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Leaf anatomical traits of non-arboreal savanna species along a gradient of tree encroachment

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ABSTRACT

In the Brazilian savanna (Cerrado of Brazil), fire suppression has transformed typical savanna formations (TS) into forested savanna (FS) due to the phenomenon of encroachment. Under encroachment, non-arboreal plants begin to receive less light due to greater tree density and canopy closure. Here we aim to evaluate if leaf anatomical traits of non-arboreal species differ according to the degree of tree encroachment at the Assis Ecological Station – São Paulo, Brazil. To this end, we evaluated leaf tissue thickness and specific leaf area (SLA) in representative non-arboreal species occurring along a gradient of tree encroachment. Leaves of TS species showed a trend towards xeromorphism, with traits reported to facilitate survival under high luminosity, such as thick leaves, thick epidermis and mesophyll, and low SLA. In contrast, FS species exhibited mesomorphic leaves, with thin mesophyll and high SLA, which are able to capture diffuse light in denser environments. Thus, non-arboreal understory species with mesomorphic leaf traits should be favored in environments with denser vegetation in contrast to typical savanna species. The results suggest that typical non-arboreal savanna species would not survive under tree encroachment due to the low competitiveness of their leaf anatomical strategies in shady environments.

Keywords: canopy closure, Cerrado, fire suppression, light, specific leaf area

Introduction

The savanna ecosystem has a large geographical distribution in different continents, and is subjected to diverse reliefs, soils and climates (Sankaran *et al*. 2005; Staver *et al*. 2011). In terms of structure, it is constituted by scattered trees growing in a very diverse continuous herbaceous-grassy layer consisting of many non-arboreal species, such as subshrubs, grasses and herbs (Scholes & Archer 1997). This structure is maintained by the constant presence of fire, in which its frequency determines the degree of tree cover (Hoffmann *et al*. 2012; Stevens *et al*. 2017).

In the absence of fire, savannas can undergo transformations and become denser or even acquire forest attributes, such as elevated basal areas, increased tree density and a high canopy cover (Durigan & Ratter 2006; Pinheiro *et al*. 2010). This is only possible because some typical forest tree species can establish in savanna sites during fire absence (Silva *et al*. 2008; Hoffmann *et al*. 2009; Geiger *et al*. 2011). The recruitment of forest species contributes to canopy closure, decreasing the total amount of light radiation reaching the understory (Pinheiro *et al*. 2016) and increasing the availability of nutrients (as P) in the superficial soils under encroachment (Blaser *et al*. 2014; Pinheiro *et al*. 2016; Rossatto & Rigobelo 2016). Changes in irradiance have been proven as a strong environmental

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filter (more than soil P) affecting the occurrence of non-arboreal savanna species, since its decrease drastically reduces species richness and diversity in encroached savanna sites (Ratajczak *et al*. 2012; Pinheiro *et al*. 2016).

In the savanna environment, light availability is variable due to the presence of trees (Peterson *et al*. 2007). This fact implies that non-arboreal plants may require leaf traits which can deal with higher (when growing between trees) or lower irradiances (when growing near the trees) (Ludwig *et al*. 2004). In contrast, non-arboreal plants living in encroached environments may show leaves with structural traits related exclusively to improving the performance of capturing diffuse light, since they establish and grow in shaded environments; responses that are well-known in forest tree species (Valladares *et al*. 2002; Santiago & Wrtight 2007), but yet not reported for non-arboreal savanna species. Among the necessary traits to survive under these circumstances, those linked with leaf structure can be considered the most important (Abrams & Kubiske 1990), since leaf structure and functioning are coupled (Sefton *et al.* 2002). In such encroached conditions, these traits may consist of larger and thinner leaves, with thin cuticles, thin epidermis and mesophyll thickness (Givnish 1988).

It is well established in the literature that woody savanna plants have leaves with xero- and scleromorphic aspects, possessing normally thick cuticles and epidermis, and large amounts of palisade parenchyma (Bieras & Sajo 2009; Rossatto *et al*. 2015); traits that are mainly found when light irradiances are high (Givnish 1988; Rossatto & Kolb 2010). The same pattern occurs in some non-arboreal species; however, there is a wide variation in leaf anatomical traits of these plants, ranging from thinner mesomorphic leaves to leaves with some xeromorphic traits (Rossatto *et al.* 2015). The study of Rossatto *et al*. (2015) evaluated such leaf anatomical traits in a typical savanna area without encroachment. However, a more detailed description of leaf structural strategies in sites with and without encroachment may shed light on the causes of the disappearance of these non-arboreal typical savanna plants given the widespread tree encroachment in savanna areas under fire absence (Stevens *et al*. 2017).

