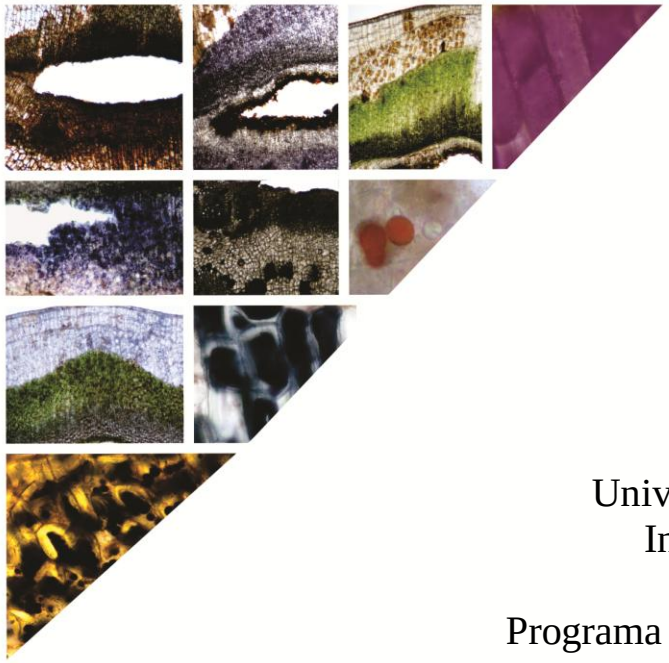

Universidade Federal de Minas Gerais
Instituto de Ciências Biológicas
Departamento de Biologia
Programa de pós-graduação em Biologia Vegetal

Gracielle Pereira Bragança

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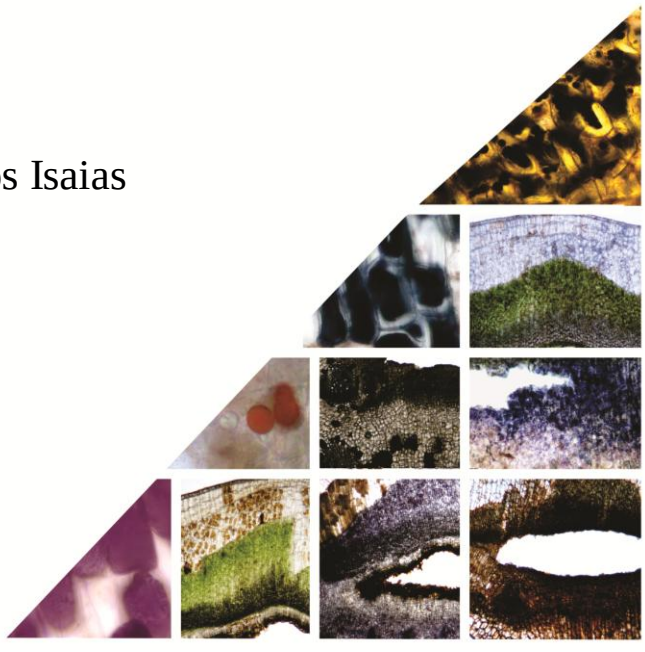

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GRACIELLE PEREIRA BRAGANÇA

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Área de concentração: Morfologia, Sistemática e Diversidade Vegetal

Orientador: Prof. Dra. Rosy Mary dos Santos Isaias

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Índice

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Resumo geral

Galhas são resultantes do estímulo de indutores específicos, os quais coordenam alterações teciduais que proporcionam ambiente favorável ao seu desenvolvimento. A relação específica entre planta hospedeira-herbívoro galhador revela características diferenciais que compõem cada sistema. *Piper arboreum* Aubl (Piperaceae) é hospedeira de um Diptera: Cecidomyiidae, o qual coordena a formação de um morfotipo intralaminar lenticular em folhas. O design morfológico, anatômico e funcional foi estabelecido mediante processos alimentares específicos do grupo galhador, corroborando padrões pré-estabelecidos. Processos de hipertrofia no plano anticlinal e hiperplasia no plano periclinal foram também determinantes para definir o design lenticular. Processos de rediferenciação celular foram essenciais para o estabelecimento neo-funcional, com tecidos especializados em nutrição e proteção. A necessidade alimentar do galhador e o desenvolvimento estrutural da galha requer armazenamento e disponibilidade de nutrientes via atividade enzimática, localizados especialmente nos tecidos internos da galha enquanto que, a localização externa de metabólitos tóxicos provenientes do metabolismo secundário podem proteger o galhador contra o ataque de inimigos naturais. No sistema *Piper arboreum*-Cecidomyiidae, a compartimentalização espacial, o acúmulo e a disponibilidade de carboidratos produzidos em resposta ao estímulo do galhador e limitações impostas pela planta hospedeira, modelaram características específicas do perfil químico dos tecidos da galha. O fornecimento de hexoses destinadas a suprir as demandas metabólicas e alimentares está associado ao aumento do estresse oxidativo no sítio da galha. Este estresse foi determinado pelo maior acúmulo de espécies reativas de oxigênio (EROs), o qual desencadeia uma cascata de sinalizações celulares favorecendo ou não o desenvolvimento da galha. Sob estímulo do galhador, foi estabelecido um gradiente centrífugo de EROs em *Piper arboreum*, desencadeando respostas citológicas diferenciais nas camadas de tecidos da galha, essenciais para manter o alto metabolismo celular. Caracteres citológicos conservativos em galhas denotaram o potencial da planta hospedeira em reduzir o impacto do estresse oxidativo e em conjunto com as características estruturais foram essenciais para manter a similaridade entre o perfil fotossintético de folhas não galhadas e galhas maduras.

Palavras chave: Anatomia foliar, anatomia da galha, histoquímica, fotossíntese, metabolismo de carboidratos

Abstract

Galls result from the stimulation of specific inducers, which coordinate tissue changes that provide favorable environment for their development. The specific relationship between host plant-galling herbivore reveals differences, characteristic of each system. *Piper arboreum* Aubl (Piperaceae) hosts a Diptera: Cecidomyiidae, which induces a lenticular intralaminar morphotype on leaves. The morphological, anatomical and functional design was established by specific food processes linked to the galling herbivore taxa, confirming pre-established patterns. Hypertrophy in anticlinal axis and hyperplasia in periclinal axia were determinant for the lenticular design of the gall. Cell redifferentiation was essential to the tissue neo-functions, such as nutrition and protection. The nutritional requirements of the Cecidomyiidae, and the gall structural development requires nutrient storage and availability via enzymatic activity, especially located in the inner tissues of the gall. The external location of toxic metabolites from the secondary metabolites may protect the gall and galling herbivore against the attack of natural enemies. In *Piper arboreum*-Cecidomyiidae system spatial compartmentalization, accumulation and availability of carbohydrates produced in response to the gall Cecidomyiidae stimuli, and restrictions imposed by the host plant, modeled specific characteristics of the gall chemical profile. The supply of hexoses designed to fit the metabolic and nutritive demands are associated with the increased oxidative stress at the gall site. The greatest accumulation of reactive oxygen species (ROS) triggers a cascade of cellular signals which may favor or not gall development. Under the stimulation of the galling herbivore, a centrifugal gradient of ROS was established in *Piper arboreum*, triggering cytological differential responses in the gall tissue layers, which were essential to maintain cell high metabolism. Cytological conservative characteristics in galls denote the potential of the host plant to reduce the impact of the oxidative stress and together with the structural features were essential to maintain the similarity between the photosynthetic profile of non galled leaves and mature galls.

Key words: Leaf anatomy, gall anatomy, histochemistry, photosynthesis, carbohydrate metabolism

Introdução Geral

Fatores abióticos e bióticos influenciam diretamente o crescimento e desenvolvimento das plantas, desencadeando respostas diferenciais que revelam o equilíbrio preciso capaz de amenizar a condição de organismo séssil (Dangl e Jones 2001). Neste contexto, galhadores, como um fator biótico, são capazes de estimular modificações nos três sistemas de tecidos vegetais (Mani 1964), convergindo para a formação de um design atípico e ao mesmo tempo característico e específico, a galha.

Piperaceae é amplamente distribuída na região tropical e subtropical, caracterizada especialmente pela diversidade da composição química dos óleos essenciais, utilizados como condimentos, inseticidas, antifúngicos e na indústria farmacêutica (Souza et al. 2009; Judd et al. 2009). Dentre as espécies dessa família, *Piper arboreum* apresenta o desenvolvimento de galhas intralaminares lenticulares (*sensu* Isaias et al. 2013) induzidas por um Diptera: Cecidomyiidae, grupo de insetos que é conhecido pela abundância de galhas na região Neotropical (Espírito-Santo e Fernandes, 2007).

As galhas são geradas através de diferentes estímulos físico-químico coordenados pelo galhador (Hori 1992). Dentre os estímulos químicos, a secreção salivar, excretas e hormônios são capazes de desencadear diferentes respostas no órgão hospedeiro (Hori 1992). Com relação aos estímulos físicos, o hábito alimentar do galhador é proposto como mecanismo crucial para a heterogenidade tecidual e estrutural apresentada pelas galhas (Rohfritsch 1992). Portanto, hábitos alimentares de grupos associados evolutivamente devem convergir para o estabelecimento de padrões de desenvolvimento, como proposto por Rohfritsch (1992) para galhas induzidas por diferentes grupos de insetos.

O design anatômico, morfológico e funcional das galhas é decorrente de inibição (Oliveira et al. 2006) ou rediferenciação da atividade celular (*sensu* Lev-Yadun, 2003), hiperplasia e hipertrofia (Isaias et al. 2011; Ferreira e Isaias 2013), e modificações no padrão de expansão e alongamento celular (Magalhães et al. 2014). Tais processos são também responsáveis por configurar os diferentes designs das galhas (Isaias et al. 2013; Isaias et al. 2014).

Além destas alterações anatômicas, o galhador também pode manipular a produção e o acúmulo de metabólitos primários e secundários (Bronner 1992). Estes metabólitos podem ser compartimentalizados nas camadas celulares da galha e denotam novas funcionalidades a estrutura, como a proteção contra inimigos naturais e a disponibilidade de recursos nutritivos (Nyman and Julkunen-Titto 2000; Stone e Schonrögge 2003). Como forma de proteção,

terpenoides, flavonoides, fenois, alcaloides (Oliveira et al. 2006) são acumulados especialmente na região externa da galha (Nyman and Julkunen-Titto 2000; Formiga et al. 2011; Isaias et al. 2014), minimizando o ataque de predadores e parasitoides (Nyman and Julkunen-Titto 2000). Nutricionalmente, compostos do metabolismo primário como carboidratos, lipídios e proteínas são armazenados nos tecidos internos das galhas, onde a atividade de enzimas específicas disponibilizam compostos tanto para a alimentação do galhador quanto para o metabolismo celular (Bronner 1992; Oliveira e Isaias 2010; Oliveira et al. 2010). A sacarose, principal açúcar translocado no floema, pode ser sintetizado por diferentes vias metabólicas (Wind et al 2010) e metabolizado especialmente pelas enzimas sacarose sintase (Susy) e invertases (Roitsch e González 2004; Koch 2004). A atividade da invertase está relacionada a clivagem irreversível da sacarose em glicose e frutose, fornecendo substrato para respiração celular e crescimento e desenvolvimento de tecidos (Roitsch e González 2004). A sacarose sintase por sua vez está relacionada a quebra reversível da sacarose em frutose e UDP-glicose em tecidos de armazenamento (Koch 2004). Em galhas, a dinâmica entre a atividade enzimática e o metabolismo de carboidratos podem estar relacionados a formação do tecido nutritivo, dieta dos insetos e estabelecimento de gradientes histoquímicos e de espécies reativas de oxigênio (EROs) (Oliveira e Isaias 2010).

