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Ferns and Lycophytes as new challenges

Alterations induced by *Tortrimosaica polypodivora* on the stems of *Microgramma vacciniifolia*: simple or complex galls?

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Abstract

Microgramma vacciniifolia, an epiphyte fern, hosts of two stem galls. One is induced by *Tortrimosaica polypodivora* (Lepidoptera), which can also induce galls in *M. squamulosa* and *M. mortoniana*. The alterations induced by *T. polypodivora* on *M. vacciniifolia* stem were compared to non-galled organs to evaluate the anatomical potentials of host ferns in response to a galling Lepidoptera. Histochemical and histometrical comparisons between galled and non-galled stems were performed to assess the processes leading to gall formation. *M. vacciinifolia* and *M. squamulosa* galls were anatomically similar, although their sizes differ, reflecting the growth potential of each host species. Simple structural alterations, such as hyperplasia of cortical and pericycle cells, occur during gall formation, while cell hypertrophy, common in more complex galls, was only detected on the pericycle. Meristele size remained unaltered in galls, but the pericycle appeared hyperplasic. The protective scales were broader in galls. A nutritive tissue with lipids, typical to Lepidoptera galls, was observed around the larval chamber, with small cells and meristematic activity. Starch, proteins, and reducing sugars accumulated in nutritive cells are uncommonly found in Lepidoptera galls. Despite simple structural alterations, *T. polypodivora* induced a gradient of primary metabolites, similar to angiosperm galls.

Key words: histochemistry, histometry, plant anatomy, plant-insect interactions, scales.

Resumo

Microgramma vacciniifolia, uma samambaia epifíta, abriga duas galhas caulinares, uma delas induzida por *Tortrimosaica polypodivora* (Lepidoptera), que também induz galhas em *M. squamulosa* e em *M. mortoniana*. Alterações induzidas por *T. polypodivora* nos caules de *M. vacciniifolia* foram comparadas aos caules não-galhados, para avaliar os potenciais anatômicos das samambaias hospedeiras em resposta a um Lepidoptera galhador. Comparações histoquímicas e histométricas foram realizadas para avaliar os processos que levam à formação das galhas. As galhas de *M. vacciinifolia* e *M. squamulosa* são anatomicamente semelhantes, embora seus tamanhos diferem, refletindo o potencial de crescimento de cada espécie hospedeira. Alterações estruturais simples ocorrem durante a formação da galha, como hiperplasia das células corticais e pericíclicas, enquanto hipertrofia celular, comum em galhas mais complexas, é detectada apenas no periciclo. Os meristelos não têm o tamanho alterado, mas o periciclo apresenta hiperplasia. As escamas são mais largas nas galhas. Ao redor da câmara larval observa-se um tecido nutritivo com lipídios, típico das galhas de Lepidoptera, com células pequenas e atividade meristemática. Amido, proteínas e açúcares redutores também se acumulam nas células nutritivas, raramente relatado em galhas de Lepidoptera. Apesar das alterações estruturais simples, *T. polypodivora* induz o estabelecimento de um gradiente de metabólitos primários, semelhante ao observado em galhas de angiospermas.

Palavras-chave: histoquímica, histometria, anatomia vegetal, interações inseto-planta, escamas.

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Introduction

Galls are neoformed structures and products of anatomical and chemical alterations that form on diverse plants induced by species-specific herbivores, the gall inducers (Mani 1964; Isaias *et al*. 2014). These neoformed structures are essential for the nutrition and protection of galling organisms (Isaias *et al.* 2014). Although little attention has been paid to insect-fern/lycophyte interactions, recent reviews reported 809 phytophagous insect species on 382 species of ferns and lycophytes. Among them, 3.1% are gall inducers (Santos *et al.* 2019a; Fuentes-Jacques *et al.* 2022). The ferns of *Microgramma* C. Presl (Polypodiaceae) have been highlighted due to numerous associations, mainly with insects. For instance, *Microgramma*-ant interactions were recorded in the presence of domatias, such as stem cavities, in *M. bifrons* (Hook.) Lellinger, *M. brunei* (Wercklé *ex* Christ) Lellinger, *M. fosteri* B.León & H.Beltránand and *M. tuberosa* (Maxon) Lellinger, while *M. megalophylla* (Desv.) de la Sota lacks myrmecodomatia (Almeida *et al.* 2021; Almeida 2018; Santos *et al.* 2019b). In addition, the interactions with gall-inducing insects, such as stem and leaf galls, were reported in *M. percussa* (Cav.) de la Sota found in Costa Rica (Santos *et al.* 2019a), *M. mortoniana* de la Sota (Lehn *et al.* 2020) in the subtropical forests, and *Microgramma squamulosa* (Kaulf.) de la Sota (Kraus *et al.* 1993; Brown *et al.* 2004; Santos & Maia 2018; Santos *et al.* 2019a) and *M. vacciniifolia* (Langsd. & Fisch.) Copel. (Maia & Santos 2011, 2015; Santos & Maia 2018; Santos *et al.* 2019a) in the Brazilian Atlantic forest ecosystems.

The neotropical species of *Microgramma vacciniifolia* are widely distributed in the Atlantic forest, mainly near the lowlands (Sehnem 1970; Almeida 2023). As an epiphyte, *M. vacciniifolia* is commonly found in Restinga (Brazilian sandy coastal plains), xeric environments associated with the Atlantic forest, where several interactions between angiosperms and insects have been reported (Maia 2001; Maia & Souza 2013; Carvalho-Fernandes *et al.* 2016). In *M. vacciinifolia*, two types of stem galls have been described, one of which is induced by *Tortrimosaica polypodivora* Brown & Baixeras, 2004 (Lepidoptera: Tortricidae) (Maia & Santos 2015) and another by *Primadiplosis microgrammae* Maia, 2011 (Diptera: Cecidomyiidae) (Maia & Santos 2011).

The lepidopteran species *Tortrimosaica polypodivora* is a gall-inducing species in the stems of *Microgramma squamulosa* (Brown *et al*. 2004) from the Brazilian southeastern Atlantic forest fragments at São Paulo state. The galling habit is unusual for Tortricidae, whose most species are stem borers, and the ferns are uncommon hosts of these lepidopterans (Brown *et al.* 2004). Although galling insects are generally species-specific, *T. polypodivora* has an oligophagous habit, inducing galls in other species, such as *M. vacciniifolia* (Maia & Santos 2015) and *M. mortoniana* (Lehn *et al*. 2020) occurring in Restinga and the subtropical forests, respectively. The occurrence of galls in closely related species of host plants may have similar biochemical interactions between the galling herbivore and host species or lower specificity when compared to other species of galling insects (Lehn *et al.* 2020).

