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Ferns and Lycophytes as new challenges

As soft as silk: structural and chemical traits can help with the identification of *Niphidium crassifolium* (Polypodiaceae) gall inducers

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Abstract

Ferns have been poorly reported as hosts of gall inducers, and their multitrophic interactions and relationships are practically unknown to science. We focused on *Niphidium crassifolium* (Polypodiaceae) that hosts globoid leaf galls. The galls on *N. crassifolium* have only reported for the South and Southeast regions of Brazil, with a discussion regarding the identity of the gall inducer: is it a Cecidomyiidae-Diptera or to Coccidae-Hemiptera? These two insect groups have distinct characteristics and consequently their galls must have distinct anatomical and histochemical traits. Such traits may work out as functional tools to be used to confirm the taxa of the associated galling herbivore and to evaluate their geographic distribution. Our study aimed to expand the known distribution of *N. crassifolium* and its interactions based on an inventory of scientific articles and on herbarium data, and also to test the usefulness of anatomical and histochemical traits for proposing the identity of the gall inducer. The geographic distribution of *N. crassifolium* galls involves five South American countries, *i.e.*, Bolivia, French Guiana, Guyana, Peru, and Brazil. The development of an outer tissue compartment with phenolic-rich cell layers and an inner nutritive tissue leads us to infer that the inducer belongs to the Cecidomyiidae family.

Key words: Diptera-Cecidomyiidae, fern galls, geographical distribution, phenolics, proteins.

Resumo

Samambaias são pouco relatadas como hospedeiras de galhas, e suas interações e relações multitroficas são comumente desconhecidas pela ciência. Atualmente, nos concentramos em *Niphidium crassifolium* (Polypodiaceae) que abriga galhas globoides nas folhas. As galhas em *N. crassifolium* são relatadas apenas nas regiões sul e sudeste do Brasil, onde também há uma discussão sobre a identidade do indutor da galha: seria um Cecidomyiidae-Diptera ou um Coccidae-Hemiptera? Esses dois grupos de insetos possuem características distintas e, consequentemente, suas galhas devem apresentar características anatômicas e histoquímicas distintas. Tais características podem ser utilizadas como ferramentas funcionais para corroborar a identificação dos táxons do galhador associado e avaliar sua distribuição geográfica. Nosso estudo teve como objetivo ampliar a distribuição conhecida de *N. crassifolium* e suas interações com base em inventário de artigos científicos e dados de herbário, e também testar a utilidade de características anatômicas e histoquímicas para propor a identidade galhador. A distribuição geográfica das galhas de *N. crassifolium* foi ampliada para cinco países da América do Sul: Bolívia, Guiana Francesa, Guiana, Peru e Brasil. O desenvolvimento de um compartimento tecidual externo com camadas de células ricas em fenólicos e um tecido nutritivo interno nos leva a supor que o indutor pertence a família Cecidomyiidae.

Palavras-chave: Diptera-Cecidomyiidae, galhas em samambaia, distribuição geográfica, fenólicos, proteínas.

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Introduction

Niphidium crassifolium (L.) Lellinger (Polypodiaceae) is a predominantly epiphytic plant that may occasionally grow on rocks or on the ground. It is distributed from Central America (Mexico to Panamá) to South America (from Colombia to the north of Peru, Brazil, French Guiana, and Guyana), and the Caribbean islands (Lellinger 1972; Moran 1995), mostly below 1,500 m. This plant hosts globoid galls with a pocket-like development and silk covering the gall aperture. Galls are new plant organs that exhibit diverse forms in nature; however, despite this morphological variation, their morphology and anatomy are related to their inducer (Mani 1964; Isaias *et al.* 2013). There is controversial information regarding gall shape, which has been previously recorded as globoid, conical, cylindrical, or clavate in Rio de Janeiro (Maia & Mascarenhas 2017, 2021; Santos & Maia 2018), and as clavate and columnar tridimensional in Rio Grande do Sul, Brazil (Farias *et al.* 2020; Cenci & Horodyski 2022). Also, there is still a lack of information regarding the presence of these galls according to host plant distribution, as well as the identification of the gall inducer at the order or species level (Maia & Mascarenhas 2017, 2021). The gall inducer was first described as an undetermined species of Coccidae-Hemiptera (Rübsaamen 1908; Houard 1933) and later on as an undescribed species of Diptera (Santos & Maia 2018). The confusion persists after a recollection and presumption of its identification as a Coccidae-Hemiptera insect (Cenci & Horodyski 2022) based on the first identification by Rübsaamen (1908) and Houard (1933) and the silk covering the gall aperture. This silk is classified as a thin white membrane that coats the abaxial surface of the gall (Rübsaamen 1908). The presence of serous secretions is common in certain Coccidae species (Gullan & Kosztarab 1997). These secretions are formed by various substances such as wax, resins, and lipids (Gullan & Kosztarabs 1997).

Galls are induced in all groups of non-vascular and vascular plants by several organisms, whose development involves hyperplasia and cell hypertrophy (Mani 1964). Even though galls are more frequent in spermatophytes, they may also be found in ferns and lycophytes (Santos *et al.* 2019), where at least 153 species hosting insect-induced galls have been reported worldwide. However, the anatomical and histochemical traits of these interactions have not been commonly described. Diptera: Cecidomyiidae are the most frequent gall inducers associated with fern species, but Lepidoptera, Thysanoptera, Hemiptera, Hymenoptera, and Coleoptera may also induce galls on leaves, shoots (erect or creeping rhizomes), and buds of ferns (Santos & Maia 2018; Santos *et al.* 2019). Recently, a review of galls on ferns and lycophytes reported 133 gall morphotypes associated with 93 host species from 41 genera, mainly in the Neotropical region, with Polypodiaceae being the most important taxon regarding the gall records (Santos *et al.* 2019). In addition, the record of ferns published in gall inventories is rare and even neglected, as also is the identity of their inducers, as mentioned for *N. crassifolium* (Polypodiaceae) (Maia & Mascarenhas 2017, 2021; Santos & Maia 2018).