Here we evaluated leaf anatomical traits and specific leaf area of representative Brazilian savanna non-arboreal species in a gradient of tree encroachment (typical savanna, dense savanna and forested savanna) in Southeast Brazil. We studied qualitative and quantitative leaf anatomy, as well the specific leaf area of non-arboreal species to evaluate differences in anatomical strategies in plants living with distinct degrees of tree density. Specifically, we tested if plants under an encroached situation would possess thinner leaves with thin mesophyll and elevated specific leaf areas in comparison with the thicker leaves with thick mesophyll and low specific leaf areas expected for plants in non-encroached conditions.

Materials and methods

Study area

We performed this study in a savanna area in Southeast Brazil at the Assis Ecological Station (AES), in Assis, São Paulo state, Brazil (22º33’20’’S 50º21’27’’W). According to the Köppen classification, AES climate is a transition between Cwa and Cfa, with a dry season occurring between May and September. The annual average temperature is 22 °C and average rainfall is 1,400 mm. Soils are sandy dystrophic Oxisoils (Juhász *et al*. 2006).

For the past 50 years, the savanna vegetation in AES is under fire absence, which has promoted increases in tree density due to the advancement of forest trees in previously savanna sites (Durigan *et al*. 1999; Durigan & Ratter 2006; Pinheiro & Durigan 2009). In this region, tree encroachment has transformed typical savanna areas into forested savanna. For this study, we selected a tree density gradient, encompassing three situations: a) typical savanna (TS): a discontinuous tree layer (785 trees ha^{-1}) with low canopy cover (LAI = 0.96 ± 0.049) and a continuous herbaceous-grassy layer, b) dense savanna (DS): a TS site, with a tree density of 1,050 trees ha^{-1} and intermediate canopy cover (LAI = 1.22 ± 0.13) and c) forested savanna (FS): originally TS, now with a continuous tree layer (1,605 trees ha^{-1}) with an elevated canopy cover (LAI = 1.90 ± 0.12) (Pinheiro *et al*. 2016).

Leaf anatomical traits and specific leaf area

To compare leaf anatomical strategies and specific leaf area of non-arboreal plants along the gradient of tree encroachment, we selected species in each physiognomy, taking into account species surveyed by Pinheiro *et al.* (2016), but also sampling species found to be common and characteristic of each encroachment situation during field observations. We collected samples from fully expanded leaves (of third or fourth node from the apex for herbs, shrubs and sub-shrubs, and of the eighth or ninth node for climbers), without disease signals, and at least from three or preferably six individuals per species (n = 3-6). We sampled 14 species, eight species for TS, six for DS and six for FS (Tab. 1). Voucher specimens were deposited at the Assisense Herbarium (HASSI).

Leaf fragments were removed from the middle region including the main vein. These fragments were fixed in formalin - acetic acid - ethanol 70 %, dehydrated in ethanol series, infiltrated and embedded in paraffin (Johansen 1940) and cut into 8 μm sections. Cross-sections were stained with Astra blue - basic fuchsine and mounted in Entellan®. This double staining can indicate the presence of cellulosic and lignified cellular walls (Kraus *et al*. 1998). In addition, the following histochemical tests were applied: Sudan IV to detect lipid substances (Johansen 1940), ferric ferricyanide to show phenolic compounds (Lison 1960), Alcian Blue 8G to detect mucopolysaccharide (Johansen 1940), hydrochloric acid to detect calcium crystals (Chamberlain 1932) and hydrofluoric acid to identify silica bodies (Yoshida *et al.* 1962). We performed the measurements of epidermis from leaf surfaces, mesophyll and total leaf thickness 10 times in each individual, for each parameter, using a 10x objective lens and the AxioVision software. Paradermic sections were obtained from the dissociation of leaf fragments in glacial acetic acid and hydrogen peroxide solution (modified from Franklin 1945). Each decoupled epidermis was washed with distilled water, stained with Safranin and mounted in glycerin for stomata and trichomes observation. Illustrations were assembled with the software Photoshop CS2 (Adobe®). Images were obtained in a Zeiss® microscope, Axio Scope A1 model, connected to an AxioCam ICc3.

The specific leaf area (SLA) was obtained by the ratio between the leaf area scanned and measured with ImageJ software and the dry mass obtained after leaf drying at 70 °C for three days.

