In leaves of *Blepharocalyx salicifolius*, total phenol (Figure 4a) and tannin

(Figure 4c) concentrations increased in +P and +NP plots, whereas in leaves of *Dalbergia miscolobium*, total phenol concentrations decreased in +P and +NP plots (Figure 4b).

Changes in total phenol and tannin concentrations across leaf growth stages were also species-specific. In the evergreen group, total phenol concentration increased in mature leaves of *Roupala montana* (+P plots) ($F = 6.8$, $P = 0.002$) and *Styrax ferrugineus* (+N plots) ($F = 16.3$, $P < 0.001$) (Supplementary material table 2), while tannin concentration decreased with leaf maturity in *Ouratea hexasperma* (+N and +P plots, $F = 15.2$, $P < 0.001$) and *Styrax ferrugineus* (all plots, $F = 28.0$, $P < 0.001$) (Figure 3f). Conversely, the brevideciduous species, *Blepharocalyx salicifolius* showed lower total phenol concentration with leaf maturity (+NP plots, $F = 5.5$, $P = 0.002$). Tannin concentration also decreased in mature leaves of *B. salicifolius* (+N, +P, and +NP plots) ($F = 24.9$, $P < 0.001$) and decreased in young leaves relative to newly sprouted leaves in *C. brasiliense* (+P plots) ($F = 212$, $P < 0.05$) (Supplementary material table 2).

Discussion

In dystrophic environments such as the Brazilian savanna woodland, slow-growing species with low foliar nutrient levels might be replaced by species with higher growth rates and greater ability to increase foliar nutrient levels under higher nutrient availability (Aerts & Chapin 2000, Bustamante *et al.* 2012).

Previous studies in the experimental site indicated more pronounced responses at community level, such as reduced woody plant diversity and increased leaf litter decomposition rates in +NP plots than in +N and +P plots (Jacobson *et al.* 2011). Two years after the first fertilization in these plots, N and P concentrations were not significantly altered in leaves of *C. brasiliense* and *O. hexasperma*, only *B. salicifolius* leaves had increased P concentrations (in +P plots) (Kozovits *et al.* 2007). Seven years after the first fertilization, N (in +N plots) and P (in +P and +NP plots) concentrations were higher in *B. salicifolius* and in *C. brasiliense* (P in +NP plots) (Saraceno 2006), demonstrating the variation of leaf nutrient concentration in savanna woodland species over time in response to fertilization.

Changes in foliar nutrient concentrations in response to fertilization seems to be more related to differences in the nutritional requirements of the species. The brevideciduous *B. salicifolius* responded more strongly to fertilization than the other studied species. Thus, changes in soil nutrient