Espécies reativas de oxigênio geradas durante o metabolismo fundamental da célula especialmente em organelas como mitocôndrias, cloroplastos e peroxissomos podem atuar como moléculas sinalizadoras de diferentes processos celulares (Bell et al. 2009; Liu et al. 2013; Møller et al. 2007). As propriedades físicas da parede celular são alteradas e influenciam no crescimento do órgão (Del Rio e Puppo 2009). As EROs oxidam diversos componentes celulares (Del Rio e Puppo2009; Møller et al. 2007); sinalizam sistematicamente respostas de defesa (Karpinski et al. 1999; Liu et al. 2013) e, conduzem a regulação gênica durante a morte celular programada (MPC) (Pitzschke et al. 2006, Bell et al. 2009; Shetty et al. 2008). Na interação planta hospedeira- herbívoro galhador, as EROs estão sendo amplamente discutidas como moléculas associadas ao estabelecimento do design final das galhas (Oliveira et al. 2010; Oliveira e Isaias 2010; Isaias et al. 2014). EROs também podem atuar na formação do gradiente citológico e histoquímico, conferindo novas funcionalidades a estrutura (Oliveira et al. 2010; Oliveira e Isaias 2010; Oliveira et al. 2014). O acúmulo de EROs, pode influenciar diretamente no perfil fisiológico da galha levando a alterações no desempenho fotossintético (Castro et al. 2012; Carneiro et al. 2014). Estas alterações em taxas fotossinéticas podem ser neutra, como nas galhas de Cecidomyiidae em

Aspidosperma spp. (Oliveira et al 2011a), positiva, como no sistema *Silphium integrifolium*–Cynipidae (Fay et al 1993) ou negativa, como nas galhas de Cecidomyiidae em *Copaifera langsdorffi* (Castro et al 2012) e de *Colopha compressa* em *Ulmus laevis* (Samsone et al 2012). O balanço devidamente ajustado entre as diferentes funcionalidades de EROs, favorece o estabelecimento da galha.

Dentre os diferentes fatores que favorecem este equilíbrio podem ser mencionados a produção de enzimas antioxidantes (Møller et al. 2007), fenólicos, flavonoides e terpenoides (Blokhina et al. 2003). As características estruturais como o contínuo morfológico com o órgão hospedeiro (Oliveira et al. 2011; Castro et al. 2012; Carneiro et al. 2014) e ultraestruturais como presença plastoglóbulos associados aos cloroplastos (Austin et al. 2006) e reciclagem do sistema de endomembranas (Levine 2002; An et al. 2006a; An et al. 2006b; Oliveira et al. 2011; Carneiro e Isaias 2014) também são determinantes para diminuir os impactos causados durante a cecidogênese (Oliveira et al. 2011; Carneiro e Isaias 2014).

O sistema *Piper arboreum* - Cecidomyiidae foi utilizado como modelo no presente estudo para avaliar respostas morfológicas, químicas, fisiológicas e citológicas, desencadeadas pelo galhador durante a interação com a planta hospedeira, visando os seguintes objetivos:

1. Descrever o desenvolvimento do órgão hospedeiro, a folha, traçando a origem e o destino de cada tecido neoformado pelo estímulo do galhador, focando em correlacionar ao padrão de desenvolvimento das galhas de Cecidomyiidae e caracterizar aspectos citométricos envolvidos no desenvolvimento do design intralaminar lenticular.

2. Avaliar aspectos metabólicos e químicos envolvidos na diferenciação de tecidos especializados em virtude da compartimentação espacial de metabólitos primários e secundários e a disponibilização de recursos energéticos sob estímulo do galhador e as limitações químicas da planta hospedeira.

3. Investigar se alterações fotossintéticas estão associadas ao acúmulo de espécies reativas de oxigênio (EROs) induzidas pelo galhador e se caracteres citológicos e estruturais podem reduzir os possíveis danos causados pelo estresse oxidativo.

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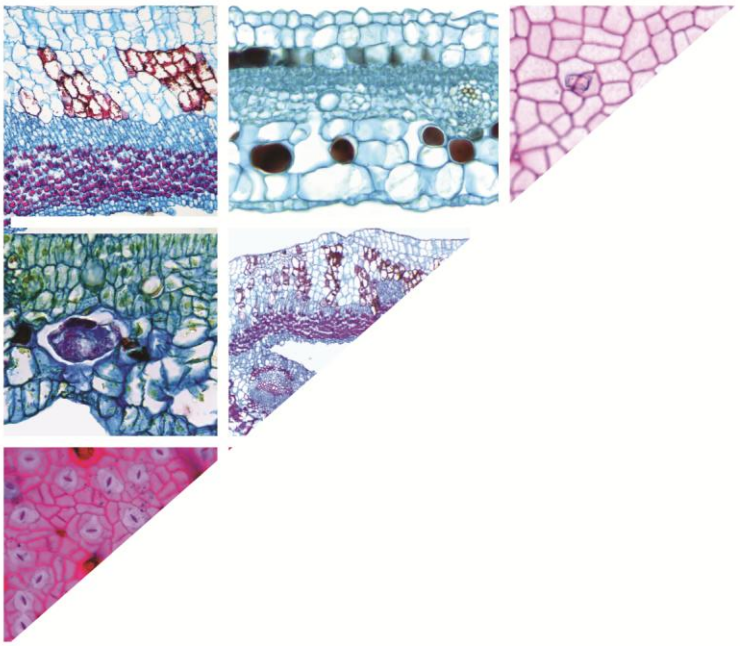

Capítulo I

Ontogenesis and redifferentiation during the establishment of the new functional and structural design on the leaf galls *Piper arboreum* Aubl. (Piperaceae)

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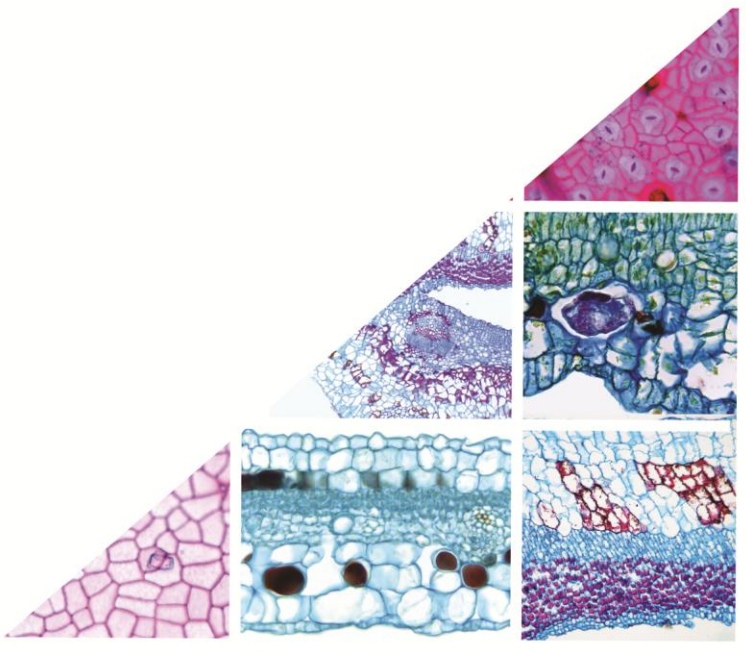

Ontogenesis and redifferentiation during the establishment of the new functional and structural design on the leaf galls *Piper arboreum* Aubl. (Piperaceae)

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Título abreviado: Ontogenesis and redifferentiation during the establishment of galls on *Piper arboreum* Aubl. (Piperaceae)

Key words: Cecidomyiidae, cytometry, intralaminar lenticular galls, patterns of development.

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Abstract

Galling herbivores can change the morphogenetic patterns of the host plants, turning their organ on specific structures, the galls, which ensure protection, nutrition, and favorable microenvironment to inductor. The objective of this study is to evaluate possible alterations on the host leaves of *Piper arboreum* Aubl. (Piperaceae) that fit the typical patterns of development for galls induced by Diptera, Cecidomyiidae, which guarantee the adaptive value the sctructure. The development, cytometric and histochemical analyses of non-galled leaves and galls at different stages of development were performed, focusing on alterations in the pattern of expansion and cell elongation allowing the acquisition of intralaminar lenticular design. The non-galled leaves followed the ontogenetical pattern described in literature for simple leaves, and the feeding activity of the galling Cecidomyiidae induced an increase in all cell areas and changes in the patterns of cell elongation essential for acquisition of new structural and functional design as well as for the formation of the typical zoning tissue in Cecidomyiidae: an outer cortex parenchymatous, a sclerenchymatic sheath and the nutritive tissue which together confer the adaptive value of the structure.

Key words: Cecidomyiidae, cytometry, intralaminar lenticular galls, patterns of development.

Resumo

Herbívoros galhadores podem alterar os padrões morfogenéticos das plantas hospedeiras, transformando seus órgãos em estruturas específicas, as galhas, que proporcionam proteção, nutrição e microambiente favorável ao indutor. O objetivo deste estudo é avaliar as possíveis alterações nas folhas hospedeiras de *Piper arboreum* Aubl. (Piperaceae), que se encaixam nos padrões típicos de desenvolvimento de galhas induzidas por Diptera, Cecidomyiidae, e que garantem o valor adaptativo da estrutura. Foram realizadas análises ontogenéticas, citométricas e histoquímicas em folhas não galhadas e galhas em diferentes estágios de desenvolvimento, com foco em alterações no padrão de expansão e alongamento celular que permitiram a aquisição do design intralaminar lenticular. As folhas não galhadas seguiram o padrão ontogenético descrito na literatura para folhas simples. A atividade alimentar do Cecidomyiidae induziu aumento em todas as áreas celulares e mudanças nos padrões de alongamento celular essenciais para aquisição do novo design estrutural e funcional, bem como para a formação do zoneamento tecidual típico para galhas de Cecidomyiidae: um córtex externo parenquimático, uma bainha esclerenquimática e um tecido nutritivo que juntos conferem o valor adaptativo a estrutura.

Palavras chave: Cecidomyiidae, citometria, galhas intralaminares lenticulares, padrões de desenvolvimento

Introduction

Galls are structures developed in response to the activity of a specific gall inducing agent (Raman 2007), which may be an insect, a nematoda, an acari, a bacteria, a fungi, an algae, or a virus (Mani 1964, Arduin and Kraus 2001). Gall phenotype is diverse, with variation in shape, size, color and anatomical structure (Rohfritsch 1992), configuring several morphotypes (Isaias et al. 2013). This variation reflects changes in the morphogenesis of plant tissues, including processes of hyperplasia and cell hypertrophy, inhibition of differentiation and redifferentiation (Kraus et al. 2002, Lev-Yadun 2003, Oliveira et al. 2006, Isaias et al. 2011). Alterations in the patterns of cell elongation and expansion can also occur, leading to the new functional and morphological design of the leaves (Isaias et al. 2011, Ferreira and Isaias 2013, Magalhães et al. 2014).

The developmental patterns reflect the complex and efficient strategies of the insect-plant interactions (Roskan 1992) the most common galling agents, which may induce, modify and maintain the neoformed organ, the gall (Oliveira et al. 2006). Cecidomyiidae galls have specialized tissues involving the galling larvae, organized in a nutritive zone, surrounded by layers of lignified cells forming a mechanical barrier, and a parenchymatous outer cortex (Rohfritsch 1992). These anatomical features guarantee nutrition, and shelter against potential predators, and abiotic factors, which consequently can guarantee the adaptive value of the gall to the galling herbivores (Price et al. 1987, Stone and Schonrögge 2003).

The genus *Piper* includes several species of economical and medicinal interest (Souza and Lorenzi 2005, Judd et al. 2009) due to the chemical composition of its essential oils. Piper species may also contend terpenoids (Silva and Machado, 1999) and also host of galling herbivores (Jeff 1996, Maia et al. 2008, Maia and Azevedo 2009, Maia 2013). *Piper arboreum* Aubl. (Piperaceae) presents a leaf gall morphotype induced by a Diptera: Cecidomyiidae, which protrudes to both leaf surfaces (Maia et al. 2008, Maia 2013) being characterized morphologically as intralaminar lenticular (*sensu* Isaias et al. 2013). According to Isaias et al. (2013), this shape represents 15.9% of the total morphotypes recorded in the inventories of Neotropical galls. The current approach evaluates cell responses to the feeding stimuli of a galling Cecidomyiidae capable of altering host cell fates. This intimate plant-insect interaction focus, by the first time, on the ontogenetic and cytometric features involved in the development of intralaminar lenticular morphotype. Our model of study is the *Piper arboreum* - Cecidomyiidae

system, and aims to answer the following questions: (i) What cell fates are altered during the cecidogenetic process? (ii) How does the new functional design confer the adaptive value of the neoformed organ? (iii) Does the final fit the pattern described in literature for Cecidomyiidae galls? And (iv) how do the new patterns of cell elongation and expansion determine the final gall shape, i.e., the intralaminar lenticular morphotype?

Material and methods

Sampling. Non-galled leaves (NGL) and galls ($n \geq 10$) of *Piper arboreum* were collected at Estação Ecológica of Universidade Federal de Minas Gerais, and at the Parque Estadual Serra Verde, units of urban conservation (Fernandes et al. 1988, Portugal-Santana and Isaias 2015) located in the state of Minas Gerais, Brazil, from March 2013 to February 2014. Some galls were dissected under a stereomicroscope (Olympus® SHZ) for sampling of gall inducers and associated guild. The five stages of development of non-galled leaves (NGL) and the three stages of development of galls: growth and development (GD), mature (MG) and senescent (SG) were determined by anatomical features.

