

Elaphoglossum (Dryopteridaceae-Fern) of Amazon Rainforest in Brazil: Anatomic Characterization and Adaptative Strategies*

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ABSTRACT

This study describes the anatomy of sterile leaves of *Elaphoglossum discolor* (Kuhn) C. Chr., *E. flaccidum* (Fée) T. Moore and *E. laminarioides* (Bory ex Fée) T. Moore, the most representative species of the genus in the Ecological Park of Gunma in Pará State. It reports the main diagnostic characters and provides new systematic data for the group. In addition, it locates the production and accumulation sites of bioactive compounds to determine possible adaptive strategies of these species in the Amazon rainforest environment. Diagnostic structural features include stoma typology, central veins and margin forms, type of mesophyll, and the presence of schlerenchymatous sheaths in the cortex, among others. Among the bioactive compounds related to defense adaptation are phenolic compounds, which occur in all three species, and alkaloids and mucilage, which are exclusive to *E. laminarioides*. Of the three species studied, *E. laminarioides* has features that make it the best suited to the rainforest environment.

Keywords: Fern; Ombrophilous Forest; Epiphytes; Comparative Anatomy; Secondary Metabolites

1. Introduction

Because many of the features of *Elaphoglossum* Schott ex Smith do not allow for the diagnosis of sterile individuals, it is one of the fern genera with the greatest need for further studies [1,2]. In fact, most infrageneric classifications are based on morphological characters such as plant size, blade form and size, and scale color and type [2-6]. However, most of these characters are only relevant when plants are in their reproductive stage [3].

Elaphoglossum grows in primary formations and is sensitive to environmental changes. Some species may be resistant for some time in human modified habitats, but different light and moisture conditions have considerable influence on their morphology [7].

Since the 1970s, these species have been mentioned in floristic studies in the Amazon [8-15]. Their constant presence in inventories is due to different adaptation strategies developed during their evolution, especially in the Amazon rainforest environment. Such strategies in-

clude various phytochemical defenses, high resistance to diseases in moist environments, high tolerance to acute nutrient imbalance in substrates, plasticity to adapt to different ecological opportunities, mycotrophic interactions [16] and the epiphytic habitat, in which *Elaphoglossum* prevails [17].

The scarcity of studies on fern anatomy hinders the understanding of their structural organization. Among the main anatomical studies carried out on this genus are [18], who analyzed rhizomes and leaves of Jamaican species; [19] who described the leaf anatomy of *Elaphoglossum* in Tucumán, Argentina and [20,21], who addressed leaf architecture of Argentinean species by focusing on their induments. None of these studies examined Brazilian species, indicating the lack of the knowledge about their anatomy and possible relationships to the environments where they grow.

The aim of this study is to characterize the anatomy of sterile leaves of *E. discolor*, *E. flaccidum* and *E. laminarioides*, listing their main diagnostic characters and providing new data on the systematics of this group, in addition to identifying production and accumulation sites

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of bioactive compounds, in order to determine possible adaptation strategies of these species in the Amazon rainforest environment.

2. Material and Methods

2.1. Plant Materials

Botanical material was collected in the Ecological Park of Gunma (PEG) located in Santa Bárbara do Pará (01°13'25"S, 48°17'40"W), Pará State, Brazil. The area is covered by lowland Amazon forest with a maximum altitude of 100 m above sea level. Adult individuals with totally unwound leaf blades were collected. The vouchers of the fertile material deposited in the herbarium MG are: *E. discolor* (MG 195547; MG 193856), *E. flaccidum* (MG 195545; MG 193858), and *E. laminarioides* (MG 195548; MG 195551; MG 195552; MG 193852).

2.2. Field Observations

Elaphoglossum discolor and *E. flaccidum* were always found and collected in shady areas within the forest, while *E. laminarioides* was found at the forest edges, more exposed to the sunlight. Although the individuals of the latter presented many leaves that seemed to have been chewed, no visitors were observed during our ten excursions to the field over two years.