When the identification of the galling insect, which demands the collection of all instars, is not possible, anatomical and histochemical gall traits can provide evidence about the taxonomic identity of the inducer since gall development usually follows conservative patterns (Rohfritsch 1992; Ferreira *et al.* 2019), with galls thus representing the extended phenotypes of their associated galling herbivores (Isaias *et al*. 2013; Carneiro *et al.* 2015). In addition, gall traits may work out as efficient tools for evaluating the geographical distribution and local richness of gall inducers by consulting herbaria collections (Arriola *et al*. 2016; Ley-López *et al*. 2019; Mertz *et al.* 2022), as herein proposed for *N. crassifolium* globoid galls. Our focus aimed to (I) expand knowledge about the distribution of *N. crassifolium* gall inducers and (II) to test the anatomical and histochemical traits of galls to help the taxonomic identification.

Material and Methods

Geographic distribution of gall records

The distribution of galls on *N. crassifolium* was checked in herbaria and in the literature. A total of 813 exsiccates from the F, NY, EFC, FURB, HUEM UEM, CRI, US, UEC, and MBML herbaria (according to Thiers, continuously updated) were checked. The first step in the search for literature reports was taken on January 13, 2023 using Google Scholar and the keyword "*Niphidium crassifolium* galls", covering the period from 1988 to 2023. As a second step, we consulted the speciesLink network (speciesLink 2023) in January 2023 to access herbarium data on *N. crassifolium* hosting galls worldwide, as proposed by Arriola *et al*. (2016). In order to study the geographical distribution of gall inducers, we analyzed georeferencing data obtained from specimens sourced from various taxonomic collections within the speciesLink database. We specifically included data points where the coordinates were labeled as "consistent" or "original". The distribution map of *N. crassifolium*

galls was constructed using QGIS version 3.10.5 (QGIS.org 2020) and the database of the Brazilian Institute of Geography and Statistics (IBGE).

Sampling

Samples of non-galled leaves (n = 10) and of mature galls (n = 10) were collected from three individuals of *N. crassifolium* at Pedra do Garrafão, Santa Maria de Jetibá, Espírito Santo, Brazil (20°10'24.5"S, 40°55'06.6"W), at 1,079 m altitude. The samples were stored in plastic bags and transported in an ice cooler to the laboratory, where the galls were opened under a stereomicroscope to check for the presence of insect larvae or nymphs. The samples of galls and non-galled leaves were immediately fixed in Karnovsky's solution (2.5% glutaraldehyde and 4.5% formaldehyde) (Karnovsky 1965, modified to 0.1 mol L^{-1} phosphate buffer, pH 7.2) for 24-48 hours and then submitted to structural and histochemical analyses.

Structural and histochemical analyses

One set of fixed samples (n = 5) of non-galled leaves and mature galls of *N. crassifolium* were dehydrated in an ethyl series and embedded in Paraplast X-TRA® at 60 °C (Kraus & Arduin 1997). The samples were sectioned with a rotatory microtome (Leica® 2035 BIOCUT) (12–14 µm) and affixed to slides using Bissing's adhesive (Kraus & Arduin 1997). The sections were deparaffinized in butyl acetate, rehydrated in an ethyl series, stained with 0.45% Astra blue and 0.05 % safranin (Bukatsch 1972; Kraus & Arduin 1997), and mounted in colorless varnish Acrilex® (Paiva *et al.* 2006).

A second set of fixed samples of non-galled leaves (n = 3) and mature galls (n = 3) was sectioned with razor blades and subjected to histochemical reactions. Several metabolites were detected with the following reagents: starch grains using Lugol (Johansen 1940), proteins using Xylidine Pounceau (Vidal 1970), lipophilic substances using Sudan black (Pearse 1972), phenolics using 10% ferric chloride (Johansen 1940), flavonoids using p-dimethylaminocinnamaldehyde (DMACA) (Feucht *et al.*1986), lignins using Wiesner's reagent (acidified phloroglucinol) (Johansen 1940), terpenoids using 1% α-naphthol, 1% dimethyl-phenylenediamine (NADI) in 0.01 M phosphate buffer, pH 7.2 (David & Carde 1964), and reducing sugars using Fehling's reagent (solution A - 7.9% copper sulfate, and solution B - 34.6% sodium potassium tartrate and 1% sodium hydroxide, 1:1) heated to pre-boiling temperature (Sass 1951). Blank sections were used as controls. Analyses and photographs were obtained using a Leica DM 500 light microscope (Leica, Wetzlar, Germany) with a coupled digital camera ICC50 HP® (Leica, Wetzlar, Germany).