The anatomical alterations induced by *Tortrimosaica polypodivora* in the stems of *Microgramma squamulosa* were described by Kraus *et al.* (1993), including maintaining a layer of cells with meristematic features lining the gall chamber. This feature is commonly reported for galls induced by Lepidoptera on the leaves and stems of angiosperms, reported as nutritive tissues with lipid accumulation (Bedetti *et al.* 2013; Ferreira & Isaias 2013; Vecchi *et al.* 2013; Ferreira *et al.* 2015, 2022; Rezende *et al.* 2019; Guedes *et al.* 2023). Other alterations reported for *T. polypodivora-M. squamulosa* galls include increased pericycle cell layers in the meristeles and sclereid-like cells lining the larval chambers in the galls in the final stages. Despite the changes Kraus *et al.* (1993) reported, which indicated a few changes in the stem organization, histochemical and histometric studies were not performed. Such kind of analyses were complementary in gall structural studies to understand other induced alterations, such as the differential accumulation of reserves in the nutritive tissues lining the gall chamber and the phenomena of cell hypertrophy and hyperplasia leading to gall formation, as reported in lepidopteran–angiosperm galls (Ferreira & Isaias 2013; Bedetti *et al.* 2013; Vecchi *et al.* 2013; Ferreira *et al.* 2015, 2022; Rezende *et al.* 2019).

Based on gall structural features induced by *Tortrimosaica polypodivora* on *Microgramma squamulosa* and the reports on the oligophagous habit of this gall inducer, we assessed the

anatomical, histometric, and histochemical changes induced by *T. polypodivora* on *M. vacciniifolia* stems. The starting point was the following hypotheses: the galls on *M. vacciniifolia* are products of structural changes similar to those induced by the same species on *M. squamulosa* but with peculiarities similar to the differences between the stems of the two host species; hyperplasia and hypertrophy in the ground and vascular tissues explained the swelling of the galled stem; histochemical changes in the accumulation of primary and secondary metabolites were similar to those described for lepidopteran galls occurring in angiosperms; and variations in stem scale dimensions are induced by *T. polypodivora*.

Material and Methods

Collections and sampling

Non-galled and galled stems of *Microgramma vacciniifolia* individuals (n = 8) were sampled at the Área de Proteção Ambiental de Maricá, Rio de Janeiro, Brazil (22º57'34"S, 42º52'17"W), from March to June 2022. The plants were collected from eight points, from distinct phorophytes (Fig. 1a), common in closed shrubby vegetation of Myrtaceae (Fig. 1b). The collection area was characterized by long sandy plains, with predominant xeromorphic vegetation under the sea (IBGE 2012; Thomazi *et al.* 2013; Melo-Júnior & Boeger 2015). From collected specimens (n = 8), we selected galls induced by *T. polypodivora* that were only observed

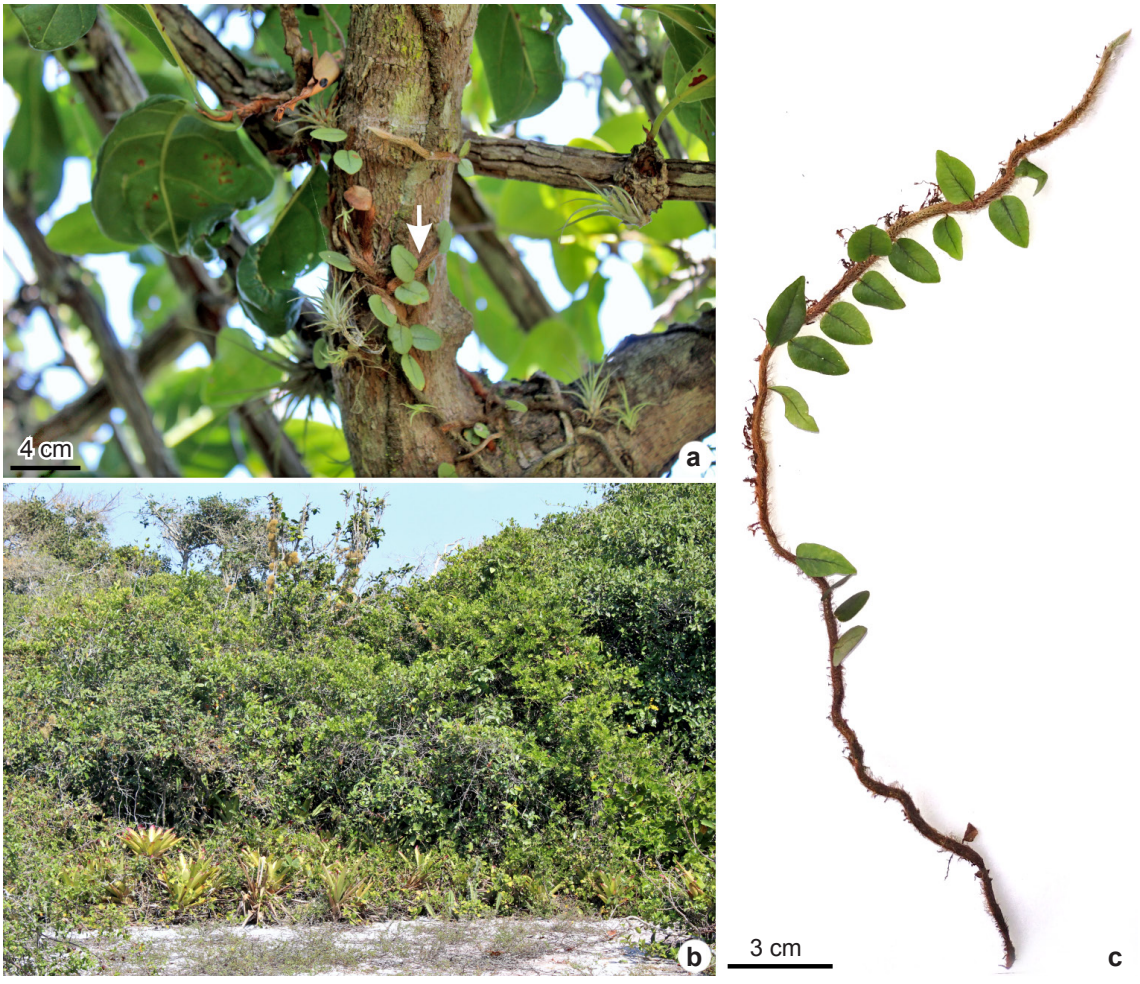

Figure 1 – a-c. *Microgramma vacciniifolia* occurring in the Restinga de Maricá, Rio de Janeiro state, Brazil – a. natural habitat, attached to the trunk of a shrubby phorophyte; b. closed shrubby vegetation of Myrtaceae, where *M. vacciniifolia* was attached to the trunks; c. general aspect of an *M. vacciniifolia* plant removed from the phorophyte.

near the stem tip and non-galled stem tips occurring in the regions corresponding to the positions of the galls, *i.e.*, at the same distance of the stem apex. The voucher specimens were deposited in the Herbarium of Museu Nacional/UFRJ (R; Thiers, continually updated) under the following collector and accession numbers: Martins 03 (R 241143), 04 (R 241144), 05 (R 241143). The length and width of non-galled and galled stems were measured with an MTX digital caliper (Mundo das Ferramentas do Brasil, Guarulhos, Brazil). The morphological terminology followed Lellinger (2002) for ferns and Isaias *et al.* (2013) for galls.