2.3. Light Microscopy and Histochemical Tests

Leaves were fixed in FAA₇₀ for 24 h [22], and in neutral buffered formalin [23] and formalin-ferrous sulphate [22] for 48 h. Leaf samples (n = 7) were dehydrated in a tertiary butanol series [22] and embedded in histological paraffin. Leaves were cut into sections with a Leica RM 2245 microtome and stained in safranin and Astra blue [24].

The following histochemical tests were applied by hand to sections of the central vein: Sudan black B [25], Nile blue sulphate [26], NADI reagent [27], copper acetate/rubeanic acid [28], PAS reaction (Periodic Acid Schiff) [29], ruthenium red [30], tannic acid/ferric chloride [31], Lugol's solution [22], ferric chloride [22], fluoroglucinol [22], vanillin hydrochloric [32], Dragendorff's [33] and Wagner's reagents [34]. Control sections without treatment were also analyzed and photographed to compare their color to that of the material fixed in FAA₇₀ and NBF. Photomicrographs were taken with a Zeiss Axiolab microscope equipped with a Canon digital camera.

2.4. Scanning Electron Microscopy (SEM)

The samples fixed in FAA₇₀ were dehydrated in an ethanol series [22], critical point dried with CO₂ [35] and metallized with gold for SEM analyses. Images were cap-

tured with an LEO 1450 VP electron microscope. Frontal views of epidermis were observed in a scanning electron microscope because chemical and mechanical dissociation techniques were unsuccessful.

3. Results

3.1. Leaf Blade

In frontal view, the epidermis of each of the three species presented sinuous, thin anticlinal walls on both faces (**Figures 1(a)** and **(b)**), with subtle cuticular striations (**Figure 1(c)**), as well as fungal hyphae along the surface. All species were hypostomatic (**Figures 1(a)** and **(b)**). Stomata are on the same level as the other epidermal cells (**Figures 3(a)** and **(e)**). In *E. discolor* and *E. flaccidum*, stomata were exclusively polocytic (**Figures 1(d)** and **(e)**), while *E. laminarioides* presented anomocytic (**Figure 1(g)**) and copolocytic stomata (**Figure 1(h)**) associated to polocytic (**Figure 1(f)**) stomata. Cross sections of the epidermis of each species showed that it consists of one layer of cells covered with a thin cuticle (**Figures 2(b)**, **(f)** and **(j)**). However, in *E. laminarioides*, the outer periclinal walls are prominent on both faces (**Figure 2(j)**).

In cross sections, the midrib differed according to species: in *E. discolor*, both the adaxial and abaxial faces (**Figure 2(a)**) are similarly concave; in *E. flaccidum* both faces were concave, but the adaxial one was more pronounced (**Figure 2(e)**) and in *E. laminarioides*, the adaxial face is flat (**Figure 2(i)**). The midrib also revealed a pattern of the structural organization of the studied species: schlerenchymatous cells subjacent to the epidermis of both faces formed an arch comprised of 2 - 6 layers (**Figures 2(b)**, **(f)** and **(j)**) with the rest filled with parenchymatous tissue. The midrib enclosed two large and one small vascular bundles, each of which was surrounded by a cortical schlerenchymatous sheath (**Figures 2(c)**, **(g)** and **(k)**), which was thicker in *E. laminarioides*. Adjacent to this sheath was the endodermis, followed by a 2-layered pericycle (**Figures 2(d)** and **(h)**). Vascular bundles were bicollateral, with curved xylem at their tips.

Mesophyll was homogenous (**Figures 3(a)**, **(e)** and **(i)**), with 4 - 6 strata of branched cells in *E. flaccidum* (**Figures 3(e)** and **(f)**) and lobed cells in the other two species (**Figures 3(a)**, **(b)**, **(i)** and **(j)**). In addition, the intracellular spaces were conspicuous with scarce collateral bundles.