Results

Geographic distribution of *Niphidium crassifolium* galls

Five scientific articles reported the occurrence of galls in *N. crassifolium* in the states of Rio de Janeiro (Maia & Mascarenhas 2017, 2021; Santos & Maia 2018) and Rio Grande do Sul, Brazil (Farias *et al.* 2020; Cenci & Horodyski 2022). In the herbaria data (Fig. 1), we found 22 records of galls on *N. crassifolium* (Tab. 1). Nineteen of these records had

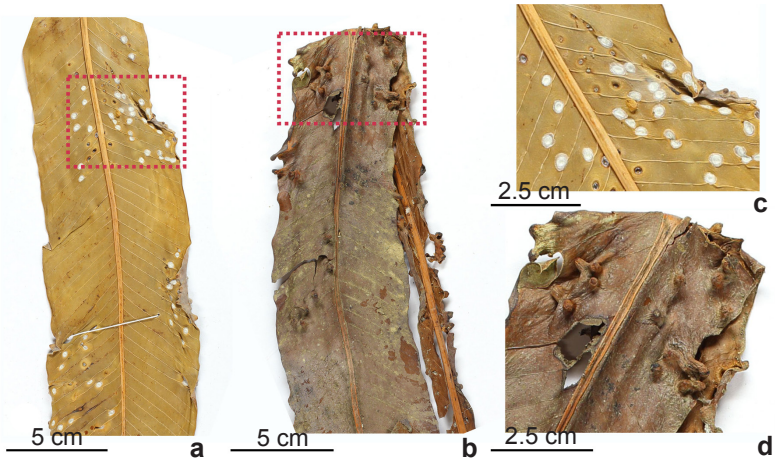

Figure 1 – a-d. Galls on *Niphidium crassifolium* records in exsiccates of virtual herbaria – a. abaxial surface of a galled leaf (*Verdi et al.* 2545 (FURB) - Santa Catarina); b. adaxial surface of a galled leaf (*Schmitt et al. 1438* (FURB - Santa Catarina); c-d. detail of the red boxes in the figures a and b.

Table 1 – Herbarium records of galls in *Niphidium crassifolium* (Polypodiaceae) from South America.

Voucher and herbarium	Region or state	Country	Collected year
Cadorin et al. 1544 (FURB)	Santa Catarina	Brazil	2010
Cadorin et al. 2370 ** (FURB)	Santa Catarina	Brazil	2010
Caglioni & Junckes 477 (FURB)	Santa Catarina	Brazil	2014
Clarke 7471 (NY)	Upper Takutu-Upper Essequibo	Guyana	1998
Dias et al. 10415 (F)	-	Peru	1999
Granville et al. 8963 (US)	-	French Guiana	1986
Henkel 2956 (NY)	Upper Takutu-Upper Essequibo	Guyana	1993
Kollmann et al. 8802 (MBML-HERBARIO)	Espírito Santo	Brazil	2006
Kort & Kniess 3759 (FURB)	Santa Catarina	Brazil	2010
Kort & Kniess 4404 (FURB)	Santa Catarina	Brazil	2010
Martins et al. 30929 ** (HUEM)	Paraná	Brazil	2016
Prado et al. 2069 (UEC)	São Paulo	Brazil	2009
Schmitt et al. 672 (CRI)	Santa Catarina	Brazil	2009
Schmitt et al. 1438 (FURB)	Santa Catarina	Brazil	2010
Schwirkowski 2854 (FURB)	Santa Catarina	Brazil	2018
Stival-Santos et al. 1776 (FURB)	Santa Catarina	Brazil	2010
Stival-Santos et al. 2418 (FURB)	Santa Catarina	Brazil	2010
Vargas Caballero 2073 (NY)	Santa Cruz	Bolívia	1993
Verdi et al. 2545 (FURB)	Santa Catarina	Brazil	2009
Verdi et al. 2960 (FURB)	Santa Catarina	Brazil	2009
Verdi et al. 5346 (FURB)	Santa Catarina	Brazil	2010
Völtz et al. 460 * (EFC)	Paraná	Brazil	2014

Legends: CRI = Herbário Pe. Dr. Raulino Reitz (Santa Catarina); EFC = Escola de Florestas (Curitiba); FURB = Herbário Dr. Roberto Miguel Klein (Santa Catarina); F = Field Museum of Natural History (Chicago); HUEM = Herbário UEM (Maringá); MBML = Herbário Mello Leitão (Espírito Santo); NY = The New York Botanical Garden (New York); UEC = Herbário da Universidade Estadual de Campinas (Campinas); US = Smithsonian Department of Botany (Washington). * = The image of the specimen is not available on the speciesLink network; ** = The presence of galls is reported, but galls are not detected on the image of the specimen in the speciesLink network.

galls on their specimens, but they were not reported in the notes of the collectors. Only one collector reported the presence of galls, but the exsiccate is not available on the speciesLink network (*Völtz et al. 460*, EFC). Two collectors also reported the presence of galls in their notes, but there were no galls on the exsiccates (*Martins et al.* 30929 (HUEM); *Stival-Santos et al. 1776* (FURB) (Tab. 1).

Based on herbaria data, we expanded the occurrence of *N. crassifolium* galls to altitudes between 50 and 2010 m in five South American countries, *i.e.* Bolivia (01), Brazil (17), French Guiana (01), Guyana (2), and Peru (01). In Brazil, the presence of galls was observed in the Southeast region (02), in the states of Espírito Santo and São Paulo, and in the South region (15) in the states of Paraná, Santa Catarina, and Rio Grande do Sul (Fig. 2).