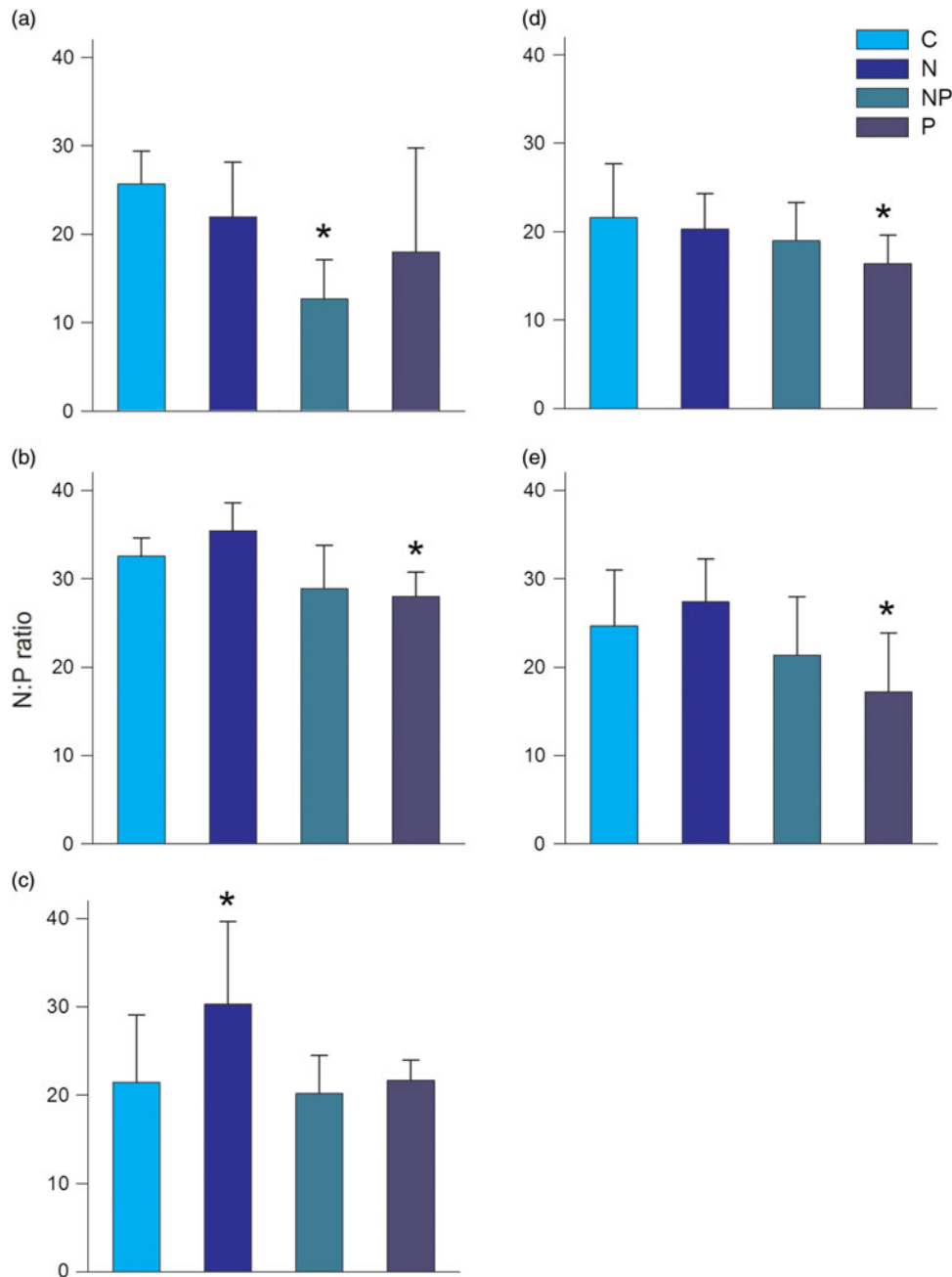


Figure 2. N:P ratio in young leaves (YL) and mature leaves (ML) in brevideciduous species *Caryocar brasiliense* (a), *Blepharocalyx salicifolius* (b), *Oureatea hexasperma* (c), *Roupala montana* (d) and *Styrax ferrugineus* (e) in control plots (C) and fertilized plots (+N, nitrogen; +P, phosphorus; and +NP, nitrogen plus phosphorus) in a savanna woodland in Central Brazil. * Indicates significant differences with control plots (Dunnett, $P < 0.05$). Error bars represent the standard deviation.

availability affect species composition (Jacobson *et al.* 2011). In our study, the lack of significant differences in foliar N concentrations between control and fertilized plots may have been due to increased nutrient demand for generation of new tissues after the accidental burning, causing decreased N leaf storage. Changes in N plant cycling due to fire were reported by Nardoto *et al.* (2006) that showed higher N resorption efficiency for Brazilian savanna tree species in burned plots compared to unburned plots. The decreased N:P ratio after P addition suggests plants absorb P at higher rates than N. Only one species (*O. hexasperma*) increased its N:P ratio after N addition, also suggesting greater P translocation efficiency (Kozovits *et al.* 2007). The higher P limitation was also reflected by differences in N and P concentrations between young and mature leaves in this study suggesting that this nutrient is retranslocated even before senescence begins.

The increased foliar S concentration was evident in all studied species in +NP plots, and in four of the six species in +P plots. This result suggests that savanna woody plants may also be limited by S, which has not been reported in the literature before. Jacobson (2009) also reported a general pattern of increased S concentration in leaf litter in fertilized plots and a significant decrease in S use efficiency compared with control plots. Sulphur might play a role in strategies to avoid excessive tissue dehydration of savanna woody plants during the dry season as primary S metabolism and associated metabolites are linked to responses to drought stress (Chan *et al.* 2013, Sieh *et al.* 2013).