Although these species had revolute leaf margins, (**Figures 3(c)**, **(g)** and **(k)**), they differed according to taxa: in *E. discolor*, they were less revolute and the epidermis had lignified walls (**Figure 3(c)**); in *E. flaccidum*, they were rostriform (**Figure 3(g)**) and in *E. laminarioides* they were more revolute, with slightly truncated extremities (**Figure 3(k)**).

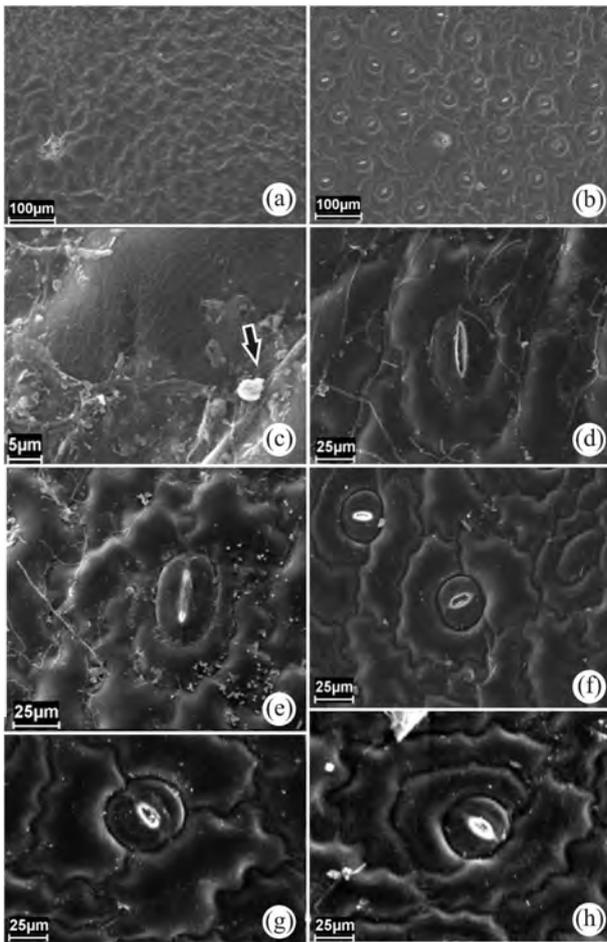


Figure 1. Scanning electron micrographs of *Elaphoglossum*. (a), (b) General view of the surface of *E. laminarioides*: (a) Adaxial face, (b) Abaxial face, (c) Epidermis with striated cuticle and fungal hyphae (arrow); (d)-(f) Details of polycytic stomata: (d) *E. discolor*, (e) *E. flaccidum*, (f) *E. laminarioides*, (g) Anomocytic stoma of *E. laminarioides*, (h) Copolycytic stoma of *E. laminarioides*.

3.2. Petiole

Cross sections of the petiole were cylindrical (Figures 4(a), (e) and (i)), but concave on the adaxial surface of *E. laminarioides* (Figure 4(i)). The epidermis of all species was formed by thin-walled rounded cells (Figures 4(b), (f) and (j)). The cortical region, which comprised 6 - 10 strata of sclerenchymatous cells, is more evident in *E. discolor* and *E. flaccidum*. Below this strata, the rest of the cortex was filled with parenchymatous tissue. Unlike *E. laminarioides* (Figure 4(k)), the cortex sheaths of *E. discolor* and *E. flaccidum* were inconspicuous (Figures 4(c) and (g)) in both the medial and basal regions of the petiole. The petiole vascular system was similar to that described for the midrib of the taxa, with the only difference being that it presented five vascular bundles at its basal portion (Figures 4(d), (h) and (l)), which merged