Statistical analyses

Data were tested for normality (Kolmogorov-Smirnov's test) and homogeneity of variances (Levene's test) and when necessary they were transformed using $\log_{10}$. To compare the values of leaf tissues and leaf thickness among physiognomies, we performed an ANOVA nested design, where we tested the effect of the species (nested in the study site) and study site (TS, DS and FS), followed by Tukey test. In all cases, we adopted $\alpha = 0.05$.

Results

Qualitative analysis along the encroachment gradient

Regardless of the site occurrence in the tree encroachment gradient, the studied species showed similar qualitative aspects in their leaf anatomy. All the species showed unisseriate epidermis in both adaxial and abaxial surfaces (Figs. 1, 2), possessing hypostomatic leaves with their stomata placed in the same level of adjacent epidermal cells. *Merostachys skvortzovii* presented papillose epidermal cells in abaxial epidermis surface (Fig. 1J) and *Psychotria hoffmannseggiana* presents this feature in both epidermal surfaces (Fig. 2A-C). Trichomes occurred in all encroached conditions, and it was observed that tector trichomes were more common than glandular (Tab. 2). The cuticle was thin for most of the studied species, with thick cuticles occurring in two species in TS, two species in DS and one species in FS (Tab. 2).

The mesophyll was dorsiventral in all eudicot species and in *Merostachys skvortzovii* and *Rhynchospora albiceps*, while in *Axonopus pressus* (Fig. 1A) and *Smilax campestris* (Fig. 1B) it was homogeneous. The general pattern for the dorsiventral mesophyll species was that the palisade parenchyma had only one or two layers of cells (Figs. 1C-J, 2). In some species, we found calcium crystal idioblasts (Fig. 1G-H), as well phenolic compounds appearing inside the epidermal and

Table 1. Non-arboreal species selected in a tree density gradient at the Assis Ecological Station, Assis – SP, Brazil. TS: typical savanna; DS: dense savanna; FS: forested savanna; HASSI: voucher number at the Assisense Herbarium. Growth form according to Durigan *et al*. (1999; 2004) and Rossatto *et al*. (2008).

Species (Family)	Physiognomy			Growth form	Voucher number
	TS	DS	FS		
Axonopus pressus (Nees ex Steud.) Parodi (Poaceae)	x			herb	HASSI 1040
Coccocypselum lanceolatum (Ruiz & Pav.) Pers. (Rubiaceae)		x		herb	HASSI 1122
Croton campestris A.St.-Hil. (Euphorbiaceae)	x			herb	HASSI 1123
Eugenia sp. (Myrtaceae)			x	subshrub	HASSI 1046
Leandra melastomoides Raddi (Melastomataceae)		x		shrub	HASSI 1120
Merostachys skvortzovii Send. (Poaceae)			x	herb	HASSI 1071
Miconia fallax DC. (Melastomataceae)	x	x		shrub	HASSI 1044
Miconia paucidens DC. (Melastomataceae)			x	shrub	HASSI 1056
Peltodon tomentosus Pohl (Lamiaceae)	x			herb	HASSI 1119
Peritassa campestris (Cambess.) A.C. Sm. (Celastraceae)	x			subshrub	HASSI 1077
Psychotria hoffmannseggiana (Willd. ex Schult.) (Rubiaceae)	x	x	x	subshrub	HASSI 1079
Rhynchospora albiceps Kunth (Cyperaceae)	x	x	x	herb	HASSI 1087
Serjania lethalis A. St.-Hil. (Sapindaceae)		x	x	vine	HASSI 1116
Smilax campestris Griseb. (Smilacaceae)	x			vine	HASSI 1092