Fixation and structural analyses. Non-galled leaves and galls were fixed in Karnovsky (5% glutaraldehyde, 4% paraformaldehyde in phosphate buffer 0.1M, pH 7.0) (Karnovsky 1965 modified), dehydrated in an n-butyl series, and embedded in Paraplast® (Kraus and Arduin 1997). The sections (12-14 µm) were obtained in a rotatory microtome (Leica® 2035 BIOCUT), deparaffinized in butanol, and stained in astra blue and safranin, 9:1 (v/v) (Bukatsch 1972, modified to 0.5%). The slides were mounted on colorless varnish Acrilex® (Paiva et al. 2006), and photographed with a digital camera (Canon Power Shot A650®) coupled to an optical microscope (Olympus BHS ®).

Filed samples were post-fixed in 1% osmium tetroxide (OsO_4) for 2h, washed in 0.1 M phosphate buffer (pH 7.2), and gradually dehydrated in an ethanol series (Johansen 1940), followed by critical point drying with CO_2 (Balzers® model CPD-020). These samples were mounted on aluminum support (stubs) and covered with 15 nm of gold sputter (Bal-Tec®, MD20 model). The adaxial and abaxial surfaces were observed, and photographed in a scanning electron microscope (LEO EVO® 40).

Filed mature galls were post-fixed in 1% osmium tetroxide, dehydrated in an ethanol series (Johansen 1940), and infiltrated in Spurr® (Lufth 1961). The material was sectioned in an ultramicrotome Leica® model UC6, contrasted in lead citrate and uranyl (Reynolds 1963), and analyzed under a transmission electron microscope (Tecnai G2-20 - SuperTwin FEI - 200 kV).

Cytometry. Measurements of area, height and width of two cell units in two sections of young NGL (n=5) and of galls at GD, and MG (n=10 per stage) were obtained on digital images, using the Axion Vision 4.9.1® software. In NGL, the cells of the adaxial epidermis, adaxial hypodermis, adaxial layer of the middle meristem located above the vascular bundles of the minor veins, abaxial layer of the middle meristem located below the vascular bundles of minor veins, abaxial hypodermis, and of the abaxial epidermis were measured. In galls, the cells of epidermis on both surfaces, of the adaxial and abaxial outer cortex, and of the inner cortex above and below the vascular bundles were measured. In MG, the cells of nutritive tissue above and below the vascular were also measured. The degree of expansion and changes in the pattern of cell elongation from NGL through GD until MG were compared. To fit the assumptions of homoscedasticity and normality, some data were transformed into a logarithmic scale. Normal data were compared by one-way ANOVA and Tukey test at 5% probability, while non-normal data were compared by Kruskal-Wallis test, followed by Dunn's test at 5% probability using the SigmaStat® and the Graphprism 5.0®software.

Histochemistry. NGL, GD, MG and SG were hand-made cross-sectioned and immersed in a solution of 1% alpha/naphthol, 1% dimethyl-p-phenylenediamine, and 0.01 M phosphate buffer, pH 7.2 (David and Carde 1964) for the detection of terpenoids. Some other sections were submitted to 1% ferric chloride for 5 minutes for the detection of (poly) phenols (Johansen 1940).

Results

Leaf and gall morphology

The leaves of *Piper arboreum* are red when young and green when mature (Fig.1A,B). The galls are induced on young leaves by an unidentified species of Diptera: Cecidomyiidae (Fig.1C) and associated to the mid, secondary and tertiary

veins. When mature, they are intralaminar and lenticular, with the absence of chlorophyll in the cells of the adaxial and abaxial outer cortices (Fig.1D).

(Figure 1)

Leaf ontogenesis. Leaf primordia is protected by a stipule, and leaf development begins with the differentiation of the protoderm, followed by that of the ground meristem, and finally of the vascular system. Leaf primordia in its first developmental stage is adaxially fold, and its cells have rough cytoplasm, evident nuclei. The marginal initials divide anticlinally to originate the protoderm, and the submarginal initials divide periclinally to originate the three initial layers of the ground meristem. The adaxial meristem (AD) is one-layered, the middle meristem (MD) is 3-layered and the abaxial meristem (AB) is 1-layered. The mid vein is poor developed, constituted of 6-11cell layers (Fig.2A).

At the second stage of development, trichomes are differentiated on the primordia, which are still protected by the stipule (Fig.2B,C). Protodermic cells divide anticlinally, while periclinal divisions of the ground meristem increase the mesophyll thickness. The AD is 2-layered, the MD is 3-4 layered, and the AB is 2-3 layered. Some cells of the AD and AB accumulate phenolics. The mid vein has 32-37 cell layers in the abaxial cortical region, and 6-8 undifferentiated procambium strands (Fig.2B). Collenchyma differentiates beneath the abaxial epidermis. The second order veins start to differentiate.

At the third stage of development, the stipules fall down. The cells of the MD have rough cytoplasm, while the other cells are more vacuolated. The number of cells is maintained, and they elongate anticlinally on the AD and AB. The MD is 4-layered, and the procambium strands differentiate the minor veins. The mid vein has differentiated xylem and phloem, surrounded by non-lignified cells. The second order veins are still differentiating (Fig.2D,E).

At the fourth stage of development, the epidermis has sac-like trichomes on the adaxial and abaxial surfaces and only non-glandular trichomes on the abaxial surface. The hypodermis differentiates on the adaxial and abaxial side of the lamina. The thickness of the mesophyll increases due to periclinal and anticlinal divisions of the MD. Idioblasts are evidenced by the elongation of some cells. The second order veins are differentiated, but the minor veins are still differentiating (Fig.2F).

At the fifth stage, the leaf lamina is mature (Fig.2G-I). The stomata are exclusive of the abaxial epidermal surface (Fig.2H,I). The non-glandular trichomes are

multicellular, aciculate, with 3-11 cells. The mesophyll is heterogeneous, and dorsiventral, with 2-3 layers of palisade, and 2-3 layers of spongy parenchymas. Phenolics accumulate in mesophyll cells, and idioblasts filled with terpenic content are interspersed to the cells of the hypodermis, of the palisade and of the spongy parenchymas. The abaxial hypodermis is interrupted by the stomata chambers (Fig.2G). In the mid vein, the cortex has a 30-layered parenchyma, and a 6-9-layered collenchyma next to the epidermis. The vascular system has individualized bundles with collateral arrangement surrounded by pericyclic fibers on the mid and secondary veins.

(Figure 2)

Ontogenesis of Cecydomyiidae galls on Piper arboreum. The first anatomical symptom of the gall induction is the anticlinal hypertrophy of the hypodermic cells. The mesophyll is hyperplasic, and the vascular system disorganizes by divisions of the vascular parenchyma cells (Fig.3A). During GD (Fig.3B), the cells at gall site increase in number and size. The dermal system is uniseriated. The adaxial outer cortex is 5-8 cell layered, and the abaxial outer cortex is 5-11 cell layered. Vascular bundles limit the 14-29 layered inner cortex, with hypertrophied cells and a hyperplasic parenchyma surrounding the larval chamber. Mature galls (Fig.3C,D) have a 5-12 cell layered adaxial outer cortex, and a 9-23 layered abaxial cortex, accumulating phenolics and terpenoids. The inner cortex is composed of 24-38 cell layers. Lignified sclereid-like cells differentiate both from the cells of the abaxial outer and inner cortices forming a mechanical sheath (Fig.3C). These cells can completely surround the nutritive tissue, or have no connection at the lateral portions. The sclereid-like cells have a non-lignified wall portion facing the larval chamber. A nutritive tissue differentiates from the parenchymatic cells of the inner cortex. Neo-formed vascular bundles are not surrounded by lignified sheaths (Fig.3D).

At senescence (Fig.3E,F), the outer cortex consists of 6-12 adaxial cell layers, and 13-18 abaxial cell layers, and the inner cortex is 20-30 cell layered. At this stage, the galling Cecidomyiidae leaves the gall by the escape channel, which may face either the adaxial or the abaxial surface. Gall surface has some necrotic spots, and the cortical and nutritive cells collapse, with suberin deposition on the larval chamber surface. The vascular system remains similar to that of the mature galls.

(Figure 3)

Cytometric analyses. The epidermal cells elongate periclinally following the development of new organ (Fig.4). Cell area increases significantly along gall

development (Fig.5A). Adaxial epidermal cells, isodiametric in NGL, become anisodiametric in galls, while the abaxial epidermal cells were anisodiametric throughout gall development. Hypodermic cells reach their maximum size at maturation stage, constituting the adaxial and abaxial outer cortices (Fig.5B). The adaxial hypodermic cells maintained the anisotropic pattern of NGL, and the abaxial hypodermic cells, that were isodiametric in NGL, elongate anticlinally becoming an anisodiametric in galls (Fig.4). All tissues originated from the middle meristem are hypertrophied during gall development (Fig.5C,D). The parenchymatic cells above the vascular bundles, have an anisotropic pattern of elongation, while the cells below the vascular bundles, have an isotropic pattern. These patterns of elongation were maintained in the inner cortex and nutritive tissue (Fig. 4).

(Figure 4 and 5)

Discussion

Alterations in cell fates and the adaptive value of the new functional design

The ontogenesis of the leaves of *P. arboreum* follows the developmental pattern of the simples leaves described by Fahn (1990), and observed in some host leaves of other galls in the Neotropics (Moura et al. 2009, Oliveira and Isaias 2010, Isaias et al. 2011, Dias et al. 2013). The protoderm originates the epidermis, the ground meristem originates the mesophyll, and the vascular system originates from the procambium strands, firstly differentiated from the middle layers of the ground meristem.

The intralaminar lenticular galls of *P. arboreum* are induced on young leaves, the preferential site of oviposition for most of the galling insects (Rohfritsch 1992, Isaias and Oliveira 2012, Isaias et al. 2014). The meristematic and consequent responsiveness of the host leaf tissues by the time of oviposition facilitates the reactions to the insect's stimuli getting to gall establishment (Rohfritsch 1992, Arduin and Kraus 2001, Oliveira et al. 2008, Sá et al. 2009). Under stimulation of the galling herbivore, plant tissues assume distinct features and functions due to the process of cell redifferentiation (*sensu* Lev–Yadun 2003). This redifferentiation is particularly conspicuous in the cells originated from the middle meristem. While the dermal and vascular systems keep great similarities to their original states, i. e., they confer protection, and water and nutrients supply to the gall, the ground system loses its primary function of photosynthesis towards conferring a favorable microenvironment,

adequate nutrition, and protection against natural enemies (Stone and Schonrögge 2003).

The peculiarities of the dermal system are responsible for regulating the transpiration, the attraction of pollinators, and the defense against potential predators (Glover 2000). The dermal system of the non-galled leaves of *P. arboreum* is one-layered since the first developmental stage, as previously described for the host plant species, *Lantana camara* (Moura et al. 2009), and *Copaifera langsdorffii* (Oliveira and Isaias 2010). Mature leaves are hypostomatic with trichomes, a common feature to other species of Piperaceae (Silva and Machado 1999, Takemori et al. 2003, Albiero et al. 2005, Gogosz et al. 2012). The occurrence of trichomes since the leaf primordial is inherent aspect of *P. arboreum*, and might be enhanced by abiotic conditions (Glover 2000, Moura et al. 2009), confering protection against to predation, as proposed by Isaias et al. (2011) for *Lonchocarpus muehlberguianus*. The characteristics of the dermal system were maintained throughout the development of the galls on *P. arboreum*, but cell elongation was necessary to accompany the structural increase in size, as reported for other insect galls (Moura et al. 2009, Isaias et al. 2011).

The presence of subepidermal cells in mature leaves is common in Piperaceae (Takemori et al. 2003, Souza et al. 2004, Albiero et al. 2005, Gogozs et al. 2012). Takemori et al. (2003) refer to this cell layer on *Peperomia catharinae, P. marginella*, *P. quadrifolia*, and *P. rotundifolia* as a multiple epidermis, for they attribute its origin from the protodermal cells. However, current data on the ontogenesis of *P. arboreum* confirm the ground meristem origin of these cells.

The hypodermis as well as the cortical layers have prominent structural and chemical alterations due to the galling stimuli. The cells of the hypodermis have reacted positively to phenolics and terpenoids, while the idioblasts located in the mesophyll of non-galled leaves have reacted positively to terpenoids, but have their differentiation blocked in galls. The non differentiation of idioblasts may have favored the establishment of the galling Cecidomyiidae on *P. arboreum*, since some terpenoids can cause damage to the herbivores (Gershenzon, 1994). However the presence of terpenes in the hypodermis of the gall may be associated with protection against natural enemies due to the potential in reduce the palatability of plant tissues (Róstas et al. 2013).