Anatomy

The samples of stem galls (SG) containing pupae or larvae of *Tortrimosaica polypodivora* and corresponding non-galled stems (NGS) were collected for anatomical analysis, n > 5 for each category. The materials were fixed in FAA_{70} (37% formaldehyde, glacial acetic acid, and 70% ethanol; 1:1:18, v/v/v) (Johansen 1940). The NGS and SG were transversely sectioned (30–50 μm) using a table microtome and a holder with an attached razor blade. The sections were clarified, washed in distilled water, and stained with 0.5% Astra blue and 0.5% safranin (9:1) (Kraus & Arduin 1997). The samples were mounted between the slide and coverslip using 50% glycerin and sealed with colorless nail polish. The slides were analyzed and photographed with a digital camera under an Olympus BX51 light microscope.

Cyto-histometry

The transversal sections of NGS (n = 3, stems of different specimens) and SG (n = 3, galls on different host plants) were photographed under a light microscope and analyzed using ImageJ 1.34a software (Abramoff *et al.* 2004) for histometric comparisons. Five sections of each NGS and SG sample (repetitions) were quantified or measured for the following parameters: (i) the number of layers of cortex and pith (in NGS, the number of cell layers in radii of the pith; in SG, the pith cell layers from meristele to larval chamber); (ii) height (anticlinal extension) of the cortex and pith cells; (iii) transverse-sectional area of cortex, pericycle, and pith cells; and (iv) transverse-sectional area of the larger (dorsal) and smaller (ventral) vascular bundles (meristeles). The statistical analyses compared the number of cell layers and cell dimensions using the Student's t-test (t value) when the data was normalized and homoscedasticity data or the Mann–Whitney test (T value), in the case of non-parametric data through the SigmaStat® software. The differences were considered significant when $P < 0.05$.

Histochemistry

The transverse sections of fresh and fixed NGS and SG (n = 5), obtained by the table microtome, were subjected to histochemical tests to compare primary and secondary metabolite accumulation. The sections were subjected to Lugol's reagent for starch detection (Johansen 1940), Sudan IV for lipophilic substances (Jensen 1962), Coomassie blue for proteins (Baker 1958; Fisher 1968), Fehling's reaction for reducing sugars (Sass 1951), and 10% ferric chloride for phenolic compounds (Johansen 1940). The blank sections and controls indicated for each test were analyzed for negative control.

Comparison of scale morphology

The NGS and SG scales were manually removed, placed on slides, photographed under a light microscope, and analyzed by ImageJ software for histometric comparisons. The scales of NGS (n = 6 specimens; 37 scales) and SG (n = 3 specimens; 26 scales) were used for calculating the mean and standard deviation of the subsequent parameters: (i) scale length (from base to apex); (ii) basal width (greater width); and (iii) width of the apical portion (lesser width). The scale dimensions were compared by the Student's t-test (t-value) when the data was normalized and homoscedasticity data or the Mann-Whitney test (T value), in the case of non-parametric data through the SigmaStat® software. The differences were considered significant when $P < 0.05$.

Results

Anatomy and histochemistry

Micrygramma vacciniifolia has a long-creeping stem, dorsoventrally flattened, densely covered with appressed, peltate, and bicolor lanceolate scales (Fig. 1c). The leaves are dimorphic, with sterile ovate to lanceolate and fertile linear-lanceolate leaves, arranged in the dorsal region (Fig. 1a,c). The NGS of *M. vacciniifolia* had an average width of 2.6 ± 0.5 mm and, in the transverse section, has an elliptical shape, dorsoventrally flattened. The NGS is covered by an uniseriate epidermis, consisting of tabular epidermal cells and peltate scales with invaginations in the epidermis (Fig.

2a). The epidermis covers a cortex formed by 9.7 ± 0.8 cell layers of homogeneous parenchyma (Fig. 2a-b). The cortex surrounded a vascular cylinder of the dictyostele type, with usually five, rarely six to nine, circular-shaped amphiphloic meristeles in the cross-section (Fig. 2b). The meristeles were surrounded by a circumendodermal band of cells showing strongly darkened U- and O-shaped thickenings, surrounding endoderm composed of flattened cells and 1–3 layers of quadrangular cells, surrounding three pericycle layers (Fig. 2c). The meristele located in the dorsal region of the stem is the largest (Fig. 2b-c). The four smallest meristeles were found in the ventral position. The dictyostele surrounds a pith with 5.5 ± 0.6 layers of homogeneous parenchyma (Fig. 2b). The histochemical tests detected moderate accumulation of starch (Fig. 2d-e), and slight accumulation of lipids (Fig. 2f), reducing sugars (Fig. 2g), and proteins in the cortex and pith of NGS and reducing sugars accumulated in the endoderm (Fig. 2g). Phenolics were detected in unevenly thickened circumendodermal band walls, cortex and pith cell walls, and scales (Fig. 2h; Tab. 1).

The SG induced by the microlepidoptera, *Tortrimosaica polypodivora*, are characterized by stem swelling due to galling larva in the stem pith (Fig. 3a-b). The stem gall has a fusiform shape (Fig. 3a), with an average length of 29.6 ± 3.1 mm and an average width of 4.5 ± 0.4 mm. As in a NGS, the galls were densely covered with scales, but frond growth was impaired. We also saw little stem branches and galls induced by the midge, *P. microgrammae*. Furthermore, galls were observed closer to the shoot apical meristem regions (Fig. 3a).

An uniseriate epidermis with tabular cells covered the SG in the transverse section, dorsoventrally flattened, and the scales were peltate, with a pedicel with an invagination. The cortex of the SG is formed by homogeneous parenchyma (Fig. 3b-c), formed by 15.9 ± 3.1 layers of cells.

Internal to the cortex of the SG, five amphiphloemic meristeles were observed surrounded by a thickened U-shaped endoderm, one dorsal, with a larger diameter, and the other ventral, with a smaller diameter (Fig. 3b-d). In some transverse sections, it was observed that the meristeles exhibited lateral expansion, merging with adjacent meristeles in oblique to transverse connections (Fig. 3b). The pericycle appeared with 2–4 layers of hypertrophied cells when compared to those of the NGSs (Fig. 3c).