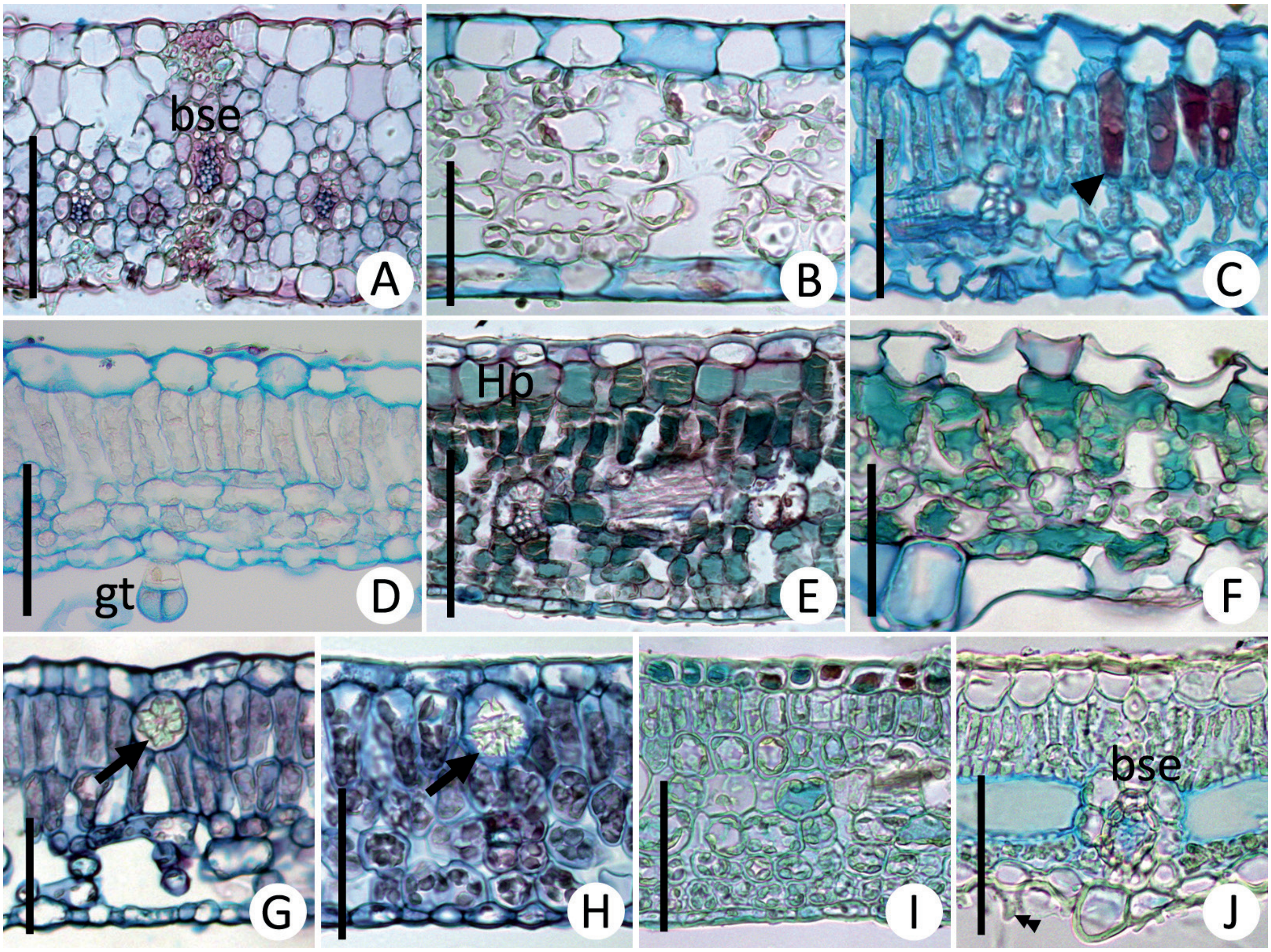

Figure 1. Leaf cross-sections of one-physiognomy non-arboreal species at the Assis Ecological Station, Assis – SP, Brazil. **A-E.** Typical savanna species: **A.** *Axonopus pressus*; **B.** *Smilax campestris*; **C.** *Croton campestris*; **D.** *Peltodon tomentosus*; **E.** *Peritassa campestris*. **F-G.** Dense savanna species: **F.** *Coccocypselum lanceolatum*; **G.** *Leandra melastomoides*; **H-J.** Forested savanna species: **H.** *Miconia paucidens*; **I.** *Eugenia* sp.; **J.** *Merostachys skvortzovii*. Arrow: calcium crystal; Arrowhead: phenolic compounds; bse: bundle sheath extension; Double arrowhead: papillae; gt: glandular trichome; hp: possible hypodermis. Scale: A, E: 100 µm; B-D, F-J: 50 µm.

mesophyll cells (Figs. 1C, 2D-H); Poaceae and Cyperaceae species presented silica bodies (Tab. 2). Mucilage was found only in *Serjania lethalis* (Fig. 2I-J). The presence of these ergastic substances in the leaves occurred regardless of the species occurring environment (Tab. 2). A bundle sheath extension was present only in monocots from Poaceae (Fig. 1A, J) and Cyperaceae (Fig. 2D-F) families.