The formation of two parenchymatic zones, with a lignified layer within, follow the morphogenetic pattern proposed by Rohfritsch (1992) for galls induced by Diptera: Cecidomyiidae. The lignification of the cell walls, in the inner and outer cortices,

confers mechanical resistance to the structure (Oliveira and Isaias 2009, Formiga et al. 2011), as well as helps the translocation of water and nutrients (Formiga et al. 2011). It also confer mechanical support to the structure, and protection against potential predators (Oliveira and Isaias 2009). However, the unequal lignification of the cell walls in the tissue layers adjacent to the nutritive ones is a peculiarity of *P.arboreum* galls, and the cytoplasmatic content confirm that these cells are alive and may potentially store nutrients.

The collateral arrangement of the vascular bundles was maintained from the non-galled state towards gall maturation, but the neovascularization seems to confer additional nutritients supply, and reinforces that the galls act as sinks of photoassimilates (Isaias and Oliveira 2012, Castro et al. 2012), corroborating the nutritional hypothesis (Price et al. 1987).

The non-lignification of the cell walls or non lignificated pericyclic fibers were evidenced on some insect galls. This morphogenetic feature was reported on ambrosia galls on *Baccharis concinna* and *Baccharis dracunculifolia* (Arduin and Kraus, 2001), and on insect galls on *Struthantus vulgaris* (Arduin et al. 1991), *Piptadenia gonoacantha* (Arduin and Kraus 1995) and *Ficus microcarpa* (Souza et al. 2000). The differentiation of the mechanical zone in *P. arboreum* should be indicative of an alternative metabolic pathway for the lignin precursors. They are synthetized and redirected to the sclereid-like cells instead of to the vascular bundle sheaths.

The nutritive tissue is the most specialized group of cells differentiated at gall sites (Isaias et al. 2014, Oliveira et al. 2014). The decrease in the number of cell layers in this tissue from maturation to senescence is consequence of the feeding activity of the galling insect, and was also observed in *Piptadenia gonoacantha* - Cecidomyiidae (Arduin and Kraus 1995), and *Lantana camara- Schismatodiplosis lantanae* (Acari) (Moura et al. 2009) systems. Cells around the larval chambers are suberized, indicating cell necrosis as a symptom of the end of the feeding stimuli by the galling herbivore. These suberized cells can protect the galls from the invasion of pathogens (Isaias and Olivera 2012) after the end of cell cycles. Suberization is not exclusive of insect galls, such as those induced by *Callophya duvauae* (Psylloidea) on *Schinus polygamous* (Dias et al. 2013), and by a Pseudophacopteronidae on *Aspidosperma spruceanum* (Formiga et al. 2011), for it has been detected on galls induced by *Aceria lantanae* (Acari) on *L. camara* (Moura et al. 2009).

The development of the lenticular intralaminar shape

The lenticular design of the galls on *P. arboreum* implies in a size increment from the growth and development to the maturation phase. This growth is product of intense hyperplasia especially the inner cortex, followed by cell hypertrophy in all tissues, especially in the adaxial and abaxial outer cortices. The processes of cell hypertrophy and tissue hyperplasia are common during gall formation (Oliveira et al. 2006, Oliveira and Isaias 2009, Formiga et al. 2011, Isaias et al. 2011, Ferreira and Isaias 2013), and the relation between its zonation and the final gall shape was firstly determined by Magalhães et al. (2014), for kidney-shaped galls on *Baccharis dracunculifolia*. In these galls, the periclinal anisotropy and cell hipertrophy allowed the folding of the host leaf lamina. According to Rohfritsch (1992), the feeding sites and consequently the shape of the larval chamber would be the pressure that generate the final shape of gall. However, the isotropic and anisotropic expansion control is essential to acquire the final shape of plant organs (Baskin 2005), which is also crucial to the acquisition of the distinct designs observed in galls (Isaias et al. 2013). In the galls on *P. arboreum*, the anisotropic anticlinal cell expansion of the hypodermal cells and those originated from the middle meristem is crucial for the development of the circular halo, while the hyperplasia in periclinal direction determines the biconvex shape, i.e., the intralaminar lenticular morphotype (*sensu* Isaias et al. 2013). Also, the shape of the larval chamber accompanies the gall final design.

Conclusions

The cell fate of the ground meristem in leaves of *Piper arboreum* Aubl. (Piperaceae) was modified by the galling stimuli, setting a new functional design that corroborates the pattern described for the Cecidomyiidae galls. This new design ensures nutrition, from the differentiation of a nutritive tissue, and mechanical protection, due to the lignified zone. The sclereid-like cells of this sheath are cytoplasm rich, which aid the transport and accumulation of nutrients. The chemical alterations of gall tissues contribute to the adaptive value of the gall by reducing the palatability, and ensuring protection against natural enemies. The determination of the intralaminar lenticular gall morphotype involves both the pressure imposed by the feeding sites of the galling herbivore and changes in the hyperplasia and pattern of cell expansion and elongation.

Acknowledgments

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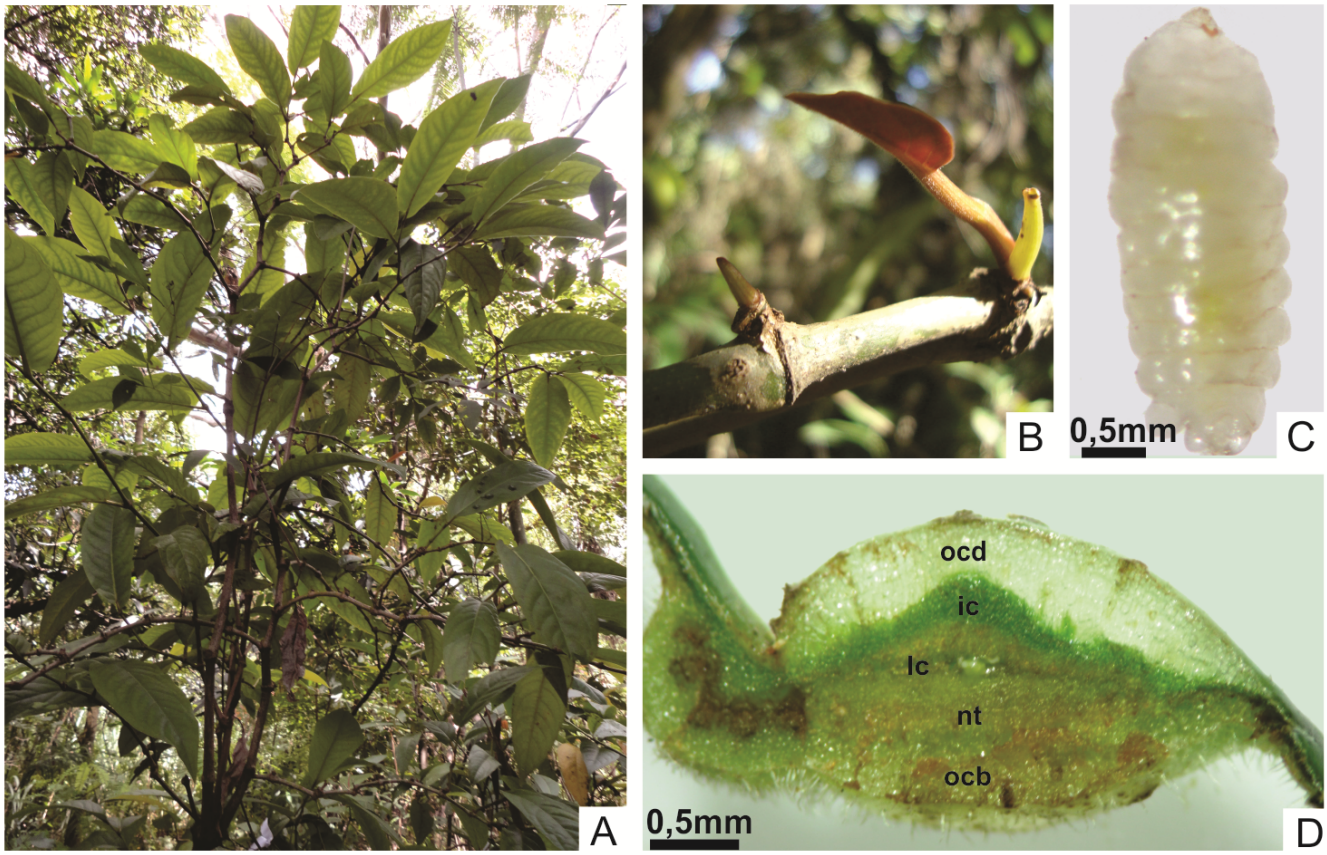

Figure 1: ***Piper arboreum*** **Aubl. (Piperaceae). A:** Habit of the plant. **B:** Detail of leaf primordia on the four stage of development. **C:** Larva of Cecidomyiidae. **D:** Cross section of a mature gall. **ocb**: outer cortex abaxial; **ocd**: outer cortex adaxial; **ic**: inner cortex; **lc**: larval chamber; **nt**: nutritie tissue.

Figure 2: Ontogenesis of the leaves of *Piper arboreum* (Piperaceae) in cross sections (A-G), and in scanning electron microscopy (H-I) of leaves. **A-E:** Overview from the first to the third stages of development showing the development of primary and secondary veins (arrows), and the midrib (dotted circle). **A:** First stage of development; **B:** Second stage of development **C:** Detail of the second stage of development. **D:** Third stage od development **E:** Detail of the third stage of development evidencing the collenchyma near the epidermis and a secondary vein. **F:** Detail of the fourth stage evidencing the increase of cell layers of the middle meristem, development of minor veins and idioblasts (*). **G:** Detail of differentiation of the pericyclic fibers in a mature leaf. **H:** Adaxial epidermis with sac-like trichomes. **I:** Abaxial epidermis showing stomata (arrow) **ad:** adaxial meristem; **ab:** abaxial meristem**; cl:** collenchyma**; md:** middle meristem; **pd:** protoderm.