The SG comprises a medullary region with isodiametric parenchyma cells surrounding a single larval chamber (Fig. 3b-e), where a *T. polypodivora* larva or pupa is observed. The gall pith had 5.1 ± 0.7 layers of cells surrounding the larval chamber. The pith cells lining the larval chamber had characteristics of meristematic cells, in division, some with prominent nuclei and dense cytoplasm (Fig. 3c-e), forming a nutritive tissue.

The histochemistry (Tab. 1) revealed an intense accumulation in SG in increasing gradients toward the meristeles (Fig. 3f). Starch grains were detected in the outer layers of pith and nutritive cells (Fig. 3g). Lipids and reducing sugars were also detected in the cortex and pith of SG (Tab. 1). An accumulation was detected in some isolated nutritive cells lining the gall larval chamber (Fig. 3h-i). In the endoderm surrounding the meristeles, reducing sugars also accumulate. An intense accumulation of reducing sugars was detected in the vascular bundles of the SG in relation to the NGS. The proteins were detected in the cortex and pith, with a high concentration in the nutritive cells surrounding the larval chamber (Fig. 3j). The phenolics were detected in unevenly thickened walls of the circumendodermal bands (Fig. 3c), in the cell walls of some parenchyma cells, and in the scales in the epidermis of galls (Tab. 1).

The pith region was consumed entirely in senescent galls, usually with a pupa, indicating a more advanced stage, with sclerenchyma-like cells lining the chamber and unevenly thickened cell walls (Fig. 3k-l). The hyperplasia and hypertrophy of pericycle cells near the gall chamber were visually evident in the vascular bundles (Fig. 3l).

Cyto-histometry

In the cortex, a significant 60% increase in the number of cell layers indicated the occurrence of hyperplasia in the SG (15.9 ± 3.1 layers) when compared to NGSs (9.7 ± 0.8 layers) ($t = -4.325$; $P = 0.003$) (Fig. 4a). The cell height was 30% higher in SG (0.86 ± 0.19 μm) compared to NGS (0.65 ± 0.03 μm), revealing a statistically significant difference ($t = -2.549$; $P = 0.034$) (Fig. 4b). The area of the cortical cells did not show a significant increase in the SG (6990.7 ± 2660.6 μm^2) in relation to the NGS (6750.5 ± 2660.6 μm^2) ($t = 0.239$; $P = 0.813$) (Fig. 4c).

No significant differences were found in the area of the dorsal meristele (the larger) in the transverse section of the SG (76778.5 ± 21297.44 μm^2) and NGS (64325.6 ± 28297.5 μm^2) ($t = 0.715$;

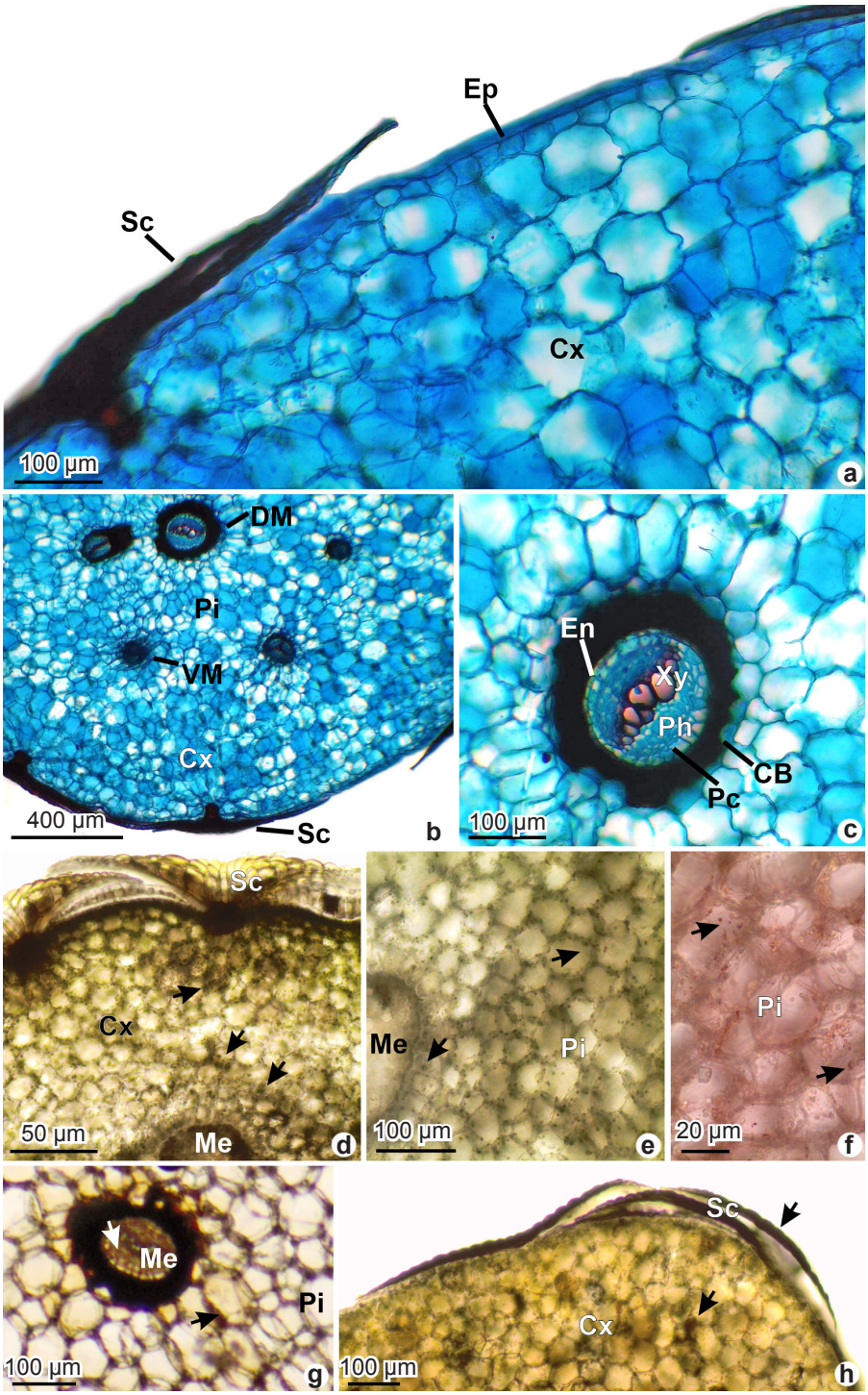

Figure 2 – a-h. Anatomy and histochemistry of *Microgramma vacciniifolia* stem in transverse sections – a. epidermis (Ep) with a peltate scale (Sc) inserted in an invagination; b. vascular cylinder of dictiostele type displaying ventral meristeles (VM) and the major dorsal meristele (DM); c. a dorsal meristele with a circumendodermal band (CB) surrounding the endoderm (En), pericycle (Pc), phloem (Ph), and xylem (Xy); d-e. starch (arrows) detected in the cortex, pith, and endoderm; f. lipids (arrows) detected in the pith; g. slight detection of reducing sugars (arrow); h. phenolics detected in the scales and cortical cells. Staining: (a-c) astra blue and safranin; (d-e) Lugol; (f) Sudan IV; (g) Fehling; (h) Ferric chloride. Abbreviations: Cx = cortex; Me = meristele; Pi = pith.