Structural and histochemical analyses

Mature galls are globoid, green, isolated or grouped, and located on the adaxial leaflet surface between the secondary veins (Fig. 3a-b). Some necrotic spots on the adaxial surface may occur (Fig. 3b). The gall has a "pocket-like development" forming a conical projection toward the adaxial leaflet surface (Fig. 3c). The abaxial surface of the gall is closed by thin silk layers produced by the

gall inducer (Fig. 3c-d). The single gall chamber hosts a larva (Fig. 3e). Analysis by taxonomists suggests that this could be an undetermined species of Diptera: Cecidomyiidae (Santos & Maia 2018; V.C. Maia 2023, personal communication).

Non-galled leaves (Fig. 4a-b) have a unistratified epidermis with periclinally elongated cells, and stomata located on the abaxial surface. The mesophyll is formed by 1–2 layers of hypodermis below the adaxial epidermis. The parenchyma is homogeneous and formed by 9–12 cell layers. Intercellular spaces are observed in the parenchyma. The vascular bundles are amphicrival and surrounded by a 1-layered endodermis with phenolics in their periclinal and anticlinal walls (Fig. 4a). In the sori, a vascular bundle with disorganized arrangement is also surrounded by a 1-layered endodermis. The receptacle is formed by 1–2 layers of parenchyma with isodiametric and highly vacuolated cells. The outermost cells of the receptacle contain phenolics (Fig. 4b). The midrib vein (Fig. 4c) is formed by 3–5 layers of lignified cells below the adaxial and abaxial epidermis. These cells surround 7–10 layers of homogeneous parenchyma. The vascular system has one or more meristele with amphicrival arrangement. The phloem cells surround the xylem. The protoxylem elements face the periphery of the organ, while the metaxylem elements are central, forming a V-shaped xylem. The outermost layer of the vascular system is formed by the pericycle with 1–2 cell layers. The endodermis has anticlinal and inner periclinal walls rich in phenolics (Fig. 4c).

Starch is detected as black-colored grains in the epidermal cells of the abaxial surface, especially in the stomata (Fig. 4d). Reducing sugars are detected as browning coloration and precipitates in the epidermal and mesophyll cells (Fig. 4e). Lipophilic substances are detected as black-colored droplets in the vascular bundles (Fig. 4f). Proteins are detected by red staining in the homogeneous parenchyma cells and phloem (Fig. 4g). Lignins are detected by red staining of the cell walls of the protoxylem and metaxylem and of the lignified cell of the midrib (Fig. 4h-i). Phenolics are detected as brown staining precipitates in epidermal, parenchyma, and phloem cells (Fig. 4j). Flavonoids are not detected in non-galled leaves.

Anatomically, galls are formed by outer (OTC) and inner (ITC) tissue compartments

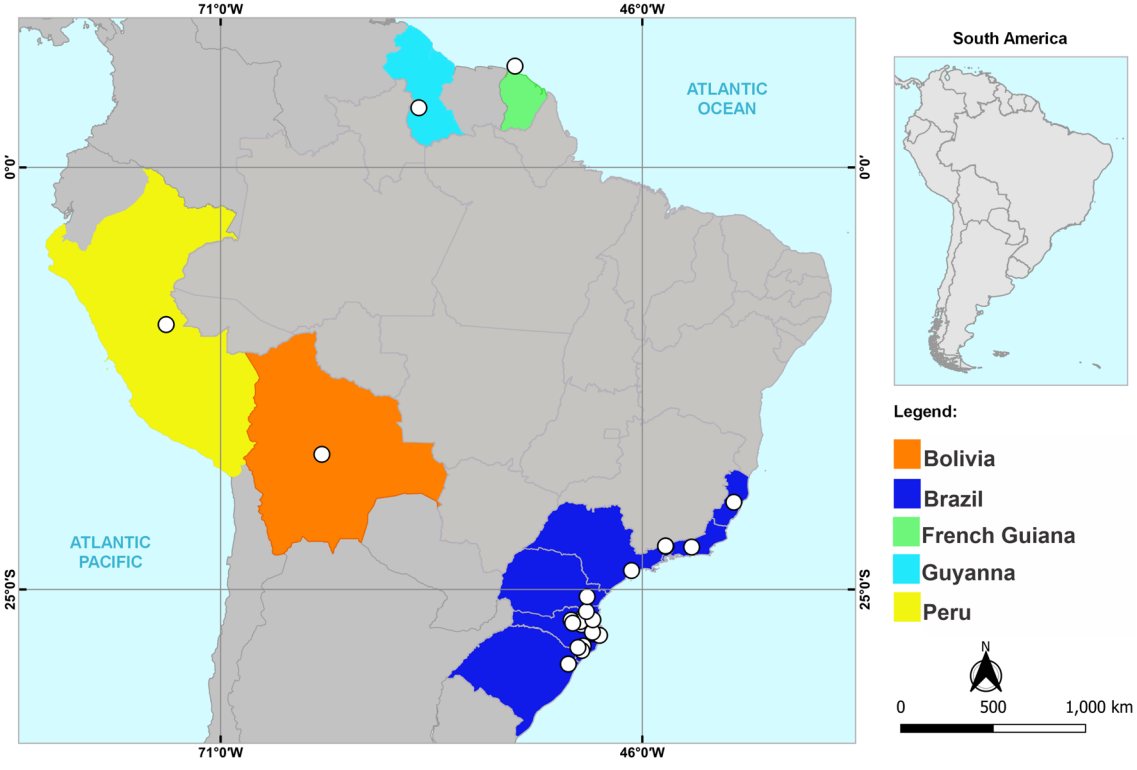

Figure 2 – Geographic distribution of *Niphidium crassifolium* (Polypodiaceae) and its galls reported in South America.