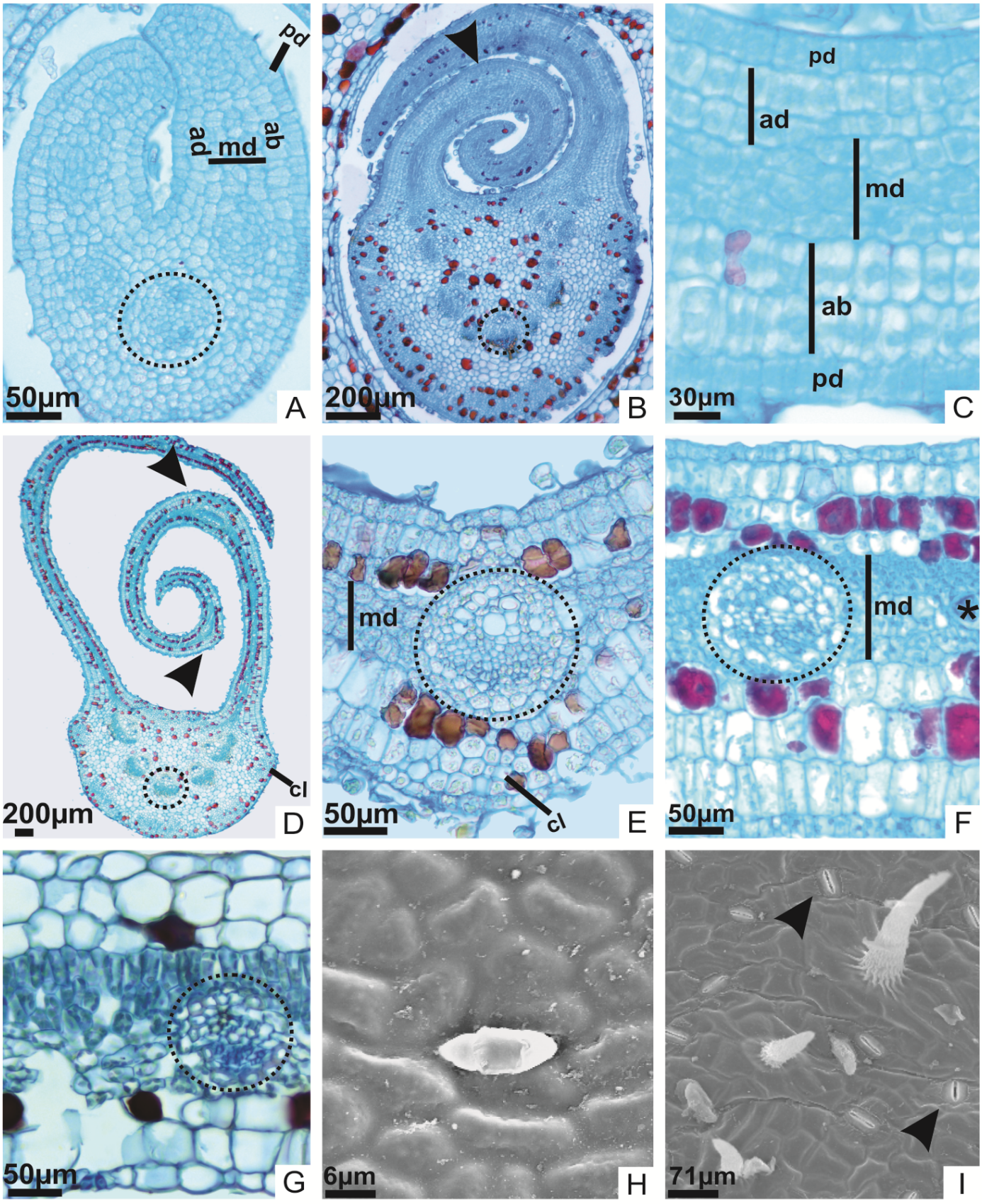
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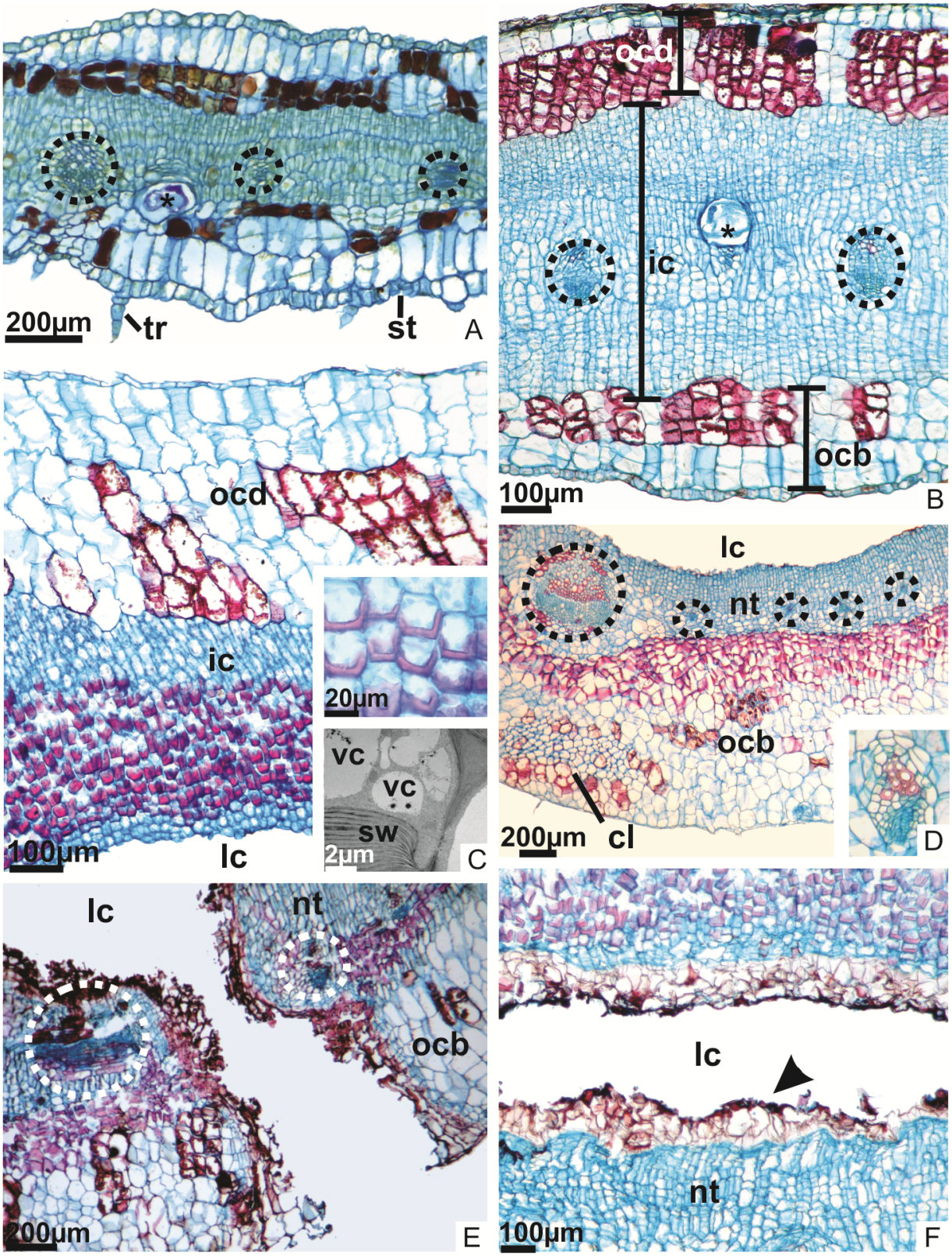
Figure 3: Ontogenesis of Cecidomyiidae galls on *Piper arboreum*. Cross section from the induction to the senescent stages. **A:** Induction phase with the galling Cecidomyiidae in the lamina leaf. **B:** Growth and development phase showing an increase in the number of cortical cell. **C:** Maturation stage showing the adaxial outer cortex and differentiation of the inner cortex. Details of the unequal lignification of the cell walls of the inner cortex **D:** Nutritive tissue and the abaxial outer cortex in the maturation stage. Detail of the vascular bundle of a minor vein without the pericyclic fibers. **E:** Gall in senescent stage evidencing the escape channel in the abaxial surface. **F:** Detail of suberization in nutritive tissue adjacent the larval chamber (arrow). **arrow:** collenchyma; **cw:** secondary wall; **dotted circle:** vascular bundle; **ic:** inner cortex; **lc:** larval chamber; **nt:** nutritive tissue; **ocb:** abaxial outer cortex; **ocd:** adaxial outer cortex; **st:** stomata; **tr:** Trichome; **vc:** vacuole.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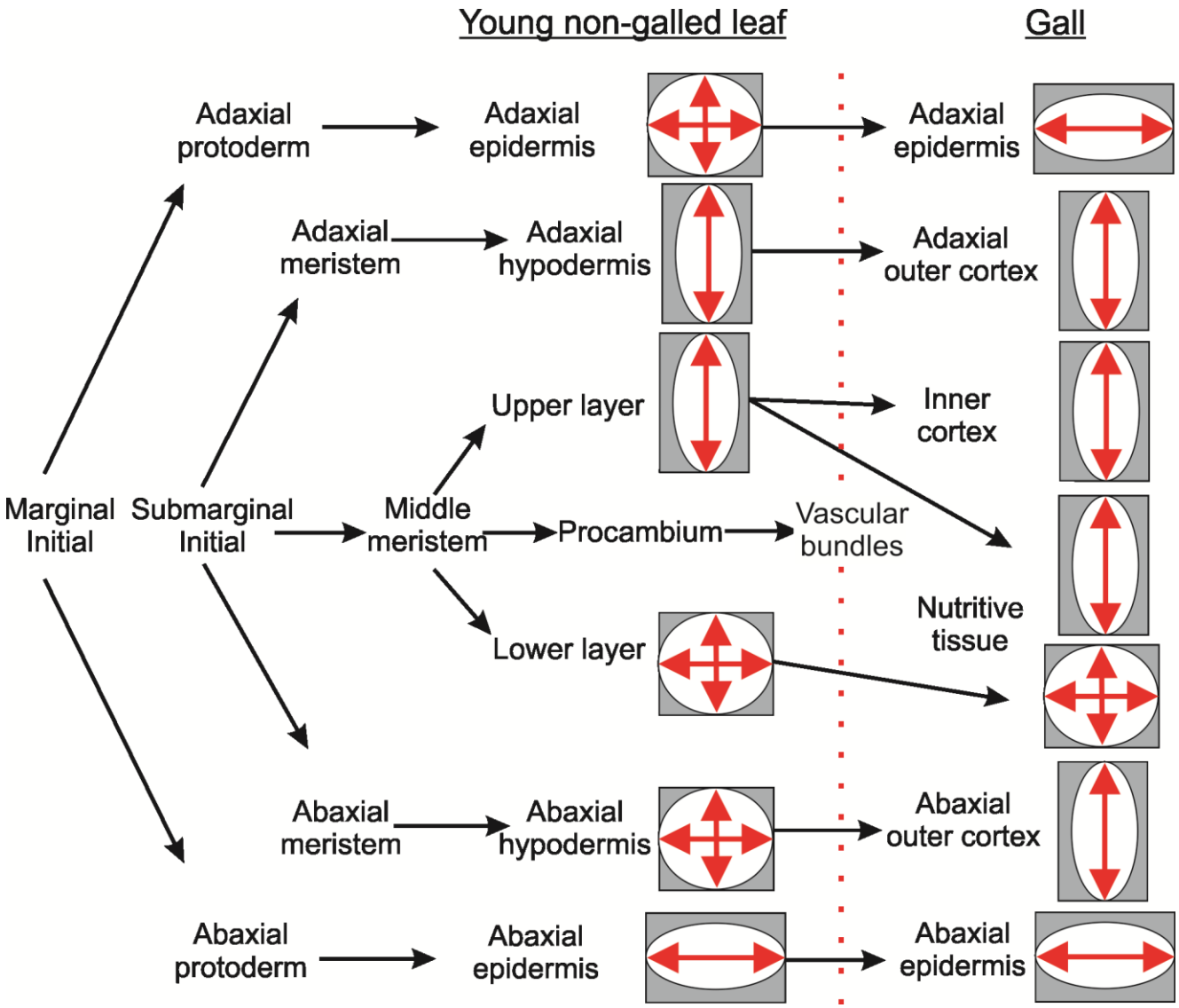


Figure 4: Diagram of the ontogeny and of the direction of cell elongation on galls induced by a Cecidomyiidae on leaves of *Piper arboreum* Aubl. (Piperaceae).