Quantitative differences along the encroachment gradient

The main differences were found in the quantitative aspects of the leaves, especially between species of typical savanna and forested savanna (Fig. 2). Concerning the quantitative aspects of leaf tissues, the species and environment had significant effects on the studied anatomical traits (Tab. 3). Among the environments, species of typical savanna presented higher thickness values for the epidermis of both leaf surfaces (Fig. 3A-B), mesophyll (Fig. 3C) and total leaf thickness (Fig. 3D), in comparison with dense and forested savanna. SLA values were significantly lower for typical savanna species compared to those obtained for species of dense and forested savanna (Fig. 3E). The two species sampled along the gradient of tree encroachment, *P. hoffmannseggiana* (Fig. 2A-C) *and R. albiceps* (Fig. 2D-F), showed greater degree of plasticity, concerning leaf thickness; while the species occurring in only two encroachment conditions (*Miconia fallax* (TS and DS) and *S. lethalis* (DS and FS)) did not show differences in leaf thickness between physiognomies (Fig. G-J).

Discussion

Accordingly with the expectations, plants under encroached conditions (FS) showed thinner leaves with elevated SLA. In contrast, in the TS, the general pattern of leaf structure was that reported to survive under elevated

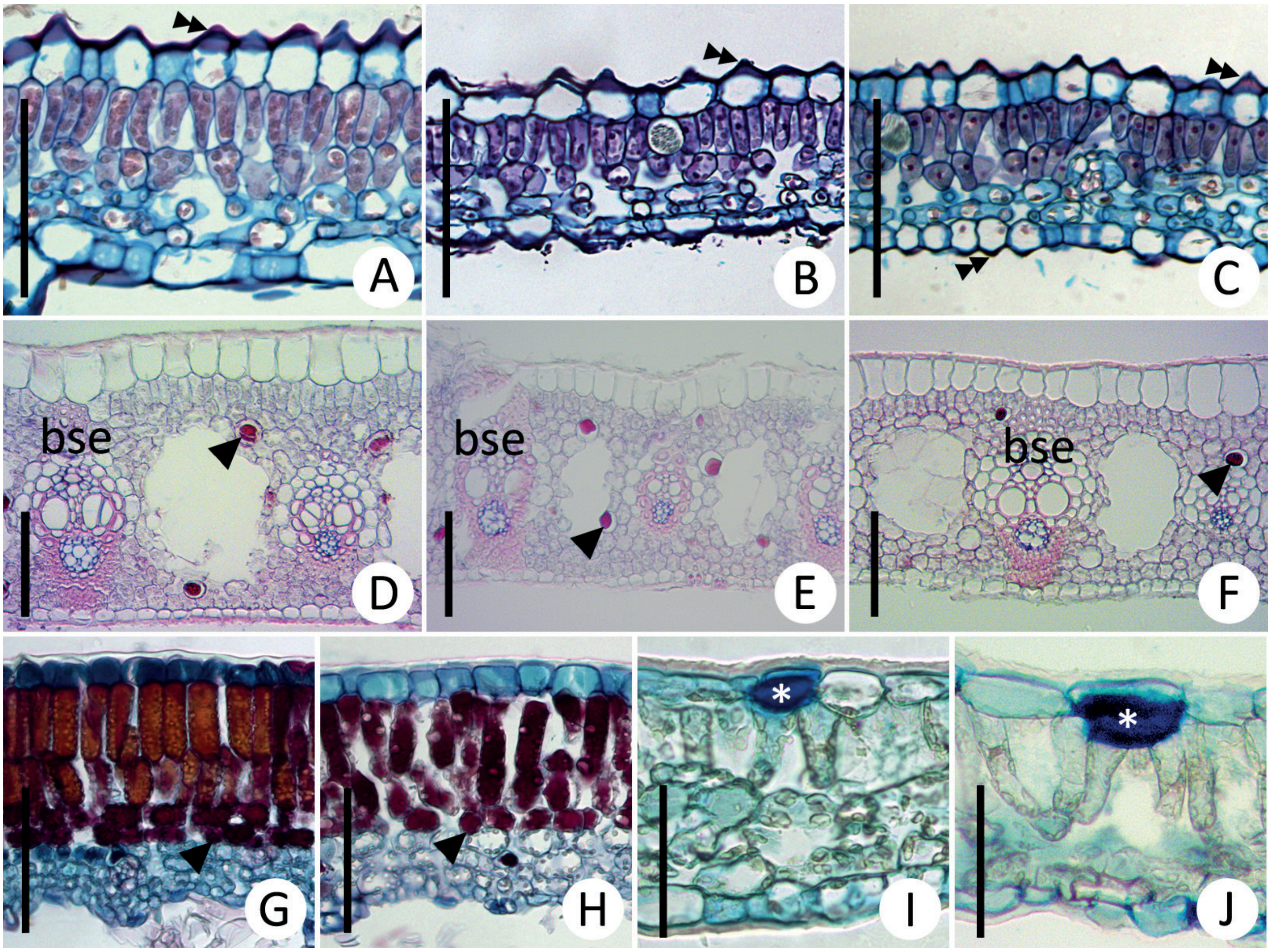

Figure 2. Variations in leaf tissues thickness of non-arboreal species in different physiognomies of a gradient of tree encroachment at the Assis Ecological Station, Assis - SP, Brazil. **A-C.** *Psychotria hoffmannseggiana*: **A.** Typical savanna; **B.** Dense savanna; **C.** Forested savanna. **D-F**. *Rhynchospora albiceps*: **D.** Typical savanna; **E.** Dense savanna; **F.** Forested savanna. **G-H.** *Miconia fallax*: **G.** Typical savanna; **H.** Dense savanna. **I-J.** *Serjania lethalis*: **I.** Dense savanna; **J.** Forested savanna. Figures of same species were obtained with similar magnification. Scale: A-H: 100 µm; I-J: 50 µm. Arrowhead: phenolic compounds; Asterisk: mucilage cell; bse: bundle sheath extension; double arrowhead: papillae.

light irradiances (thicker leaves with thick mesophyll and low SLA). The qualitative analysis did not provide any evidence which could differentiate between the studied conditions. In contrast, quantitative aspects (thickness of tissues) and SLA varied between the sites along the tree encroachment gradient, increasing from TS to FS.