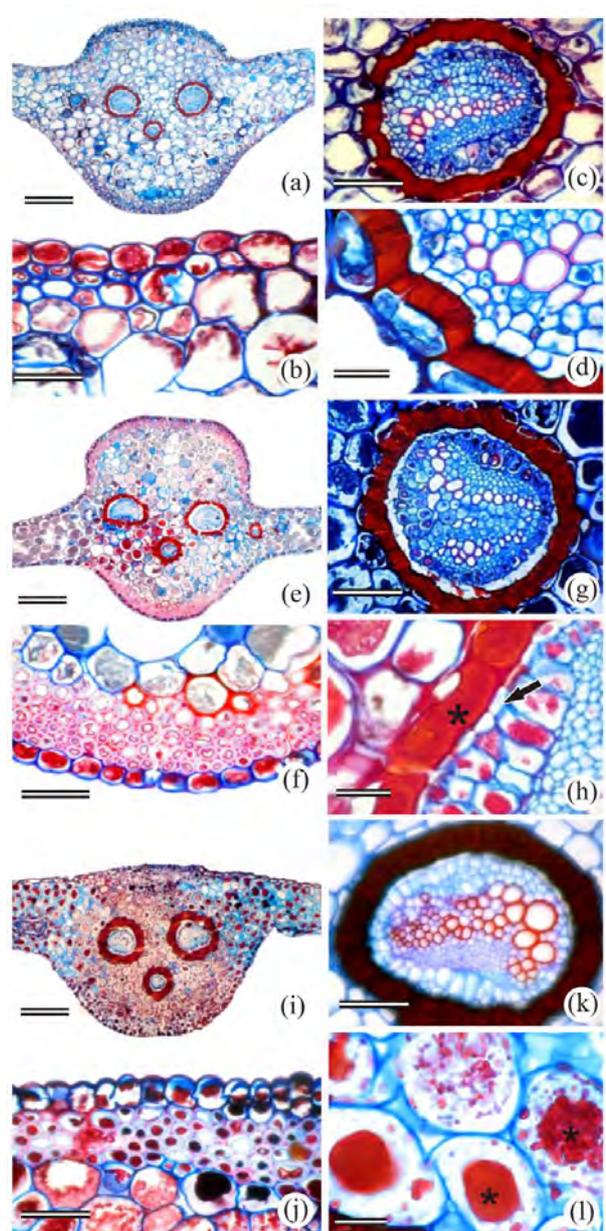


Figure 2. Cross sections of the central vein of *Elaphoglossum*. (a)-(d) *E. discolor*; (e)-(h) *E. flaccidum*; (i)-(l) *E. laminarioides*. (a), (e), (i) General view; (b), (f), (j) Detail of the epidermal cells; (j) Cells with prominent outer periclinal walls; (c), (g), (k) Vascular bundles; (d), (h) Detail of sclerenchymatous sheath in the cortex and endodermis; (l) Cells with granulose, compact content (asterisk). Bars: (a), (e), (i) = 250 µm; (b), (c), (f), (g), (j), (k) = 50 µm; (d), (h), (l) = 20 µm.

to three bundles as they approached the midrib median region.