Although comparison of polyphenolic concentrations across studies is hampered by differences in methods (Escarpa & González 2001, Yu & Dahlgren 2000), the foliar concentrations of total phenols and tannins in this study (3–26.5% for total phenols and 1.8–11% for tannins) are within the ranges found in

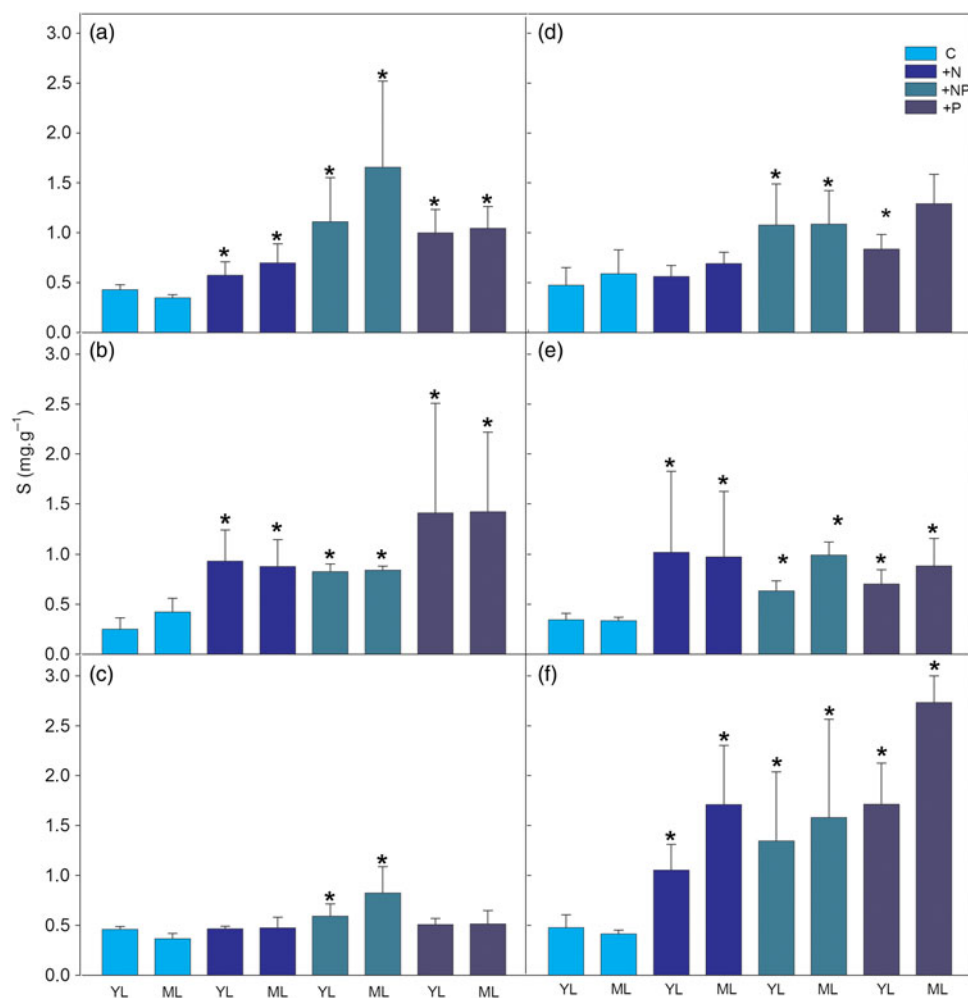


Figure 3. Sulphur concentration (mg g^{-1}) in young leaves (YL) and mature leaves (ML) in brevideciduous species *Caryocar brasiliense* (a), *Blepharocalyx salicifolius* (b), *Dalbergia miscolobium* (c) and in evergreen species *Ouratea hexasperma* (d), *Roupala montana* (e) and *Styrax ferrugineus* (f) in control plots (C) and fertilized plots (+N, nitrogen; +P, phosphorus; and +NP, nitrogen plus phosphorus) in a savanna woodland in Central Brazil. * Indicates significant differences with control plots (Dunnett, $P < 0.05$). Error bars represent the standard deviation.