Figure 3 – a-e. *Niphidium crassifolium* (Polypodiaceae) globoid galls – a. adaxial surface of the mature leaf with galls; b. detail of galls distributed on the adaxial surface of a mature leaf; c. hemisection of a mature gall exhibiting the "pocket-like development" and the gall opening; d. detail of the silk covering produced by the gall inducer; e. gall inducer.

(Fig. 5a). The OTC is formed by a uniseriate epidermis with periclinally elongated cells, 13–15 layers of vacuolated parenchyma cells whith thin and sinuous cell walls. Intercellular spaces are observed in this parenchyma. The 5–7 parenchyma cells around the nutritive cells have phenolics in the periclinal and anticlinal walls (Fig. 5b). The amphicrival vascular bundles are located in the OTC, surrounded by the endodermis, and have an accumulation of phenolics. The ITC surrounds the larval chamber and is formed by 7–9 periclinally elongated cell layers with dense cell content and an evident nucleus (Fig. 5c), forming a nutritive tissue (NT). Phenolics are detected as brown staining precipitates in the vacuoles of the epidermal and subepidermal layers of the OTC (Fig. 5d), and as brown staining in the cell walls of parenchyma around the nutritive and endoderm cells. Flavonoids are also detected as blue staining contents in the cytoplasm of the epidermis and in subepidermal cells (Fig. 5e). Lignins (Fig. 5f), terpenoids (Fig. 5g), and lipophilic substances

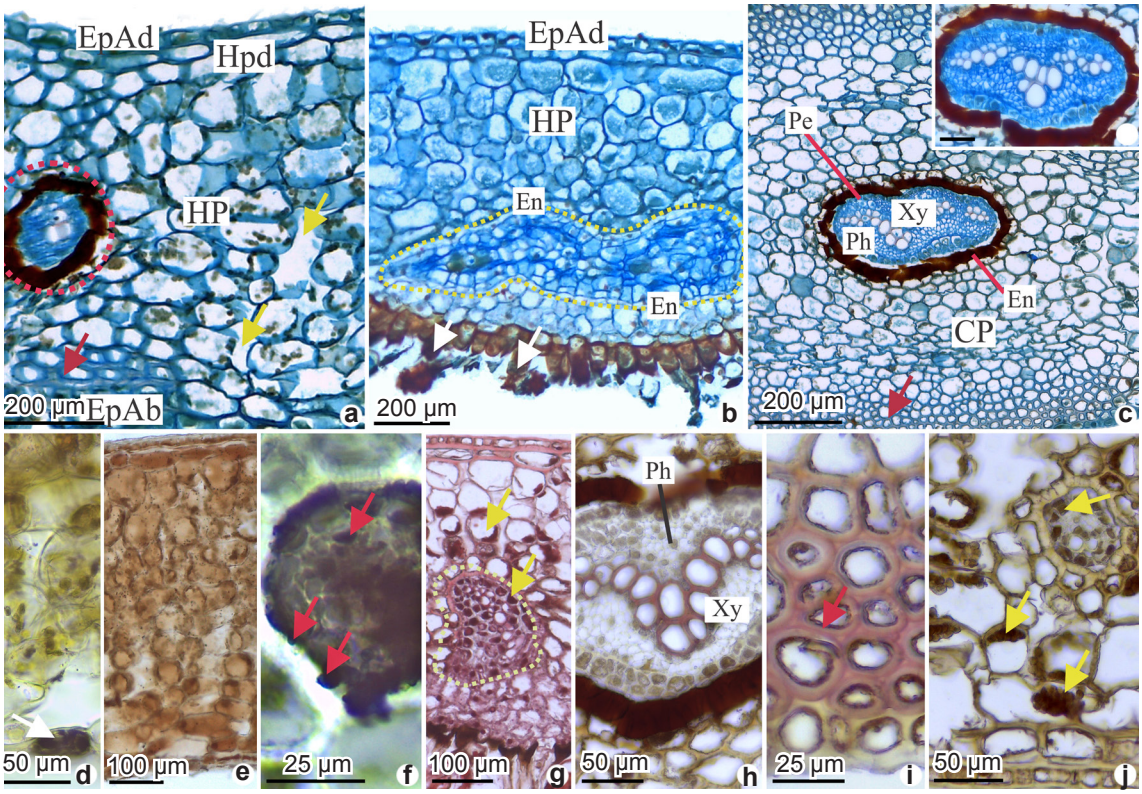

Figure 4 – a-j. Structural and histochemical profiles of *Niphidium crassifolium* (Polypodiaceae) leaves – a-c. transverse section of a leaf lamina stained with Astra blue and safranin – a. detail of the mesophyll with uniseriate epidermis on the adaxial (EpAd) and abaxial (EpAb) surfaces, hypodermis (Hpd), homogeneous parenchyma with intercellular spaces (yellow arrow), and vascular bundle (red circle). The endodermis has phenolic deposition on the cell walls and surrounds the vascular bundle. Lignified cells are located below the vascular bundle (red arrow); b. detail of the sori with the vascular bundle (yellow dotted) surrounded by the endodermis (En). Two parenchyma layers form the receptacle. The outermost cells of the receptacle contain phenolic impregnation (white arrows); c. detail of a midrib vein with lignified cells (red arrow) surrounding the cortical parenchyma (CP). The vascular system is amphicrival with the phloem (Ph) surrounding the xylem (Xy). The endodermis has phenolics in the anticlinal and periclinal walls forming a V-shaped structure; d-i. histochemical tests – d. starch grains detected in the stomata of the abaxial epidermis (white arrow); e. reducing sugars detected by browning coloration and precipitates in cytoplasm cells of the mesophyll; f. lipids detected as droplets in the vascular bundle; g. proteins detected in the cell walls and cytoplasm of parenchyma cells, and phloem in the sori; h-i. lignins detected in the xylem cells and lignified cell walls of the midrib vein; j. phenolics detected in the cytoplasm of parenchyma cells.