3.3. Secretory Tissues

In all the species analyzed, the secretory tissues were epi-

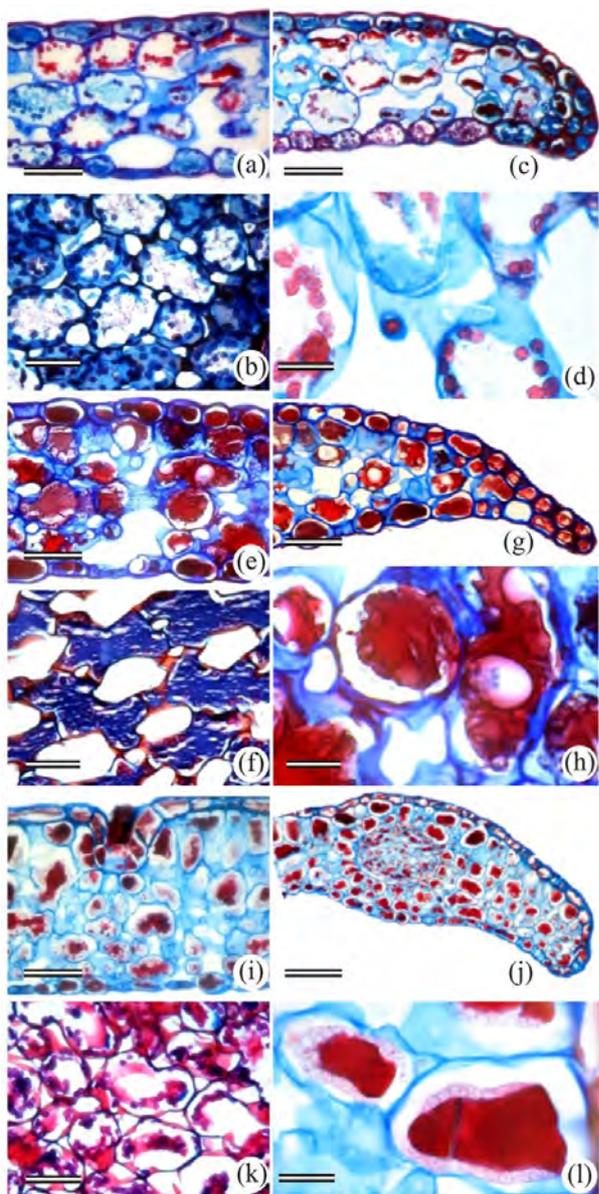


Figure 3. Sections of the leaf blade of *Elaphoglossum*. (a)-(d) *E. discolor*; (e)-(h) *E. flaccidum*; (i)-(l) *E. laminarioides*. (a), (e), (i) Cross sections of the blade showing homogeneous mesophyll; (b), (f), (j) Longitudinal sections of the mesophyll; (b), (j) Mesophyll with lobed cells; (f) Mesophyll with branched cells; (c), (g), (k) General view of the revolute margins; (d), (h), (l) Detail of the cells with content; (l) Cell with dense content (asterisk) and adjacent starch grains (arrow). Bars: (a), (e), (i) = 50 μm ; (b), (d), (f), (h), (j), (l) = 20 μm ; (c), (g), (k) = 200 μm .

dermis (**Figures 2(b), (f), (j), 3(e) and (i)**), parenchyma (**Figures 3(d), (h) and (l)**) (including that of the xylem and phloem), and pericycle. No exudate was released into the environment. Independent of the individuals or parts, the exudate had a compact or granulose aspect, but it may appear differently in neighboring cells (**Figure**