Considering qualitative traits, the non-arboreal species evaluated here showed leaves with thin cuticle and unisseriate epidermis, presence of ergastic substances, sclerenchyma and trichomes. Similar qualitative patterns were described in previous studies dealing with trees in Southeastern Brazil (Bieras & Sajo 2009), and for non-arboreal plants of a savanna site in Central Brazil (Rossatto *et al.* 2015). These aspects may suggest a conservative pattern in the qualitative aspects of leaf structure in angiosperms, as previously reported by Brodribb *et al.* (2010).

The main difference found concerns the degree of leaf tissue thickness, which varied remarkably along the tree encroachment gradient. The lower values of SLA obtained for the typical savanna species suggest a more conservative use of resources, since leaves with lower SLA survive for longer periods of time in comparison with leaves showing higher SLA (Wilson *et al.* 1999). These lower SLA values are the result of the increased investment in the epidermis and mesophyll thickness, leading to the construction of leaves with elevated thickness (Vile *et al.* 2005) compared with plants under shade. The literature reports that elevated tissue thicknesses are related to adaptations to water economy and protection against high irradiances (Fahn & Cutler 1992; Voguelmann 1993), situations that are commonly reported for Brazilian savannas (Bieras & Sajo 2009; Ferreira *et al.* 2015). Leaves with lower SLA values are also capable of performing higher photosynthetic rates in a leaf area basis, a situation found in many non-arboreal plants in savannas of Central Brazil subjected to natural

Table 2. Leaf anatomical traits of non-arboreal species in a tree density gradient at the Assis Ecological Station, Assis – SP, Brazil. AB: epidermis of the abaxial surface; AD: epidermis of the adaxial surface; BSE: bundle sheath extension; Cry: calcium crystal; Muc: mucilage; Phe: phenolic compounds; Si: silica body; VB: vascular bundle; - absence of the characteristic.

Species	Thick cuticle	Ergastic substance	Tector trichome	Glandular trichome	Sclerenchyma
Typical savanna					
Axonopus pressus	-	Si	AB	-	BSE
Croton campestris	-	Phe, Cry	AD, AB	-	-
Miconia fallax	AD	Phe, Cry	AB	-	-
Peltodon tomentosus	-	-	AD, AB	AD, AB	-
Peritassa campestris	AD, AB	Phe, Cry	AB	-	VB
Psychotria hoffmannseggiana	-	Cry	AB	-	-
Rhynchospora albiceps	-	Phe, Si	AB	-	BSE
Smilax campestris	-	-	-	-	VB
Dense savanna					
Coccocypselum lanceolatum	-	Phe, Cry	AD, AB	-	-
Leandra melastomoides	-	Cry	AB		-
Miconia fallax	AD	Phe, Cry	AB	-	-
Psychotria hoffmannseggiana	-	Cry	AB	-	-
Rhynchospora albiceps	-	Phe, Si	AB	-	BSE
Serjania lethalis	AD, AB	Phe, Muc	-	AD, AB	VB
Forested savanna					
Eugenia sp.	-	Phe, Cry	AB	-	VB
Merostachys skvortzovii	-	Si	AB	-	BSE
Miconia paucidens	-	Cry	AB	-	-
Psychotria hoffmannseggiana	-	Cry	AB	-	-
Rhynchospora albiceps	-	Phe, Si	AB	-	BSE
Serjania lethalis	AD, AB	Phe, Muc	-	AD, AB	VB