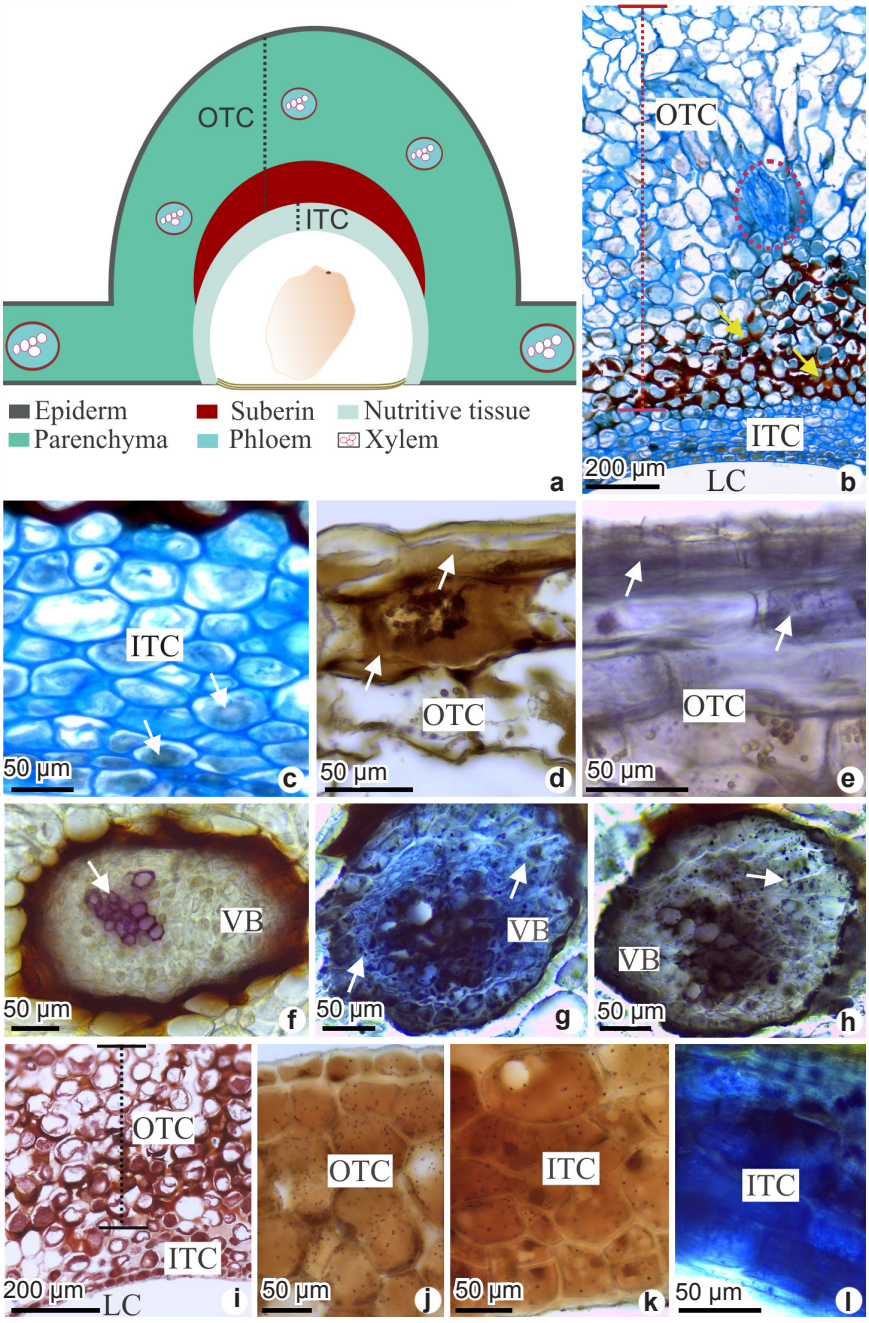

Figure 5 – a-l. Structural and histochemical profiles of *Niphidium crassifolium* (Polypodiaceae) galls – a. diagram of a transverse section of the globoid gall indicating the outer (OTC) and inner (ITC) tissue compartments; b-c. transverse section of a gall stained with Astra blue and safranin – b. the OTC is formed by parenchyma cells, vascular bundles (red circle), and phenolic-impregnated cells (yellow arrows); c. the ITC is formed by a nutritive tissue with periclinally elongated parenchyma cells and dense cellular content; d-h. histochemical tests in the outer tissue compartment – d. phenolics (white arrow) are detected in the epidermal and subepidermal cell layers of the OTC; e. flavonoids (white arrow) are detected in the epidermal and subepidermal cell layers of the OTC; f. lignins (white arrow) are detected in xylem vessel elements of the vascular bundles (VB); g. terpenoids (white arrow) are detected in vascular bundles (VB); h. lipids (white arrow) are detected as droplets in the vascular bundles (VB); i. proteins are observed in the cell walls and cytoplasm of OTC and ITC cells; j-k. reducing sugars detected by browning coloration and precipitates detected in the cytoplasm of OTC and ITC cells; l. terpenoids are detected in the nutritive tissue of the ITC.