Table 1 – Comparative cyto-histochemistry of non-galled stems of *Microgramma vacciniifolia* (Polypodiaceae) and stem galls induced by *Tortrimosaica polypodivora* (Lepidoptera: Tortricidae). (-) = negative reaction; (+) = moderately detected; (++) = intensely detected.

	Starch	Lipids	Proteins	Reducing sugars	Phenolics
Non-galled stem					
Epidermis	+	+	-	-	+
Cortex	+	+	+	-	-
Vascular bundles	-	++	+	+	+
Pith	++	+	+	-	-
Stem gall					
Epidermis	+	+	-	-	+
Cortex	++	+	+	-	-
Vascular bundles	-	+	+	+	++
Pith	+	+	+	+	-
Nutritive tissue	++	+	++	+	-

P = 0.502) (Fig. 4d). Similarly, no significant differences were found in the size of the ventral (smaller) meristeles of SG (22085.4 ± 9078.8 μm^2) and NGS (16036.1 ± 9628.7 μm^2) (t = -0.715; P = 0.189) (Fig. 4e).

The area of pericycle cells revealed that the pericycle cells of NGS were smaller (188.38 ± 95.65 μm^2) than those of SG (277.90 ± 124.35 μm^2), confirming that the pericycle cells suffered cell hypertrophy in the *T. polypodivora* galls (t = 2.210; P = 0.035).

The SG pith had 5.1 ± 0.7 layers of cells surrounding the larval chamber, a value statistically similar to the NGS pith (5.5 ± 0.6 layers) (T = 33.50; P = 0.222) (Fig. 4f). However, it is essential to consider that these cells were constantly consumed by the lepidopteran larva, and indicated that this was a hyperplasic tissue. The SG pith parenchyma cells had 0.19 ± 0.06 μm in height, approximately 80% smaller in relation to NGS pith cells (0.33 ± 0.06 μm), showing a significant reduction in the galls (t = 3.815; P = 0.005) (Fig. 4g). The mean area of the SG pith cells was 2141.2 ± 927.3 μm^2, showing a reduction of approximately 70% in relation to the NGS (6348.1 ± 2298.98 μm^2), a statistically significant value (T = 342.00; P < 0.001) (Fig. 4h).

Scales

The comparison of the scales revealed anatomical similarities between the NGS (Fig. 5a) and SG (Fig. 5b). The scales were peltate, lanceolate, with slightly thickened cell walls in central and non-thickened walls in the marginal cells. The bicolored scale apex was acute to caudate, with elongated, brown-colored central cells with cylindrical to slightly curved walls. The marginal cells were hyaline and had sinuous walls (puzzle-shaped cells) (Fig. 5c). The NGS scales were 4.80 ± 0.70 mm long, with a basal width of 0.74 ± 0.16 mm, and a distal width of 0.05 ± 0.02 mm. The SG scales were 5.33 ± 1.11 mm long, with a basal width of 0.89 ± 0.20 mm and a distal width of 0.11 ± 0.17 mm. Statistical analysis revealed that the length of the NGS and SG scales was similar (T = 698.00; P = 0.062). However, the scales of the SG were broader in the basal portion (t = 3.415; P = 0.001) and the distal portion (T = 990.00; P = 0.028) compared to NGS.

Discussion

The galls induced by *Tortrimosaica polypodivora* in *Microgramma vacciniifolia* are formed due to simple structural alterations compared to other lepidopteran galls described in angiosperms (Ferreira *et al.* 2019). The anatomical alterations were similar to *T. polypodivora* galls on *M. squamulosa* (Kraus *et al.* 1993), as an expected similar influence of the galling stimuli on congeneric host plant species. Histometric comparisons confirmed the occurrence of hyperplasia in the cortex and pericycle, cell hypotrophy in the pith