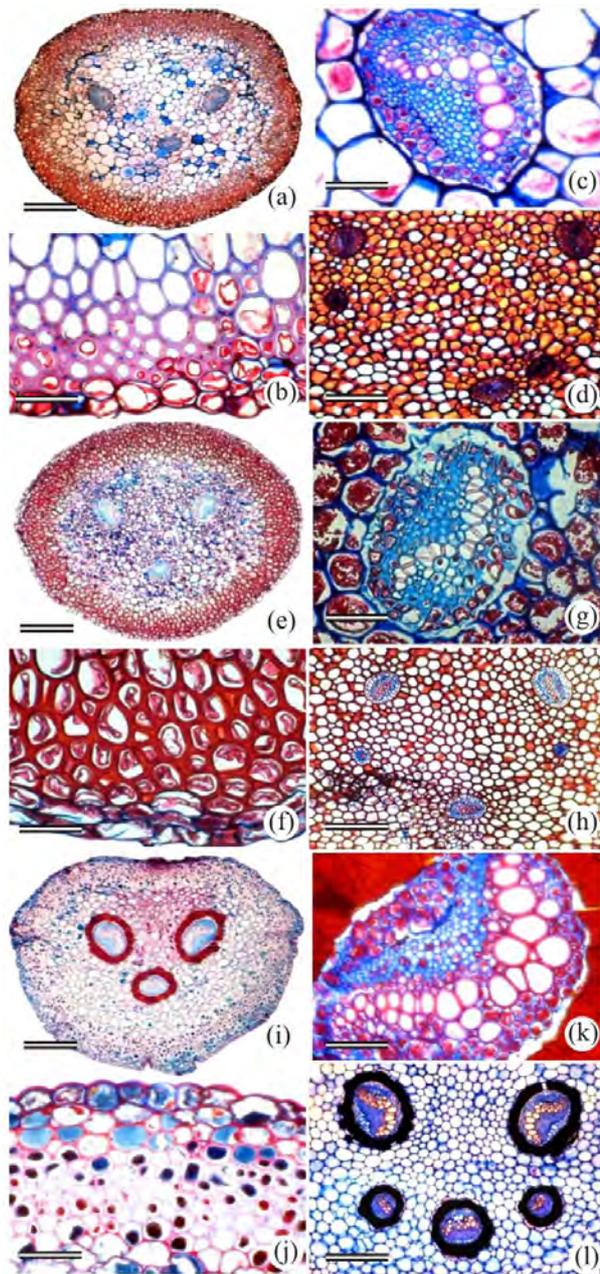


Figure 4. Cross sections of the petiole of *Elaphoglossum*. (a)-(d) *E. discolor*; (e)-(h) *E. flaccidum*; (i)-(l) *E. laminarioides*; (a), (e), (i) General view; (b), (f), (j) Detail of the epidermis and adjacent sclerenchymatous cells; (c), (g), (k) Detail of the vascular bundles in the median region; (d), (h), (l) Vascular bundles in the basal region. Bars: (a), (e), (i) = 250 μm ; (b)-(d), (f)-(h), (j)-(l) = 50 μm .

2(l)), or even in the same cell (**Figure 3(l)**). In the material fixed in either FAA or NBF, exudate color varied from yellow to light brown (**Figures 5(a) and (b)**). Exudate was more abundant on leaf blades and more conspicuous in *E. laminarioides*. The results of the histochemical tests are shown in **Table 1** and **Figures 5(a)-(t)**.

4. Discussion

The species analyzed showed significant variation in their structural characters, especially leaf blades and histochemical features. Based on our results, *E. laminarioides* was the most different in terms of structural variation and the highest number of bioactive compounds. Some of the leaf structural features studied, such as sinuous epidermis and hypostomatic leaf blades, concur with the descriptions by [18] for *E. latifolium*, *E. muscosum*, *E. pallidum* and *E. villosum* and by [20,21] for *E. crassipes* (Hieron.) Diels, *E. gayanum* (Fée) T. Moore, *E. lindbergii* (Mett. ex Kuhn) Rosenst., *E. lorentzii* (Hieron.) H. Christ, *E. pachydermum* (Fée) T. Moore, *E. piloseloides* (C. Presl) T. Moore and *E. yungense* De la Sota.

While polocytic stomata predominated in all species, they were associated to copolocytic and anomocytic stomata in *Elaphoglossum laminarioides*. Although polocytic and copolocytic stomata are among the five types that can be found in *Elaphoglossum* [36], anomocytic stomata are not described for this genus. [37,38] did not correlate this stoma type with those found in *E. laminarioides*, but Sen and De (1992) asserted that polocytic stomata are never associated to anomocytic ones. Nevertheless, latter studies carried out by [20,21] identified anomocytic stomata in Argentinean species of *Elaphoglossum*. This variation in stomata typology is relevant since

it can be used as an infrageneric diagnostic feature.

As only *E. flaccidum* presented branched cells and rostriform margins, mesophyll and leaf margins are also useful infrageneric features. [39] stated that homogeneous mesophyll with branched cells occurs only in Pteridaceae species that grow in shady habitats. Since this is the case of *E. flaccidum*, the present study corroborates their assertion.

One of the most common features mentioned in anatomical studies on ferns is their lignified schlerenchymatous sheath in the cortex [40-46], which only encloses the petiole vascular bundles in *E. laminarioides*. It is considered a specialized character found in specimens of most of the derived families, such as Dryopteridaceae [45].

Although no visitors were observed *in loco*, many leaves were chewed and pathogens such as fungal hyphae were observed on leaf epidermis. This is likely due to the presence of lipids, which are considered nutritionally important for different classes of visitors and pathogens [47].

The starch grains observed on the leaf blade of the studied species are believed to be transitorily stocked in the chloroplast during the day and degraded at night to maintain plant metabolism [48].