literature (Hättenschwiler & Vitousek 2000, Hättenschwiler *et al.* 2003, Kraus *et al.* 2003). Regarding the second hypothesis, only two (both brevideciduous) out of six species showed significant changes in foliar polyphenol concentration in response to fertilization. P and NP addition increased total phenol and tannin concentrations in young *B. salicifolius* leaves but decreased total phenol concentration in young *D. miscolobium* leaves. Total phenol concentration increased in mature leaves with P addition only in *B. salicifolius*. In the brevideciduous species *C. brasiliense* and *B. salicifolius*, total phenol concentration reached a quarter of dry leaf weight, indicating that these species allocate much of their resources toward total phenol production. Production of polyphenolic compounds is thought to be an evolutionary advantage for species growing in acidic and highly weathered soils (Chapin 1995, Northup *et al.* 1995b, 1998). Wright *et al.* (2010) hypothesized that N availability has more influence on polyphenol production than P availability because N limitation decreases protein production and thus competition for phenylalanine, a precursor of many phenolic compounds. P acts as a recyclable co-factor in these reactions, allowing protein and phenolic production to continue under low P conditions. However, we found that P addition had greater effect on foliar polyphenol concentrations than N addition. The production and allocation of polyphenols in leaves in response to increased nutrient availability differed among species, even within the same phenological group. Increased nutrient availability will likely have little effect on this

genotypic trait in such a short period, as observed with four species in this study. The species with altered polyphenol concentrations in response to nutrient addition likely have high nutritional requirements, such as *B. salicifolius*. This finding reinforces the idea that the conceptual models (e.g. Carbon Nutrient Balance Hypothesis (Bryant *et al.* 1983) or Resource Availability Hypothesis (Coley *et al.* 1985)) are insufficient to explain variability in plant polyphenol concentrations (Nitao *et al.* 2002), especially in ecosystems with extremely high biodiversity, such as the Brazilian savanna.

Contrary to our prediction, the results showed no common pattern for total phenol and tannin concentrations across leaf growth stages. Decreases in total phenol and tannin concentrations during leaf maturation are common in other ecosystems (Covelo & Gallardo 2004), consistent with the results for brevideciduous species *C. brasiliense* and *B. salicifolius* in this study.

The observed decrease of T:TP ratio (the ratio between tannin and total phenols) over time in evergreen species may be the result of lower phenol polymerization during leaf maturation. Leaves of evergreen species have longer lifespans than those of deciduous and brevideciduous species (Franco *et al.* 2005, Lenza & Klink 2006). A lower polymerization rate can maintain polyphenolic compounds longer to protect against oxidation during senescence (McKee *et al.* 2002). The low specific leaf area of evergreen species may also mitigate the leaching of low-molecular-weight phenolic compounds, since leaves with high leaf mass per area typically have a thicker cuticle to slow water and solute loss (Villar & Merino 2001).