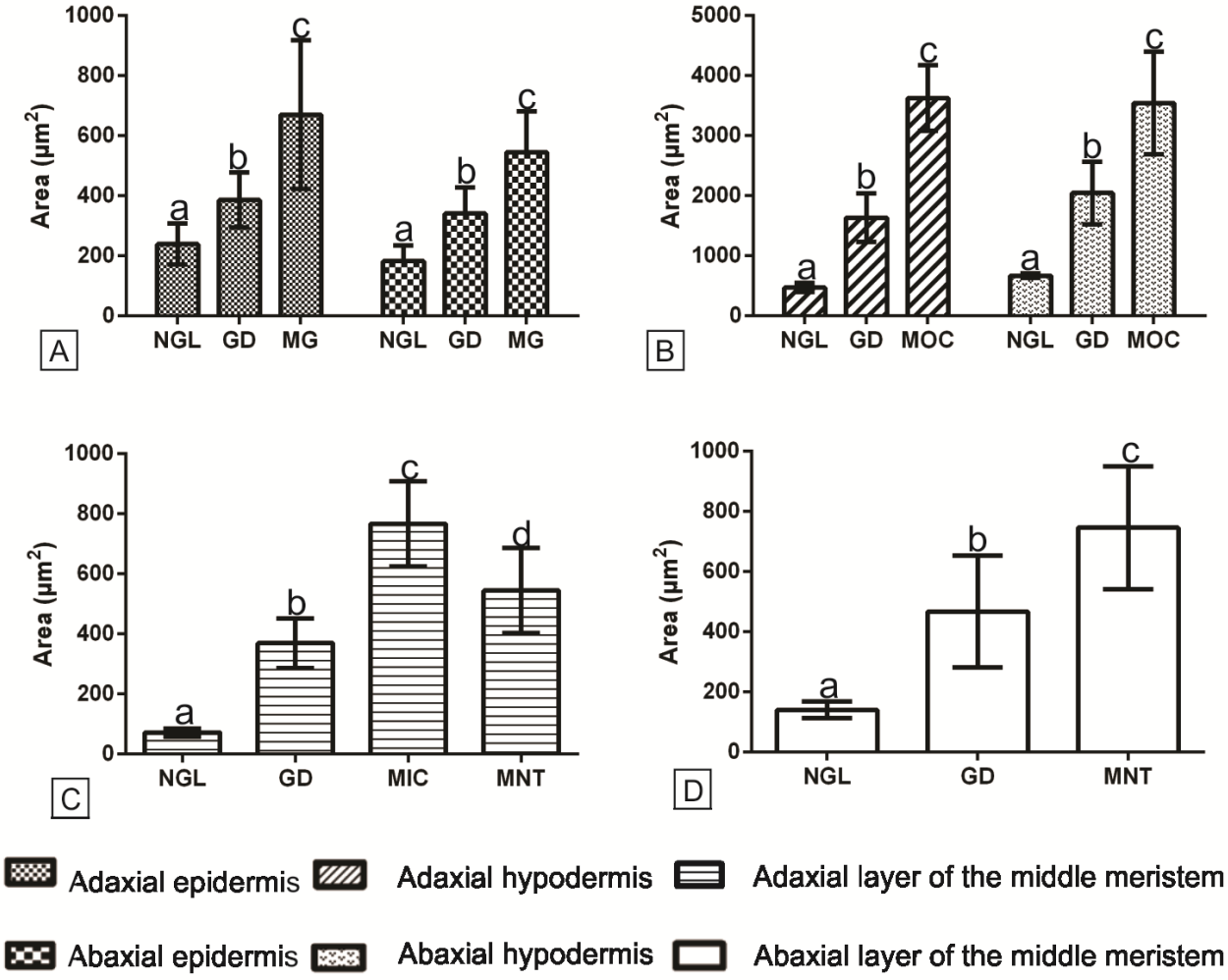
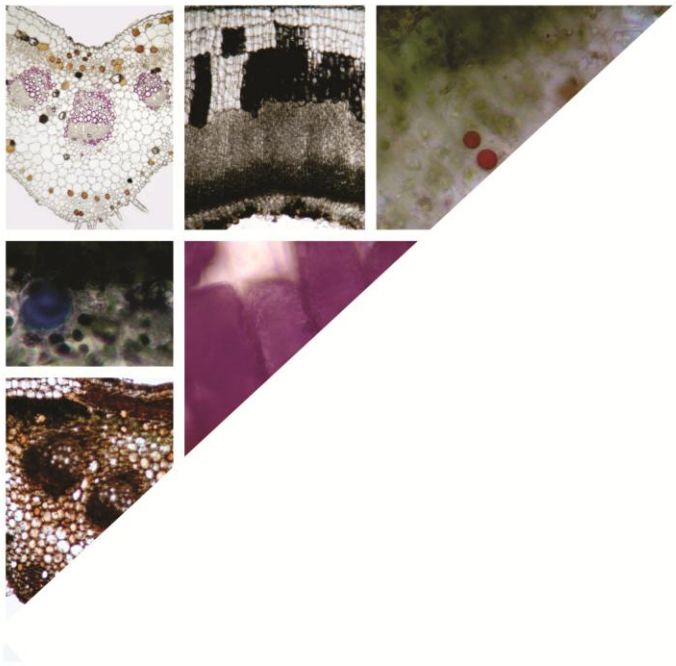


Figure 5: Cytometric analysis of tissues in non-galled leaves and galls induced by Cecidomyiidae on *Piper arboreum* Aubl. (Piperaceae) in different stages of development (n = 10, mean ± standard deviation). **NGL:** Non galled leaves, **GD:** Galls at growth and development stage, **MG:** Mature gall, **MOC:** Outer cortex of mature gall, **MIC:** Inner cortex of mature gall, **MNT:** Nutritive tissue of mature gall. Means followed by the same letter are not statistically different bars for the same variable ($p < 0.05$).



Capítulo II

Enzymes activity in nutritive cells and metabolites compartmentalization in Cecidomyiidae galls on *Piper arboreum* Aubl. (Piperaceae)

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Enzymes activity in nutritive cells and metabolites compartmentalization in Cecidomyiidae galls on *Piper arboreum* Aubl. (Piperaceae)

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Abstract

Galling insects commonly change the chemical profile of their host plant tissues by inducing galls, neoformed structures that guarantee their food and protection by accumulating a wide range of metabolites. Nutritive cells, responsible for the storage and availability of nutrients via enzymatic activity, are necessary for gall development and insect feeding. In addition, toxic metabolites should accumulate in the cells of the gall outer cortex, and confer chemical defense against natural enemies. All the specialized cells in galls, with their metabolic apparatus, should differentiate under the chemical constraints of each host plant-galling herbivore interaction. This premise is herein addressed by the investigation of the histochemical profiles of non-galled leaves and galls induced by an Diptera: Cecidomyiidae on *Piper arboreum*. The spatial compartmentalization of the nutritive and defensive metabolites should be related to the new functions assumed during the redifferentiation of the host plant cells. The primary metabolites are dynamically metabolized by enzymes, such as sucrose synthase and invertases, towards either the nutritive requirements of the galling herbivore or the structural maintenance of the gall. Secondary metabolites restrict the chemical protection to the tissue layers not involved in nutrition, but which should otherwise attract predators or parasitoids. Current results systematically documented metabolites compartmentalization, evidenced the blockage of toxic compounds in cells surrounding the larval chamber, as well as, the redirection of nutritive substances to this site. The nutritive tissue is the restrict compartment of sucrose synthase in *Piper arboreum*, which is proposed to be a cytological metabolic pattern for Cecidomyiidae galls.

Key words: Chemical profile, enemy hypothesis, galling insect diet, gall metabolism, nutritive hypothesis

Key message: Cell compartmentalization of nutritional and defensive metabolites, and enzyme activity, reveals peculiar steps of carbohydrates metabolism, and chemical constraints in *Piper arboreum*-Cecidomyiidae system.

Introduction

The galling insects change the morphogenetical patterns of their host plant organs by inducing cell redifferentiation (*sensu* Lev-Yadun 2003), division and growth (Oliveira and Isaias 2010a; Isaias et al. 2011; Isaias et al. 2014; Magalhães et al 2014). The new patterns generate specialized cells and tissues at gall site, whose chemical and functional features are distinct from those of the host organ (Oliveira et al. 2011; Castro et al. 2012;Carneiro et al. 2014). In galls, primary and secondary metabolites can be compartmentalized, enhancing new functionalities, such as the availability of nutritional resources for the galling herbivore feeding, and the protection against natural enemies (Price et al. 1987; Stone and Schonrögge, 2003). In addition, the carbohydrates drained to gall site are responsible for cell machinery support (Castro et al 2012; Oliveira et al 2006), and can also act as potential signaling molecules for cell division and growth (Koch 2004; Wind et al 2010). Thus, two important hypotheses are discussed and reveal the adaptive value of the gall tissues for the galling herbivore (Price et al. 1987). The nutritional hypothesis postulates that proteins, lipids, reducing sugars or starch accumulate in the gall inner cortex, i. e., in the cells next to the larval chamber, and can provide resources for the galling insect (Price et al. 1986; Price et al. 1987; Bronner 1992). Nevertheless, in some systems, the availability of these metabolites depends of the enzymatic activity that should break down large molecules, such as the polysaccharides into smaller sugars, improving gall metabolism and the diet of the galling herbivore (Bronner 1992; Oliveira et al. 2010; Oliveira and Isaias 2010b). The patterns of enzymatic activity depends on the mouth apparatus of the galling insects (Bronner 1992) and, the chemical profile of the host plants (Oliveira et al. 2010; Oliveira and Isaias 2010b; Oliveira et al. 2014). The enemy hypothesis postulates that defense compounds from the secondary metabolism, such as alkaloids, flavonoids, phenolics and tannins (Oliveira et al. 2006), commonly accumulate in the gall outer cortex (Nyman and Julkunen-Titto 2000; Formiga et al. 2011; Isaias et al. 2014), and can protect the galling herbivore against the attack of parasitoids and predators, and gall structure against the cecidophagous (Price et al. 1987).

Galls are considered sink of photoassimilates that drain sugars from other autotrophic parts of the host plant (Abrahamson and Mccrea 1986; Bagatto and Shorthouse 1994; Bagatto

et al. 1996; Rehill and Schultz 2003; Castro et al. 2012). In some galls, the metabolism of these carbohydrates must be mediated by enzymes activity, which has been documented just for four galling herbivore-host plant systems in the Neotropics (Oliveira and Isaias 2010b; Oliveira et al 2010; Oliveira et al. 2011; Caneiro et al. 2014). Thus, as non-reducing sucrose is the main transported carbohydrate in higher plants, we here in study its metabolism in a Cecidomyiidae gall on *Piper arboreum* in order to improve the knowledge on the metabolic aspects of the redifferentiation of nutritive cells and galling insect`s diet. The sucrose can be synthesized in the cytosol from photosynthetically fixed carbon, starch reserves or lipids metabolism (Wind et al 2010), and is transported via phloem to other plant parts. The main enzymes responsible for sucrose metabolism are the sucrose synthase (SuSy) and the invertases, which catalyse the conversion of sucrose into glucose and fructose (Koch 2004). In general, SuSy activity is associated to sink tissues and starch accumulation, while invertases mediate cell respiration, tissue growth and development (Koch 2004; Wind et al 2010). In galls, the metabolism of these enzymes is also related to the formation of histochemical gradients responsible for galling nutrition and maintenance of gall tissues (Oliveira and Isaias 2010).

Bronner (1992) was the first to propose the formation of a gradient of metabolites in Cecidomyiidae galls. In this pattern, starch accumulates in a centrifugal gradient, decreasing towards the larval chamber. Reducing sugars and proteins are evidenced in the nutritive tissue, where lipids do not accumulate. However, studies on the diverse flora of the Neotropical region, have revealed exceptions to Bronner's patterns, as observed in the *Aspidosperma spruceanum*-Cecidomyiidae (Oliveira et al. 2010) and *Copaifera langsdorffii* – Cecidomyidae (Oliveira et al. 2011) systems. These two galls are induced by non-chewing herbivores and consequently, the carbohydrates assessment needs enzymatic mediation, such as the activities of acid phosphatase, phosphorylase, invertase, and sucrose synthase, especially in the nutritive tissues (Oliveira et al. 2010; Oliveira and Isaias, 2010b; Oliveira et al. 2011; Oliveira et al. 2014). Also, Oliveira et al. (2006) reported neo-accumulation of oxonium crystals just in the tissues of *C. langsdorffii* galls, which remains unique for insect galls.

The intralaminar lenticular galls (*sensu* Isaias et al. 2013) induced on the leaves of *P. arboreum* (Piperaceae) have nutritive cells limiting the larval chamber (Bragança et al. 2015, submitted). These cells should accumulate carbohydrates, as previously observed in some other Neotropical Cecidomyiidae galls (Oliveira et al. 2010; Oliveira et al. 2011). Also, a

relation between the gradients of starch and sugars and the enzymatic activity should be detected in the inner cells around the larval chamber; while secondary metabolites, if present, should accumulate in the outer cortical cells. This spatial compartmentalization of nutritive and defensive metabolites at gall site are investigated in *Piper arboreum*-Cecidomyiidae system, focusing on the following questions: (I) is there a spatial compartmentalization of primary and secondary metabolites involved in the differentiation of nutritional and protective cells in gall site? (II) Is there neo-accumulation of metabolites in the cells of *P.arboreum*, under the stimulation of the galling Cecidomyiidae? And (III) should carbohydrate enzymatic mediation in the galls on *P. arboreum* corroborate a pattern for the redifferentiation of nutritive cells on Cecidomyiidae galls and/or be an imposition of the insect`s diet?

Materials and methods

Non galled leaves (NGL) and mature leaf galls (MG) induced by an unidentified species of Diptera: Cecidomyiidae on *Piper arboreum* were collected from September 2013 to March 2014 at Estação Ecológica da Universidade Federal de Minas Gerais in Belo Horizonte, Minas Gerais state, Brazil.

Histochemical assays. The samples were free-hand sectioned and submitted to histochemical tests with the following reagents: saturated solution of sudan Red B in 70°GL ethanol during 5 min to detect lipids (Brundett et al. 1991); Fehling's reagent (Solution "A" - 7.9% copper sulfate, and solution "B" - 34.6% sodium potassium tartrate and 1% sodium hydroxide) heated to pre-boiling temperature for reducing sugars (Sass, 1951); Lugol's reagent (1% potassium iodine–iodide solution) during 5 min for starch (Johansen 1940); 0.1% Bromophenol blue in a saturated solution of magnesium chloride in ethanol during 15 min, and later washed in acetic acid and water, for proteins (Baker 1958); 1% ferric chloride during 5 min, for phenolic compounds (Johansen 1940); Dragendorff's reagent (Solution "A"" - 12.5% bismuth nitrate in 25% acetic acid, and solution "B" 40% potassium iodide) during 5 min for alkaloids (Wagner and Bladt, 1996); Wiesner's reagent (2% phloroglucinol in acidified solution) during 5 min for lignins (Johansen 1940); fixation in 0.5% caffeine sodium benzoate in 90% butanol, followed by1% *p*-dimethylaminocinnamaldehyde (DMACA) during 30 min for flavonoids (Feucht et al. 1986); 1% alfa-naftol and 1% dimethyl-*p*-phenylenediamine in phosphate buffer (pH 7.2) (NADI) during 30 min for terpenoids (David and Carde 1964) and Lieberman-Buchard's reagent (concentrated solution of sulfuric acid and acetic acid 1:1 v/v) during 1 min for triterpenes (Wagner et al. 1984). The sections were washed in water and photographed

under an optical microscope (Zeiss Primo Star®) with a digital camera (Canon Power Shot A 630®). Blank sections were used for the comparison of results.