(Fig. 5h) are detected in vascular bundles as red, blue, and black staining, respectively. Proteins are revealed as red-colored content in all the cells of the gall outer and inner tissue compartments (Fig. 5i). The presence of reducing sugars is indicated by browning coloration and precipitates observed in OCT and ITC cells (Fig. 5j-k). Terpenoids are detected as blue-colored content in the cytoplasm of NT cells (Fig. 5l). Starch grains are not detected in gall tissues.

Discussion

The gall systems found in ferns provide intriguing models for studying multitrophic interactions in these plants, revealing a field of plant interactions that remains largely unexplored. The scope of these interactions can be expanded through the analysis of virtual collections, as observed here for *N. crassifolium*. Two insect families, Diptera: Cecidomyiidae and Hemiptera: Coccidae, are suggested as the inducers of *N. crassifolium* galls. Taxonomists suggest that the inducer could be an undetermined species of Diptera: Cecidomyiidae (Santos & Maia 2018; V.C. Maia 2023, personal communication). The analysis of the structural and histochemical traits of gall tissues supports the suggestion that Diptera: Cecidomyiidae is the family of the gall inducer. The tissue compartmentalization is similar to that observed in Cecidomyiidae galls on angiosperms (Bragança *et al.* 2017; Ferreira *et al.* 2019). The gall-inducing herbivore feeds directly from the nutritive tissue, which has intense metabolic activity and accumulates carbohydrates, lipids, sugars, and proteins as food resources. Starch grains, commonly found in galls induced by Cecidomyiidae and Hemiptera, were not observed in *N. crassifolium* galls. Instead, reducing sugars and proteins are released and metabolized to sustain the gall's metabolism.

Geographic distribution of *Niphidium crassifolium* galls in South America

Since the relationship between gall inducers and their host plants is species-specific (Isaias *et al.* 2013), we assume that the occurrence of globoid galls on *N. crassifolium* follows the geographic distribution of their host plant, as partially corroborated by the analysis of virtual collections. The distribution of the galling herbivores associated with *N. crassifolium* was expanded here to four South American countries (Bolivia, French Guiana, Guyana, and Peru). In addition, the distribution of the galling herbivore was expanded to the Espírito Santo, São Paulo, Paraná, and Santa Catarina states of Brazil. Despite the wide distribution of *N. crassifolium* in the Amazon, Caatinga, Cerrado, Atlantic Forest, Pampa, and Pantanal in Central and South America (Lellinger 1972; Croat 1978; Moran 1995; Almeida & Oliveira 2023), the records of galls are limited to few regions. In the 813 exsiccates analyzed, only 22 records of galls were observed, demonstrating that the galls may have been avoided by the collectors and that their distribution could be wider. The low number of records of galls on *N. crassifolium* reveals the importance of annotating and scanning galls in herbarium collections. These annotations, accompanied by images, are important for analyzing plant distribution and the interactions with galling herbivores (Arriola *et al.* 2016). Furthermore, herbarium sheets (exsiccates) represent an excellent gall curation technique, allowing scanning and preservation (Mertz *et al.* 2022), especially regarding smaller galls like those induced on leaves of *N. crassifolium*.

Structural and chemical traits of *Niphidium crassifolium* galls associated with its inducer

Two insect families are suggested to be the inducers of *N. crassifolium* galls: Diptera: Cecidomyiidae (Santos & Maia 2018; V.C. Maia 2023, personal communication), which feeds by sucking disrupted-cell fluids from the nutritive tissues, and Hemiptera: Coccidae (Rübsaamen 1908; Houard 1933; Cenci & Horodyski 2022), phloem-sucking insects. Although *N. crassifolium* galls are open and characterized by a silk deposition protecting the body of the inducer, a common trait of Coccidae (Gullan & Kosztarab 1997; Gonçalves *et al*. 2005), the structural and chemical features of gall tissues, *i.e.*, the two tissue compartments (outer and inner), a layer with protective cells with accumulation of phenolics, and the differentiation of a nutritive tissue accumulating proteins and sugars represent evidence that a Diptera: Cecidomyiidae insect is the gall inducer. The silk deposition in the form of filaments or films protecting the cocoon before pupation has been reported in Cecidomyiidae-induced galls, occurring on true galls induced on fungi (Evans 1970), gymnosperms (Tripp 1955),

angiosperms (Caresche & Wapshere 1975; Censier *et al*. 2014), and herein on galls induced on a fern. In addition, other insects, including inquilines (Psocoptera, Sciaridae: Diptera, and *Corythaica cyathicollis* Costa, 1864), Tingidae: Hemiptera, parasitoids (Platygastridae: Hymenoptera), and an unidentified Diptera larva (probably the gall inducer) have been found inside *N. crassifolium* galls in field studies (Maia & Mascarenhas 2017; Santos & Maia 2018). The presence of Platygastridae: Hymenoptera also helps the identification of the inducer since some of these wasp species parasitize galling Cecidomyiidae (Johnson *et al*. 2013).