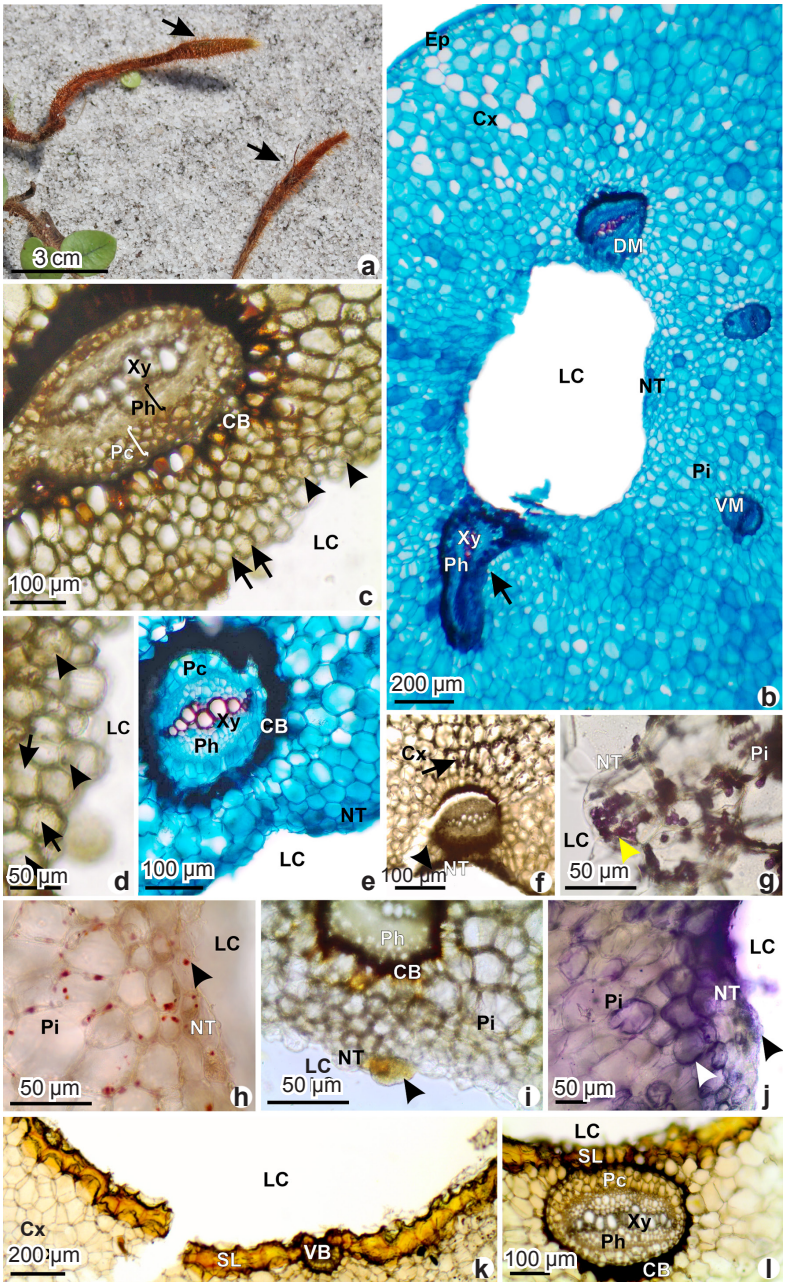

Figure 3 – a-l. Anatomy and histochemistry of the galls induced by *Tortrimosaica polypodivora* on the stem of *Microgramma vacciniifolia* – a. macrograph of fusiform stem galls (arrows); b-j. light micrographs of transverse sections – b. general view, with an obliquely disposed meristele (arrow); c. meristele with hypertrophied and hyperplasic pericycle; d. detail showing the nutritious cells lining the gall chamber under divisions (arrows) and with evident nucleus (arrowheads); e. meristele, pith, and nutritive tissue; f. a gradient of starch toward the meristeles (arrow) and nutritive cells (arrowhead); g. starch in the nutritive tissues (yellow arrow); h. lipid droplets (arrow) in the nutritive tissues; i. reducing sugars (arrow) detected in isolated cells of the nutritive tissues; j. proteins accumulated in the nutritive tissues (black arrowhead) and adjacent pith cells (white arrowhead); k-l. senescent galls with consumed pith and sclerenchyma-like tissue formed. Staining: (b,e) Astra blue and safranin; (c,d) Ferric chloride; (f-g) Lugol; (h) Sudan IV; (i) Fehling; (j) Coomassie blue; (k-l) blank sections. Abbreviations: CB = circumendodermal band; Cx = cortex; DM = dorsal meristele; Ep = epidermis; LC = larval chamber; NT = nutritive tissue; Pc = pericycle; Pi = pith; Ph = phloem; SL = sclerenchyma-like tissue; VB = vascular bundle; VM = ventral meristele; Xy = xylem.

cells, and hypertrophy in the width of the scales. SG revealed simple structural alterations, but the histochemical gradients demonstrated that despite maintaining the types of substances stored in the stem, the galling organism potentiates and induces the redistribution of the accumulated lipids and reducing sugars toward the larval chamber, essential for nutrition, as observed in other lepidopteran-induced galls (Bedetti *et al.* 2013; Ferreira & Isaias 2013; Vecchi *et al.* 2013; Rezende *et al.* 2019; Ferreira *et al.* 2019, 2022). However, some compounds were not found in the nutritive tissues in all Lepidoptera galls, such as proteins and starch, demonstrating that the specific metabolism of the host species is essential for determining the histochemical features in nutritive cells (Guedes *et al.* 2023).

Unlike what was observed for *Tortrimosaica polypodivora* (Brown *et al.* 2004; Maia & Santos 2015; Lehn *et al*. 2020), the galling insects were mostly species-specific, affecting one host plant species (Fernandes *et al.* 2014). However, the dimensions observed in the *T. polypodivora* galls on *M. squamulosa* stems (Kraus *et al.* 1993) and *M. mortoniana* (Lehn *et al.* 2020) were distinct for the size of *T. polypodivora* galls induced on *M. vacciniifolia.* This indicated that, despite the similar anatomical alterations between *M. vacciniifolia* and *M. squamulosa*, the extent of gall growth was related to the different growth potential among different host species of *T. polypodivora*.

Regarding structural features, many similarities were observed between the stems of *M. vacciniifolia* and the galls of *T. polypodivora*, as described by Kraus *et al*. (1993) for *M. squamulosa* NGS and SG, respectively. In addition, the stem structure was simple in both species, with similarities across all tissue systems. The presence of a larger dorsal meristele and smaller ventral meristeles was reported by Hirsch & Kaplan (1974) for *M. vacciniifolia* and *M. squamulosa*. These authors observed that the two smaller ventral meristeles vascularized the adventitious roots while the two lateral ones were connected to the leaves.

The present study demonstrated that the shape of the galls was determined by hyperplasia in the stem cortex, a process involved in gall development in general (Mani 1964; Ferreira *et al.* 2019). Although pith cells in SG and NGS were statistically similar, it is essential to consider that the pith region (when we also include the larval chamber) was more significant in SG than in NGS because it is constantly consumed by *T. polypodivora* larva. Therefore, the pith cells