Enzymatic activity. For detection of the activity of acid phosphatase, sections obtained from fresh samples were incubated in 0.012% lead nitrate and 0.1M potassium sodium glicerophosphate in 0.5M acetate buffer (pH 4.5) for 24 hours at room temperature. The sections were washed in distilled water and subjected to reaction in 1% ammonium sulfate for 5 min. As a control, the samples were not submitted to potassium sodium glicerophosphate (Gomori 1956). For the detection of phosphorylase activity, the sections were incubated for two hours in 1% glucose-1-phosphate in 0.1M acetate buffer (pH 6.0) at room temperature, and subsequently subjected to Lugol's reagent for 5 min. For control samples were not incubates in glucose-1-phosphate (Jensen 1962). For observation of invertase activity, sections were incubated for 3 hours at room temperature in a solution containing 0.024% tetrazolium blue (NBT), 0.014% phenazin methosulfate, 30U of glucose oxidase and 30 mM of sucrose, 0.38mM sodium phosphate buffer (pH 7.5). The control was subjected to the reaction without the presence of sucrose (Zrenner et al. 1995; Doehlert and Felker1987). For detection of sucrose synthase activity, samples were fixed in 2% paraformaldehyde with 2% polyvinylpyrrolidone and 0.005M of dithiothreitol (pH 7.0) for 1 hour at 4°C. Later they were incubated in solution containing 5µL of 150 mM NADH, 5µl (1U) of phosphoglucomutase, 5µl of 3mM glucose 1,6-bisphosphate, 5µl (1U) of glucose-6-phosphate dehydrogenase, 5µl (1U) of UDPG pyrophosphorylase, 280µL of 0.07% aqueous solution of blue tetrazolium (NTB), 350 µl of buffer and 50µL substrate during 30 minutes. The buffer contained 10 mM $MgCl_2$, 2 mM EDTA, 100 mM HEPES, 0.2% BSA and 2mM EGTA (pH 7.4). The substrate consisted of 15 mM UDP, 0.75mM sucrose, 15mM pyrophosphate. Two controls were used. In the first control, the glucose 1,6-bisphosphate and pyrophosphate were not added, and for the second control, the sucrose was suppressed in the solution (Wittich and Vreugdenhil 1998).

Histochemical tests for Reactive Oxygen Species (ROS). ROS were detected by 0.5% of 3.3'-diaminobenzidine (DAB) during 15 - 60min for reactive oxygen species (Rosseti and Bonatti 2001). The sections were washed in water and photographed under an optical microscope (Zeiss Primo Star®) with a digital camera (Canon Power Shot A 630®). Blank sections were used for the comparison of results.

Results

General features. *Piper arboreum* host galls induced by an unidentified species of Diptera: Cecidomyiidae resulting in an intralaminar and lenticular structure projected to the adaxial and abaxial leaf surface (Fig. 1a-c).

Histochemical tests for primary metabolites. Starch grains were detected in dark blue or black color in the cells of non-galled leaves (NGL) and mature galls (MG). In the NGL, starch grains were detected in the palisade and spongy parenchyma, while in MG, the detection more intense in the cell layers next to the larval chamber, increasing laterally towards the non-galled region. Its presence was also detected in the lignified cells of the inner and outer cortices (Fig. 2a,3). Lipids were detected by the red color in the droplets in the adaxial and abaxial hypodermis of NGL. The lipidic droplets were detected in the cells of the abaxial and adaxial outer cortex, and of the nutritive tissue of MG (Fig.2b,3). Reducing sugars form red precipitates in the cells of the adaxial and abaxial hypodermis of NGL (Fig.2c,3), and of the adaxial and abaxial outer cortices of the MG. Proteins were detected by the dark blue color in the nutritive tissue (Fig.2d,3).

Enzymatic activity. The activity of invertases formed a dark blue precipitate in the hypoderm and parenchymatic cells of the veins of the NGL. At the MG, the activity of invertases was detected in the cells adjacent to the larval chamber, forming a centrifugal gradient towards the non-galled region (Fig.2e,3). The activity of sucrose synthase (SuSy) was detected as a purple colored precipitate in the vascular bundles of the NGL. In the MG, the activity of SuSy was detected homogeneously throughout the nutritive tissue and vascular bundles (Fig.2f,3). The activity of phosphorylase and acid phosphatase was not observed in the non-galled and galled tissues.

Histochemical tests for secondary metabolites. Lignins were evidenced by the red color in the cell walls of the xylem and of the pericyclic fibers, both in NGL and MG. Their presence in MG was also observed in the xylem and sclerenchymatic sheath surrounding the nutritive tissue (Fig.3,4a). Phenolic compounds, flavonoids and alkaloids were identified in the cells of the adaxial and abaxial hypodermis of the NGL. These compounds were detected in the adaxial and abaxial outer cortices of the MG (Fig.3,4b,c,d). Terpenoids were detected by the blue color in the cells of the adaxial and abaxial hypodermis, and also in the idioblasts located

between the palisade and spongy parenchyma of the NGL. In MG, these compounds were evidenced only in the adaxial and abaxial outer cortices (Fig.3,4e). Triterpenes were detected by the red color in the cells of the adaxial and abaxial hypodermis of the NGL.

Histochemical tests for Reactive Oxygen Species (ROS). The ROS were observed in the epidermis and chlorophyll parenchyma of the NGL. In MG, ROS accumulated in a centrifugal gradient, decreasing towards the outer cortical tissue layers (Fig.3,4f).

Discussion

Spatial compartments of primary and secondary metabolites

The intralaminar leaf galls on *P. arboreum* have two compartments regarding the accumulation of primary and secondary metabolites. The presence primary metabolites has been especially detected in the internal tissues, while the accumulation of secondary metabolites is restricted in the outer cortical parenchyma, which corroborates the expected spatial functional division of gall tissues. The compartmentalization of metabolites is product of the conversion of the photosynthetic and respiratory roles of the host leaves into the gall new defensive and nutritional functions. The detection of the activity of sucrose synthase in gall tissues is related to the sink profile of the nutritive tissue; mean while the development of a centrifugal gradient of starch and invertases is product of the high cell respiratory metabolism, and consequent increased oxidative stress.

The inner compartment of the galls on *P. arboreum*, i.e., the nutritive tissue, accumulates proteins, similarly to the galls on *Aspidosperma spruceanum* (Oliveira et al. 2010). More than a cell response to the increased oxidative stress established during the cecidogenetic process, as proposed by Schönrogge et al. (2000), proteins also provide nutrition to the galling herbivore. Together with the oxidative stress caused by the feeding activity of the galling insect, the increased levels of proteins in the nutritive tissue can alter the sugar and enzymatic metabolism (Sturn and Tang 1999). Also, another cell response to the high respiratory activity and oxidative stress in gall tissues is the increasing levels of hexoses (Roitsch and Gonzalez 2004), products of the activity of invertases. Invertases also quickly metabolize sucrose and activate the mechanism of plant defense by increasing the synthesis of secondary metabolites (Wind et al 2010; Sturn and Tang 1999). The secondary metabolites commonly occupy the outer compartment of the galls, i.e., the outer cortex (Nyman and Julkunen-Titto 2000; Formiga et al. 2011; Isaias et al. 2014), where they are intimately related

to the chemical defense against natural enemies (Hartley 1998; Nyman and Julkunen-Titto 2000, Price et al. 1987; Isaias et al. 2014). In addition, they may help eliminating reactive oxygen species (Blokhina et al. 2003). Plant defensive compounds include the alkaloids, phenolics and terpenes, which were detected in the outer cortices of the intralaminar gall on *P. arboreum*. The alkaloids and phenolics may deter or discourage the attack predators, due to their toxic mechanisms of action (Rodhes 1994), as well as may be involved in antioxidant mechanisms (Blokhina et al. 2003; Detoni et al. 2011).

The phenols have fundamental roles in many mechanical protection and structural support, confered by cytological processes culminating in cell lignification (Isaias et al. 2000). Recently, the co-occurrence of phenols and indole-3-acetic acid (IAA) was histochemically documented by Bedetti et al. (2014) in galls on *Piptadenia gonoachanta*, and furnished strong evidence that their accumulation can increase the concentration of auxins by inhibiting the IAA-oxidases, allow cell expansion, and confer the new structural design of the gall.

The accumulation of terpenes is exclusive of the outer compartment of *P. arboreum* galls. The NGL accumulation pattern seems to have been partially blocked in the MG, where the single detection site was the outer cortical cells. The blockage of the differentiation of idioblasts in the nutritive tissue seems to favor the gall inducer, which did not come into contact with the toxic potential of the terpenes, and their anti-herbivore properties (Gershenzon 1994). The different classes of terpenoids may interfere with the establishment of galls as documented by Moura et al. (2009) in the *Aceria lantanae-Lantana camara* system. In this system, the non-detection of monoterpenes and sesquiterpenes, coupled with low trichome density, determines the choice of the plants of *L. camara* with red flowers for oviposition. Currently, the blockage of triterpenes accumulation in the MG denotes that the galling herbivore stimuli totally inactivate these substances, which helps the success of gall development.

Our results evidence primary and secondary metabolites in distinct compartments of the gall, but no neo-accumulation is detected. On the other hand, the blockage of terpenoidic derivatives under the Cecidomyiidae stimuli seems to be crucial for the gall development.

Enzymatic mediation of carbohydrates accumulation

The activity of two from the four investigated enzymes is detected in the nutritive tissue of *P. arboreum* gall, sucrose synthase (SuSy) and invertases. Both enzymes have been previously detected in galls induced by Pseudophacopteronidae and Cecidomyiidae in

Aspidosperma spp. (Oliveira and Isaias 2010, Oliveira et al. 2010), and the current results indicate a common site for the activity of SuSy only for Cecidomyiidae galls, once in the Pseudophacopteronidae galls on *A. australe*, the activity of SuSy was restricted to the vascular bundles. These distinctive sites of detection coincide with the feeding sites of the two inducers, where they must find the expected carbohydrates. The activity of SuSy is responsible for the reversible cleavage of sucrose into fructose and UDP-glucose (Amor et al. 1995; Roitsch and Gonzalez 2004; Koch 2004), and may be especially related to the synthesis of starch observed in the nutritive tissue of the galls on *P. arboreum*. Furthermore, this enzyme provides substrate (UDP-glucose) for the formation of cell wall polysaccharides (Nolte and Koch 1993; Amor et al. 1995; Salnikov et al. 2003), whose dynamics, together with the carbohydrates metabolism, have crucial roles in the development of gall structure (Formiga et al. 2013; Isaias et al. 2014), and consequently in the survival of the galling herbivore. In terms of cell metabolism, the intralaminar gall on *P. arboreum* functions as a new organ, in a strict continuum with its host tissues, establishing a sink of photoassimilates, which culminate in the accumulation of metabolites involved in its own development and in the feeding activity of the galling herbivore (Rehill and Schultz 2003; Castro et al. 2012). The nutrients can be redirected and compartmentalized into the nutritive tissue by two pathways. The first one is the transport of sucrose, via phloem, from the non-galled portions of the host leaf towards the gall site, where it is promptly metabolized and converted into starch. The conversion of sugars into starch occurs in SuSy dependent via, which produces UDP-glucose as demonstrated by Baroja-Fernandez et al (2009) in tubers of potato. In the second pathway, the sucrose is irreversibly cleaved by the activity of invertases into glucose and fructose, which are used in cell respiration (Koch 2004) and/or in the galling insect`s diet. The invertases are, indeed, the major sucrose degrading enzyme in plants, as demonstrated in *Arabidopsis*, where their low levels affects plant growth (Barrat et al 2009). The force of the sink towards the nutritive tissues of insect galls seems to be maintained by the high cytological metabolism and the dynamics between the activity of SuSy and invertases (Oliveira et al. 2010).