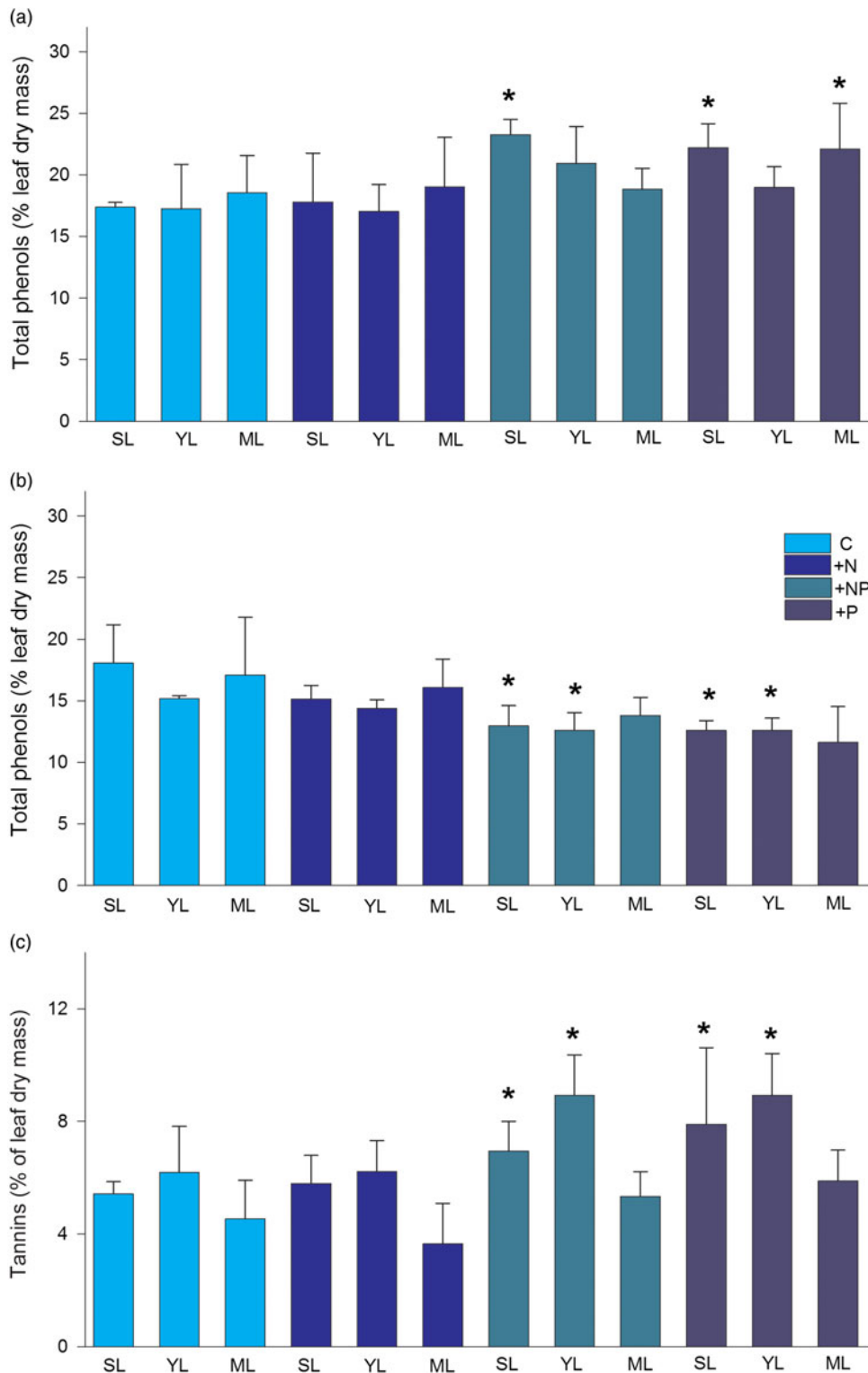


Figure 4. Total phenol concentration (% dry leaf mass) in newly sprouted leaves (SL), young leaves (YL) and mature leaves (ML) in breviceduous species *Caryocar brasiliense* (a) and *Blepharocalyx salicifolius* (b) and tannin concentration (% dry leaf mass) in *Blepharocalyx salicifolius* (c) in control plots (C) and fertilized plots (+N, nitrogen; +P, phosphorus; and +NP, nitrogen plus phosphorus) in a savanna woodland in Central Brazil. * Indicates significant differences with control plots (Dunnett, $P < 0.05$). Error bars represent the standard deviation.

The cost of leaf construction is a key factor in determining the specific competitive characteristics of a phenological group. Evergreens have the advantage of a longer photosynthetic season, whereas deciduous species normally have the advantage of leaves with a high specific area, offsetting the cost of foliar N and P with a higher rate of photosynthesis per unit leaf area (Franco *et al.* 2005, Montes & Medina 1977). Lower investment in lignin and cuticle and greater investment in cheaper structural

compounds such as cellulose and hemicellulose allows breviceduous species to invest more energy in polyphenol production. Our study showed consistent patterns in terms of leaf chemical composition when phenological groups of savanna woodland woody species are compared. However, their responses to nutrient additions showed considerable variations, showing that variability within functional groups should be considered in modelling efforts to understand how tropical and species-rich

ecosystems will respond to environmental changes, with consequences for ecosystem function and resilience. In addition, this study suggests that besides the co-limitation by N and P, savanna woodland tree species are also limited by S, indicating that the multi-element limitation deserves further investigation in these ecosystems.

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