Although we know that epiphytes are more vulnerable to water stress [49], *E. laminarioides* has always been collected in areas more exposed to the sun. The mucic

Table 1. Results of histochemical tests on secreting of *Elaphoglossum*.

Metabolites		Tests	Species		
			<i>E. discolor</i>	<i>E. flaccidum</i>	<i>E. laminarioides</i>
Lipids	Total lipids	Sudan black B	-	-	+
	Acids and neutral lipids	Nile blue sulphate	-	-	++
	Free fatty acids	Cooper acetate/rubeanic acid	-	-	+
Terpenoids	Essential oils and oil-resin	NADI reagent	-	-	-
		FFS	+	+	++
Phenolic compounds	Total phenolic compounds	Ferric chloride	+	+	+
		Tannins	-	-	-
		Lignins	-	-	-
Alkaloids		Wagner's reagent	-	-	++
		Dragendorff's reagent	-	-	++
Polysaccharides	Starch	Lugol's solution	++	++	++
	Mucilage	Ruthenium red	-	-	-
	Total polysaccharides	PAS reaction	-	-	++
	Mucilage	Tannic acid/ferric chloride	-	-	+

Notes: ++ = high positive; + = positive; - = negative.



Figure 5. Histochemical characterization of the secretion in leaf blade cross sections of *E. laminarioides*. (a), (b) Sections without treatment, note yellow to light brown color; (c), (d) Detection of lipoic acid with Nile blue, note the secretion in the cell (asterisk); (e), (f). Positive result for fatty acids with copper acetate/rubeanic acid. (g)-(j) Positive reaction for phenolic compounds: (g), (h) Detection with FFS, note the secretion in the cell (asterisk), (i), (j) Detection with ferrous chloride; (k), (l) Positive result for alkaloids: (k) Detection with Wagner's reagent, (l) Detection with Dragendorff's reagent; (m), (n) positive reaction for starch grains; (o)-(r) Detection of polysaccharides with PAS reaction, note the secretion (asterisk) and starch grains (arrow) in the cell; (s), (t) Positive reaction for mucilage with tannic acid/ferric chloride. Bars: (a), (c), (e), (g), (i), (k), (m), (o), (s) = 150 μm ; (b), (f), (j), (l), (q), (t) = 50 μm ; (d), (h), (n), (p), (r) = 20 μm .

liginous compounds found exclusively in this species thus serve to retain water and protect the plant against herbivory [50], since some chewed leaves were observed. When [51] studied fungus-insect-plant interactions, they observed that insects feed on the fungal hyphae present on the plants (mycophagy), which could explain the oc-

currence of both chewed leaves and fungal hyphae on leaf surfaces.

Phenolic compounds are known components of defensive adaptations. According to [52], they present pharmacological and antinutritional properties that inhibit lipid oxidation and the proliferation of fungi. [53] asserted that

they are involved in adaptation processes as a defense against radiation and pathogenic aggressions.

Likewise, alkaloids, found only in *E. laminarioides*, are toxic and act as a defense against herbivores and parasites, in addition to playing an allelopathic role [54]. Although no *Elaphoglossum* monospecific formations were observed in the field (which could lead us to induce some kind of dominance), the potential allelopathic activity of many fern genera has been proved, e.g. by [55] for *Pteridium* Gled. ex Scop., and [56] for *Dicranopteris* Bernh., *Gleichenia* Sm. and *Sticherus* C. Presl.

Thus, the structural data reported constitute new characters of the representatives of *Elaphoglossum* in vegetative stage. The bioactive compounds identified *in situ* also provide important unpublished data, adding information on the species and suggesting the development of adaptive strategies. This could be observed especially on *E. laminarioides*, which in addition to the compounds of communal occurrence on the species studied, was the only species presenting alkaloids and mucilage. These compounds possess peculiar properties related to herbivore and pathogen avoidance, demonstrating that *E. laminarioides* is possibly better adapted to its environment, producing secondary metabolites that are more varied and directly related to adaptive success.

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