The structure and metabolic compartmentalization of the galls are determined by the chemical potentialities of the host plant and the feeding habit of the gall inducer (Rohfritsch 1992; Bronner 1992; Ferreira *et al.* 2019), which helped the identification of the galling herbivore on *N. crassifolium*. In Angiosperms, the outer tissue compartment of galls induced by Diptera: Cecidomyiidae has been reported to be composed of parenchyma cells and a mechanical zone consisting of lignified cells (Rohfritsch 1992; Bedetti *et al*. 2017; Ferreira *et al*. 2019). The vascular bundles can occur in the gall inner or outer tissue compartments (Bragança *et al.* 2017, 2021; Costa *et al.* 2018, 2022). In *N. crassifolium* galls, the accumulation of phenolics in the outer tissue compartment may exert a protective function similar to the lignification observed in Cecidomyiidae galls on Angiosperms (Bragança *et al.* 2017). The phenolics and flavonoids detected in the outer tissue compartment of galls indicate the manipulation of secondary metabolites toward a protective role for the galling herbivores (Kuster *et al.* 2020). These metabolites are commonly detected in the outer tissue compartment of both Cecidomyiidae (Suzuki *et al.* 2015; Bragança *et al.* 2017; Kuster *et al.* 2020) and Hemiptera galls (Kuster *et al.* 2020; Silva *et al.* 2019), where they can protect gall tissues against oxidative damage (Isaias *et al.* 2015), as well as block the AIA-oxidases, favoring cell expansion (Bedetti *et al.* 2017; Bragança *et al.* 2020).

Interestingly, terpenoids were detected in the inner tissue compartment of *N. crassifolium* galls, in contact with the galling herbivore. Despite the antiherbivore characteristics of terpenoids (Sharma *et al.* 2017), they are commonly detected in the galls induced by both Cecidomyiidae (Bragança *et al*. 2017; Kuster *et al*. 2020) and Hemiptera (Kuster *et al*. 2020; Rand *et al*. 2014; Davidovich-Rikanati *et al*. 2022). Some insects, however, can alter their toxic action (Milles 1989; Himmelsbach *et al*. 2016; Borges 2018) and accumulate or exude complex metabolites (Züst & Agrawal 2016; Ameixa *et al.* 2022). This may be a metabolic impairment occurring in *N. crassifolium* galls, a hypothesis to be tested.

In *Niphidium crassifolium*, the galling herbivore feeds directly from the nutritive tissue, whose cells have an intense metabolism typical of Cecidomyiidae galls (Bronner 1992; Rohfritsch 1992; Bragança *et al*. 2017; Costa *et al*. 2021). These cells usually have a dense cytoplasm and evident nuclei and accumulate starch, lipids, sugar, and proteins (Bronner 1992; Bragança *et al.* 2017; Ferreira *et al.* 2019; Costa *et al.* 2022) that are consumed by the sucking disrupted-cell fluid activity of the gall inducer (Ferreira *et al.* 2019).

Starch grain accumulation is expected in both Cecidomyiidae-induced galls and Hemiptera-induced galls (Bronner 1992; Oliveira *et al.* 2020; Silva *et al.* 2019). Starch grains are metabolized by different enzymes, and the sugars are released for the feeding of the galling insect and the gall energetic metabolism (Oliveira *et al.* 2011; Carneiro *et al*. 2014; Bragança *et al*. 2017). Although they are a characteristic of both taxa, starch grains were not observed in *N. crassifolium* galls, as similarly reported for other Cecidomyiidae (Arriola *et al.* 2018) and hemipteran galls (Carneiro *et al.* 2014). In these galls, reducing sugars may be released by the action of acid phosphatases (Carneiro *et al.* 2014), which may also occur in *N. crassifolium* galls. In addition, starch grains may have been degraded to lower sugar molecules to maintain gall metabolism, making their detection by histochemical tests unfeasible by the time of sample collection. A high protein accumulation in phloem cells is more characteristic of Hemiptera-induced galls (Kehr 2006; Carneiro *et al.* 2014; Silva *et al*. 2019), but proteins can also be detected in the nutritive cells of Cecidomyiidae galls (Bragança *et al.* 2017; Costa *et al*. 2021). In *N. crassifolium*, the site of protein detection, *i.e.*, the cells of the nutritive tissue, indicates that they are the nutritive resource for the gall inducers. These proteins can be metabolized through enzymes present in the saliva of diverse herbivores (Kehr 2006) including those feeding by sucking disrupted-cell fluids (Zhang *et al.* 2010).

The geographic distribution of *N. crassifolium* galls covers five countries in South America: Bolivia, French Guiana, Guyana, Peru, and Brazil. In Brazil, the previously known distribution in Rio de Janeiro and Rio Grande do Sul states was expanded to Espírito Santo, São Paulo, Paraná, and Santa Catarina states by herbaria records. In addition, the distribution of the gall and its gall inducer may be even wider since *N. crassifolium* also occurs in Central America and in the Caribbean islands. Herbarium data have been demonstrated to be an excellent tool for studies of the geographical distribution of galls associated with their hosts, such as *N. crassifolium* galls. Therefore, we encourage taxonomists and all those who deposit material in the herbaria to report the occurrence and characterization of galls, which is crucial for the knowledge of biodiversity. Soft functional traits such as anatomy and histochemistry are commonly described in galls induced in angiosperms as important adaptive strategies to increase larval protection, survivorship, and nutrition in multitrophic interactions, aspects that are also true for galls induced on ferns. Furthermore, the structural and histochemical patterns observed in the galls induced on *N. crassifolium*, such as the the outer tissue compartment of galls composed of phenolic-rich cell layers, a nutritive tissue with proteins and reducing sugars, as well as the silk covering of the gall aperture, indicate that the gall inducer may belong to the Diptera-Cecidomyiidae family.

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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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