Table 3. Results of ANOVA nested design for effect of species (nested in study site) and study site (typical, dense and forested savanna) for leaf structure parameters of non-arboreal species in a tree density gradient at the Assis Ecological Station, Assis – SP, Brazil.

Parameter	Species (study site)		Study site	
	F	*P*	F	*P*
Adaxial epidermis thickness	27.358	< 0.0001	41.233	< 0.0001
Mesophyll thickness	13.305	< 0.0001	5.511	< 0.01
Abaxial epidermis thickness	15.933	< 0.0001	11.550	< 0.0001
Leaf thickness	15.035	< 0.0001	10.088	< 0.001
Specific leaf area	9.077	< 0.0001	16.995	< 0.0001

irradiance conditions (Rossatto & Franco 2017).

Despite the fact that this pattern was clearly observed in the set of typical savanna species studied here, their values of SLA were higher and their tissue thicknesses were lower than those reported for typical non-arboreal savanna species in Central Brazil (see the study by Rossatto *et al*. 2015). This difference may have arisen because the savannas in Southeast Brazil have a lower degree of seasonality and more nutrients in their soils, a condition that can select less scleromorphic leaves (Souza *et al*. 2015).

Contrasting to the previous reported pattern, plants under an encroached situation showed thinner leaves, where tissues were thinner than those found for typical savanna species, leading to higher SLA values. This set of traits improve the capture of diffuse light in forest environments (Voguelmann 1993; Valladares & Niinemets 2008). Plants in dense savanna tended to show intermediate values of leaf thickness. This may be explained by the fact that in the dense savanna, plants from typical and forested savanna communities can co-exist due to the variability of available light niches (Pinheiro *et al*. 2016).

Considering these results in the context of forest expansion over savannas (Stevens *et al*. 2017) and the ecophysiology of non-arboreal savanna plants (Rossatto & Franco 2017), it can be postulated that the persistence of non-arboreal plants in encroached situations may occur only with the set of leaf anatomical strategies presented here by the FS plants. As the TS species showed thicker leaves, with anatomical traits that are advantageous to survive under elevated light irradiances (Rossatto *et al*. 2015; Rossatto & Franco 2017), this may imply that they cannot thrive under shaded conditions, which in turn can explain why