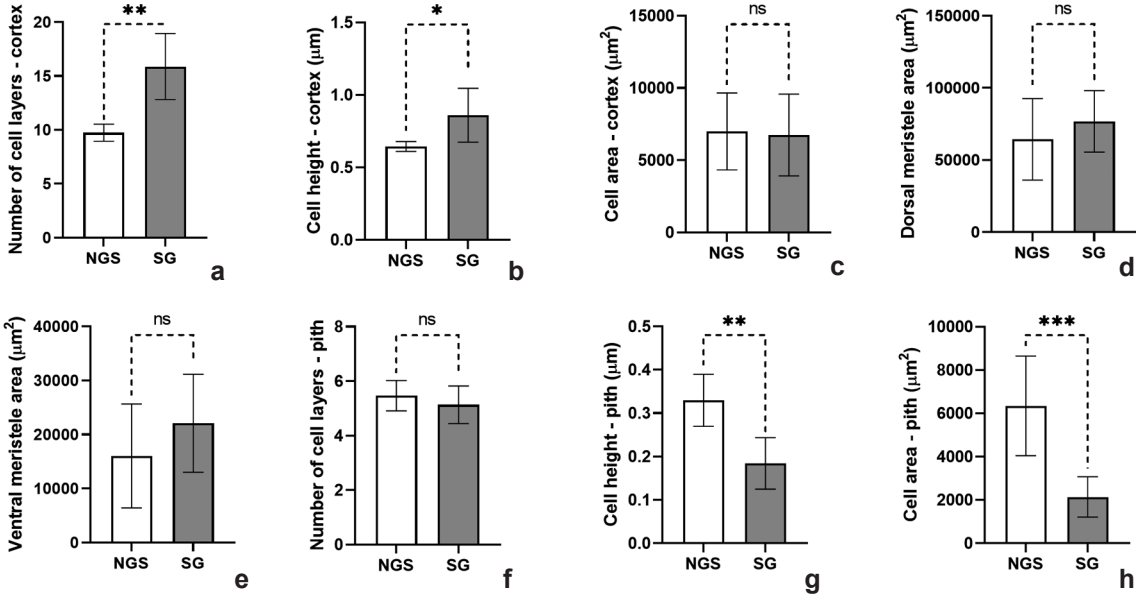

Figure 4 – a-h. Cyto-histometry of the stem of *Microgramma vacciniifolia* and the fusiform stem galls induced by *Tortrimosaica polypodivora* – a-c. cortex – a. the number of cell layers; b. cell height; c. cell area; d-e. meristeles – d. dorsal meristele; e. ventral meristele; f-h. pith – f. the number of cell layers; g. cell height; h. cell area. NGS = non-galled stem; SG = stem gall. Levels of statistical significance: (ns) = non-significant; (***) = $P < 0.001$; (**) = $P < 0.01$; (*) = $P < 0.05$.

in SG consumed by the larva were replaced by the meristematic activity of nutritive cells. Cell hypertrophy was not detected in the cortical and pith parenchyma cells of the studied galls, different from what was commonly reported for galls in angiosperms, where cellular hypertrophy was common, mainly in the external layers of the galls (Isaias *et al.* 2011, 2014; Ferreira & Isaias 2013; Ferreira *et al.* 2019). Cell hypertrophy and hyperplasia occurred in pericycle cells, distinct from the histometrical observations of cortical and pith parenchyma cells. However, the meristeles between NGS and SG were statistically similar in size. Visual changes in the number of cell layers and cell sizes in the pericycle of galls were described in *M. squamulosa-T. polypodivora* galls (Kraus *et al.* 1993). In addition, as demonstrated for *M. vacciniifolia*, oblique and transverse meristele fusions have been described in the galls of *M. squamulosa* (Kraus *et al.* 1993).

In the region surrounding the larval chamber of mature galls, the pith cells maintained, on average, the number of layers compared to the NGS. The innermost pith layers, formed by the nutritive cells, have meristematic features and were destroyed by the chewing activity of *T. polypodivora* larva. The feeding activity of *T. polypodivora* stimulated continuous divisions leading to a significant reduction in pith cells in height and area, in addition to presenting

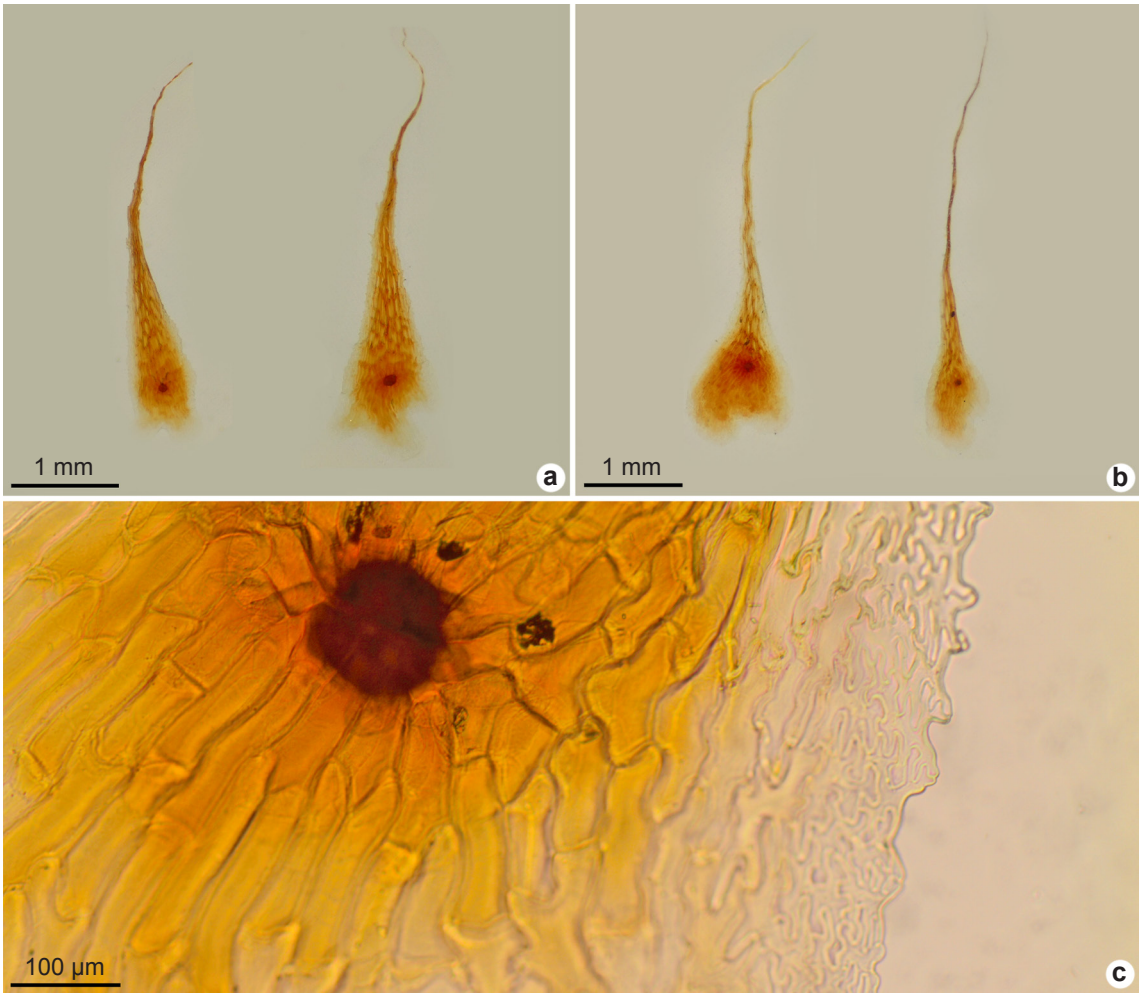

Figure 5 – a-c. Frontal view of scales on the non-galled stems of *Microgramma vacciniifolia* and galls induced by *Tortrimosaica polypodivora* – a. non-galled stem scales; b-c. gall scales; c. detail of a scale of the gall, showing brown-colored cells with cylindrical to curved walls in the center and hyaline cells with conspicuous curved walls in the margin.

meristematic characteristics, described by Kraus *et al.* (1993). Accordingly, the feeding activity was an essential stimulus for nutritive tissue formation in galls (Kostoff & Kendall 1929; Larew 1981; Bronner 1992; Ferreira & Isaias 2013). The features of nutritive cells lining *T. polypodivora* galls were similar to typical nutritive tissues described in other gall systems, as meristem-like cells, with dense cytoplasm, storage accumulation, and evident nucleus (Ferreira *et al.* 2017). Nutritive cells induced in *T. polypodivora-M. vacciniifolia* galls accumulated starch, lipids, reducing sugars, and proteins. The lipid accumulation in the nutritive tissues was a pattern in Lepidoptera galls since it was detected in Lepidoptera-angiosperm galls (Guedes *et al.* 2023). However, the proteins were reported only in Lepidoptera galls induced on *Macairea radula* (Bonpl.) DC. (Melastomataceae) (Rezende *et al.* 2019), *Schinus engleri* F.A.Barkley (Ferreira *et al.* 2022), and *Schinus polygama* (Cav.) Cabrera (Anacardiaceae) (Guedes *et al.* 2023), while starch was only reported in the galls of *Parthenium hyserophorus* L. (Asteraceae) (Raman & Dhileepan 1999), reinforcing that the metabolic features of the host plants are essential in determining gall chemical features (Guedes *et al.* 2023). Starch is detected only in the outer layers of galls in general, related to the tissues' metabolic maintenance (Ferreira *et al.* 2017). In conclusion, chewing insect galls, including those occurring in ferns, have nutritive cells around the larval chamber, responsible for providing the necessary nutritional support for the galling organism (Bronner 1992; Ferreira *et al.* 2017).

Current results demonstrated that senescent *Tortrimosaica polypodivora* galls had no pith cells and were consumed by the larvae up to the area in contact with the circumendodermal band. At this stage, the gall chamber was lined by a sclerenchyma-like tissue with unevenly thickened cell walls (Kraus *et al.* 1993; current results). Although in several galls, a sclerenchyma or mechanical layer was observed in the mature stages and considered a protective tissue to the galling larva (Rezende *et al.* 2019; Ferreira *et al.* 2019, 2022), the current results in *M. vacciniifolia* and those in *M. squamulosa* gall (Kraus *et al.* 1993) indicated that such sclerenchyma-like tissues were formed as a wound reaction at the end of *T. polypodivora* larval stage. The wound tissues were studied in several ferns, and although suberization may be observed in some cases, a typical phellogen such as those of angiosperms was not observed (Holden 1912). The most common response to wounds in the ferns is the formation of an adjacent layer of evenly or unevenly thickened cell walls by depositing cellulose, lignins, and/or tannins and adding to the accumulation of gums in the protoplast (Holden 1912). The wound cells observed in senescent *T. polypodivora* galls represented a typical response of ferns to injuries. In the galls, they can avoid the entry of pathogens and fungi after the gall inducer exits.

Furthermore, the maintenance of an intact meristele is related to the phenolic accumulation in the circumendodermal band walls, which were usually related to the defense against herbivores and pathogens (Feeny 1976; Tempel 1981), and removal of excessive reactive oxygen species (Tuladhar *et al.* 2021), avoiding premature cell senescence in the galls (Isaias *et al.* 2018; Ferreira *et al.* 2018; Guedes *et al.* 2022). A previous comparison of phenolic substances that accumulated in the NGSs and galls induced by *T. polypodivora* revealed that they had similar accumulated phenolic substances (Santos *et al.* 2022). On the other hand, in the same comparisons, the galls induced by *P. microgrammae* showed differences with the NGSs (Santos *et al.* 2022), revealing that the latter species induced alterations in polyphenol biosynthesis in the same host plants.

In addition to the alterations investigated in the tissues of ground and vascular systems of *Microgramma vacciniifolia* stems induced by *Tortrimosaica polypodivora,* we expected that the changes in gall indumenta would occur, especially in scale size, since it was reported for other lepidopteran-induced galls (Ferreira & Isaias 2013; Rezende *et al.* 2019). Although the scale length was similar between SG and NGS, the scales in galls were significantly broader than those of NGS. Fern scales may help in water absorption, an adaptive trait reported for some Polypodiaceae species, emphasizing epiphyte species (Pandé 1935; Lagoria *et al.* 2018). However, Starnecker & Winkler (1982) reported no water absorption by stem scales of *M. vacciniifolia* and *M. squamulosa*, and adequate water absorption by those of *Pleopeltis hirsutissima* (Raddi) de la Sota, *Pleopeltis mínima* (Bory) J. Prado & R.Y. Hirai, and *Pleopeltis angusta* Humb. & Bonpl. *ex* Willd. (Polypodiaceae). In the case of both *Microgramma* species, they reflect excessive light and reduce water transpiration (Starnecker & Winkler 1982). In the galls, such scales play a protective role against natural enemies and hygrothermal stress,

representing an additional adaptive value to the galling organisms inhabiting *M. vacciniifolia* stems, especially in xeric environments, such as the *restinga*, where this fern is found.

Concluding remarks

Fern gall features were explored anatomically, histometrically, and histochemically in the *Microgramma vacciniifolia-Tortrimosaica polypodivora* system, revealing the extent of developmental alterations during gall formation on such plants. Although there were simple structural changes with the absence of cell hypertrophy in the gall cortex and pith, a gradient of primary metabolites was found in the nutritive tissues, commonly observed in other gall systems. Additionally, the scales on galls were broader than those of NGSs. As we expected, the galls induced on *M. vacciniifolia* stems were anatomically similar to those described in *M. squamulosa*, even though they have distinct dimensions, which seem to be imposed by host plant growth. In the pith region, the cells showed meristematic characteristics common to nutritive cells in angiosperm galls. Histochemical analyses revealed similar gradients and accumulation of proteins, lipids, starch, and reducing sugars in these nutritive cells when compared to angiosperm galls induced by Lepidoptera, even though starch and protein accumulation was not commonly reported as a nutritional resource of other galling Lepidoptera. Despite these similarities, only hyperplasia responses were detected in the galls studied, which diverged with the common processes of angiosperm gall formation, where cell hypertrophy was quite common in cortical tissues. The hyperplasia and cell hypertrophy of pericycle and transverse fusions among meristeles are other notable features of the currently studied galls. Distinct from what was familiar to the other galls, the *T. polypodivora-Microgramma* spp. galls in the final stage of development revealed the formation of an unevenly thickened sclerenchyma layer due to a wound process commonly described in the ferns, protecting the host plant after the exit of the gall inducer. As models of developmental studies, other galls induced in ferns need to be studied to reveal divergent cellular processes that were not reported in other groups of vascular plants.

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Data availability statement

In accordance with Open Science communication practices, the authors inform that all data are available within the manuscript.

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