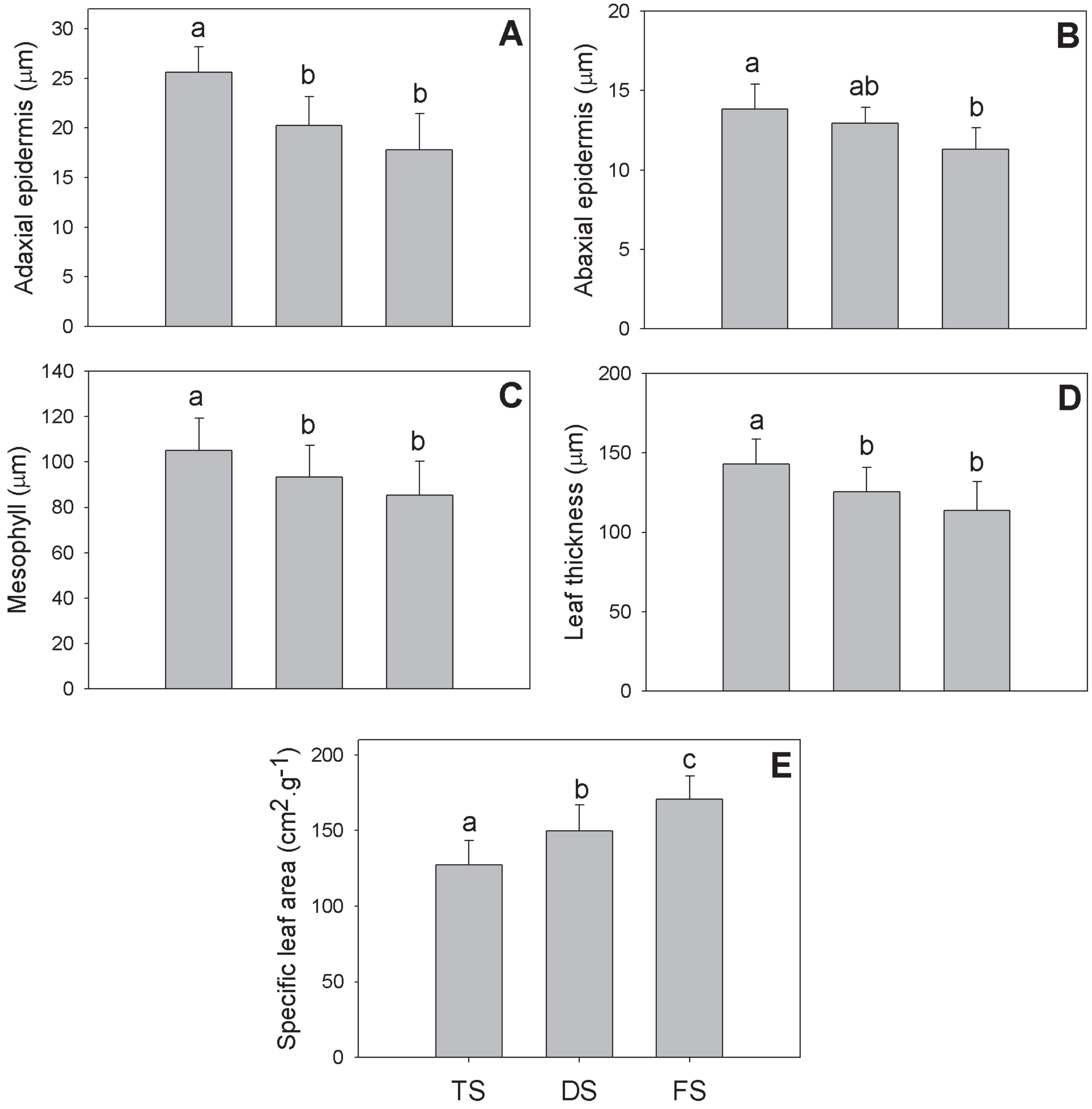

Figure 3. Leaf anatomical traits of non-arboreal species of typical savanna (TS), dense savanna (DS) and forested savanna (FS) at the Assis Ecological Station, Assis – SP, Brazil. Different letters indicate significant differences according to the Tukey test (α = 0.05). Error bars: standard error.

the diversity of such species drastically decrease from the TS to the FS (Pinheiro *et al*. 2016). The disappearance of such typical savanna species may occur because their set of anatomical leaf traits are not specialized in capturing low and diffuse light; this can drastically affect the carbon balance, probably leading plants to carbon starvation (Sevanto *et al*. 2014). Studies dealing with carbon exchange rates are necessary to provide better insights into the role of carbon starvation for typical savanna species disappearance.

Few non-arboreal typical savanna species can survive this encroachment, as reported by Pinheiro *et al*. (2016). However, this fact may happen if the species has the necessary degree of plasticity to adjust their leaves to a more shaded condition. Bedetti *et al*. (2011) showed that a common savanna shrub (*Miconia albicans*) has leaf plasticity probably related to its occurrence in open and closed environments.In our study, we found *P. hoffmannseggiana* and *R. albicepsgrowing* in all conditions of tree density, with their leaves showing distinct patterns under high light availability (thicker leaves with low SLA) and low light availability (thinner leaves with high SLA).

In conclusion, the results of this study corroborate the hypothesis that plants found under encroachment differ in their leaf anatomical strategies in comparison with plants under non-encroached situations. Thus, typical savanna species presented leaf anatomical traits tending to xeromorphism; these traits should be related mainly to high luminosity of this environment. In contrast, species growing under encroachment possessed traits linked to mesomorphism. Therefore, in a scenario of continuous and fast encroachment of savannas, mainly in South America (Stevens *et al*. 2017), typical non-arboreal savanna species may be threatened, due to the low competitiveness of their leaf anatomical traits in a shady environment, promoting their disappearance because of tree densification. Thus, studies integrating morpho-anatomical and physiological traits are needed to evaluate the survival and responses of typical non-arboreal savanna species to environmental changes caused by tree encroachment.

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