Although there was a high activity of SuSy and invertases in the nutritive tissue of the galls on *P. arboreum*, the potential accumulation of reducing sugars originally detected in the hypodermis of the NGL, and in the adaxial and abaxial gall outer cortices, was suppressed in the nutritive tissue. The non-accumulation of reducing sugars strongly indicates that the sucrose is promptly metabolized by SuSy to starch synthesis at gall site. Also, the sucrose can

be converted to monosaccharides used in cell respiration and growth, or to the galling insect feeding. The increase of cell respiration is corroborated by the accumulation of ROS in the nutritive tissue of the MG on *P. arboreum*.

Due to its direct involvement in insect`s nutrition, a gradient of carbohydrates was expected (Bronner 1992; Oliveira et al. 2010; Oliveira et al. 2014). The non-accumulation of reducing sugars, and the high accumulation of ROS in the nutritive tissue is a novelty, and denote a distinct metabolic pattern for Cecidomyiidae-induced galls, which is yet to be investigated.

Neo-accumulation of lipids in gall site

Lipid droplets were detected in some Cecidomyiidae galls in the Neotropics, such as those of *Aspidosperma spruceanum* (Oliveira et al. 2010), *Copaifera langsdorffii* (Oliveira et al. 2011), and *Marcetia taxifolia* (Ferreira and Isaias 2014). This accumulation has been detected on host plants with the potential for the accumulation of lipids, as is *P. arboreum*. Lipids are referred to as precursors in different routes of plant metabolism (Heldt and Piechulla 2011). They are related to energy storage and mobilization during plant cell differentiation and growth (Begun et al. 2010), as well as gall development (Oliveira et al. 2006; Oliveira et al. 2010; Oliveira et al. 2011; Vechi et al. 2013). In the galls of *P. arboreum*, lipid droplets are detected in the cells of cortical parenchyma, the intrinsic locations in the NGL, which were maintained in the galls. Lipids accumulation in the gall outer cortical cells may confer energy resources for the high rates of cell hypertrophy (Bragança et al. 2015, submitted). On the other hand, the nutritive tissue is a new site of lipids accumulation. This inner compartmentalization of lipids is another metabolic step, with specific enzymatic requirements to be elucidated on *P. arboreum*. These metabolites may represent reserves to be used as a energetic resource for the galling insect`s nutrition and development.

Final considerations

Even though metabolites compartmentalization has been discussed before, this is the first time that the metabolites compartmentalization is sistematically are documented and discussed in terms of the new functionalities, blockage, and neo accumulation in gall sites. In the intralaminar galls on *Piper arboreum*, two metabolites were deviated of their standard compartments: the reducing sugars, expected in the nutritive tissue, but detected only in the outer cortical parenchyma, and the terpenoids blocked in the inner gall tissues. A consequence

of the new sites of accumulation is a deviation of functions. The new site reducing sugar accumulation implies its role in energy providing for the hypertrophy processes as well as their involvement in ROS metabolism. The terpenoids on nutritive cells could intoxicate the galling larvae, and prejudice its development.

The outer compartmentalization of secondary metabolites is a pattern of *P. arboreum* leaves kept at gall sites, where the metabolites can favor the protect the gall against natural enemies. The inner compartmentalization of the activity of two investigated enzymes was herein documented at gall sites and reflects two important metabolic steps of gall development: the sink of photoassimilates and storage of starch mediated by Susy activity, and the high respiratory metabolism with cell growth and divisions mediated by invertases activity. Moreover, this is the second host plant-Cecidomyiidae system where the detection of SuSy activity occurred in the nutritive tissue, which indicates the conversion of sucrose into monossacharides as a cell response to the Cecidomyiidae stimuli.

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Author Contribution Statement

GPB and DCO conducted experiments. GPB, DCO and RMS analized datas. GPB, DCO and RMS wrote the manuscript. All authors read and approved the manuscript.

Conflict of interest

The authors declare that they have no conflict of interest.

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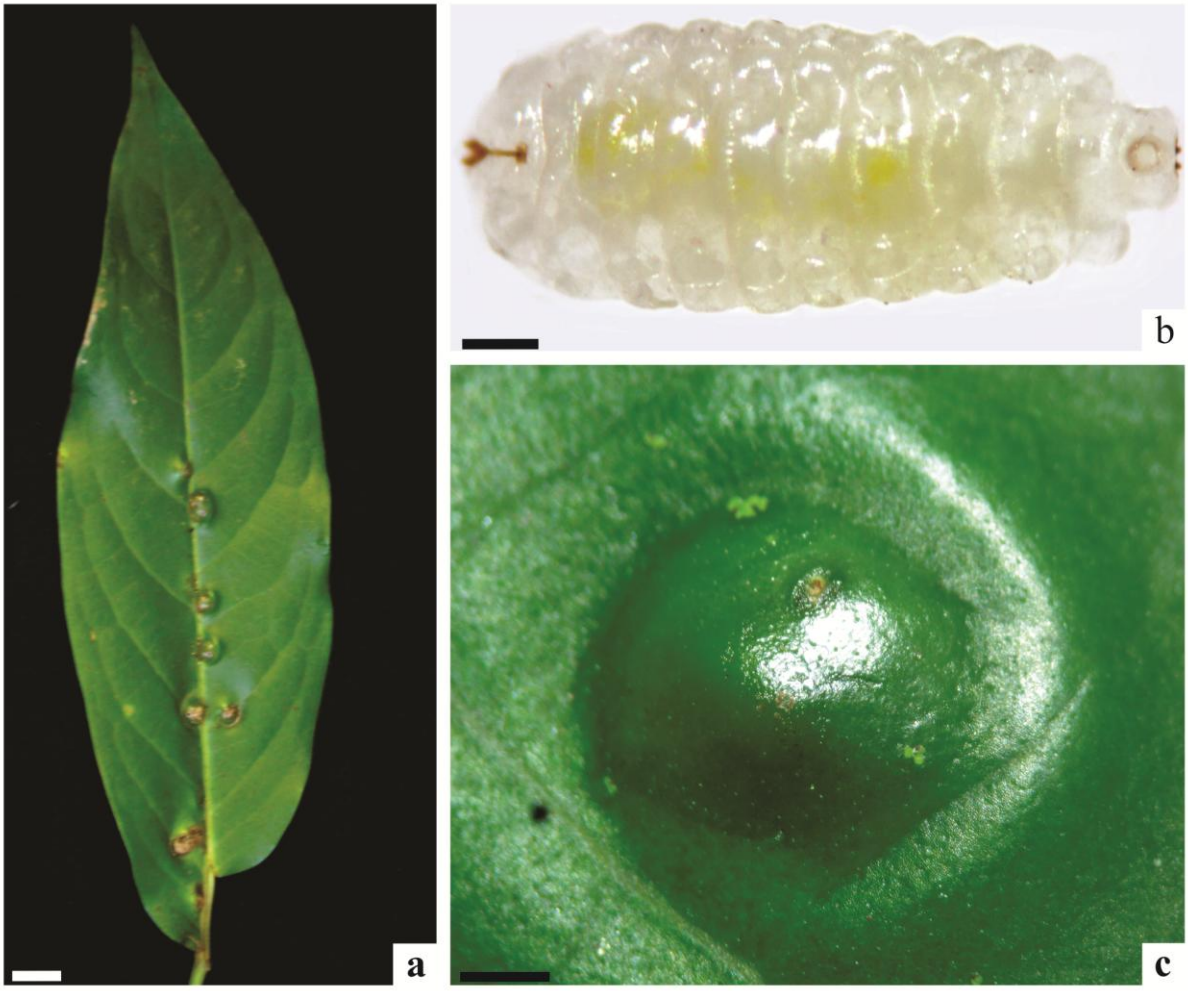
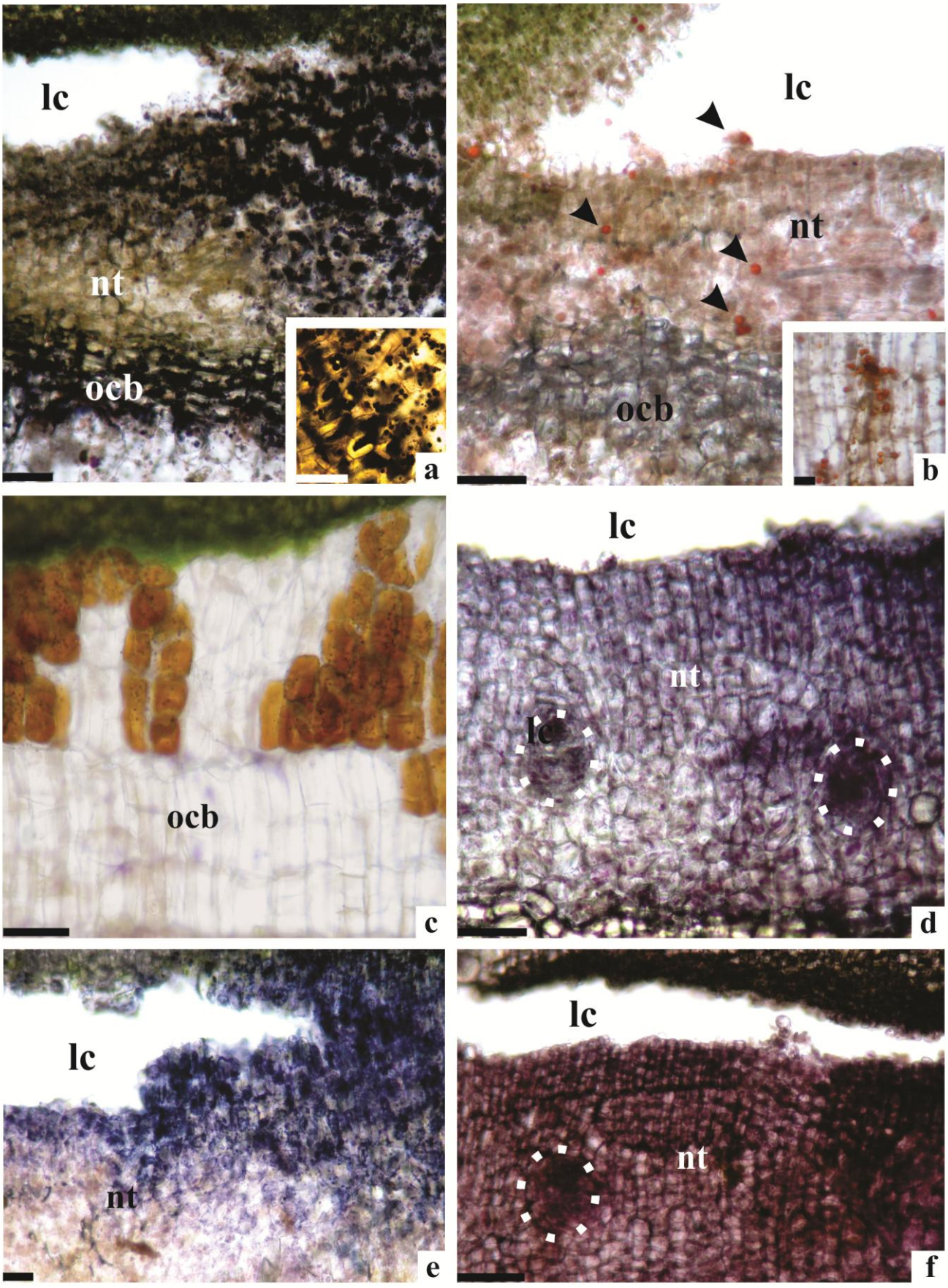


Fig. 1 Morphology of the galls induced by Cecidomyiidae in *Piper arboreum* Aubl. (Piperaceae**). a** Galls viewed by the adaxial surface of a *Piper arboreum* (Piperaceae) leaf. **b** Cecidomyiidae larva. **c** Adaxial surface of the mature gall. Bars **a** = 1cm; **b** = 0,2mm; **c** =1mm

Fig. 2 Histochemical tests in Cecidomyiidae galls on *Piper arboreum* Aubl. (Piperaceae). Primary metabolites and enzymatic activity. **a** Reaction with Lugol's detecting the presence of starch in the nutritive tissue and lignified cells (detail). **b** Sudan red demonstrating the presence of lipid droplets (arrows) in the nutritive tissue and abaxial outer cortex (detail). **c** Reducing sugars evidenced by Fehling`s reagent in the abaxial outer cortex. **d** Bromophenol blue indicating the presence of total proteins in the nutritive tissue. **e** Activity of invertases in the nutritive tissue. **f** Activity of sucrose synthase in the nutritive tissue. **lc** larval chamber; **nt** nutritive tissue; **ocb** abaxial outer cortex; **dotted circle** vascular bundle. Bars **a, c, d, f** = 100µm; **e** = 50µm; **b** = 25µm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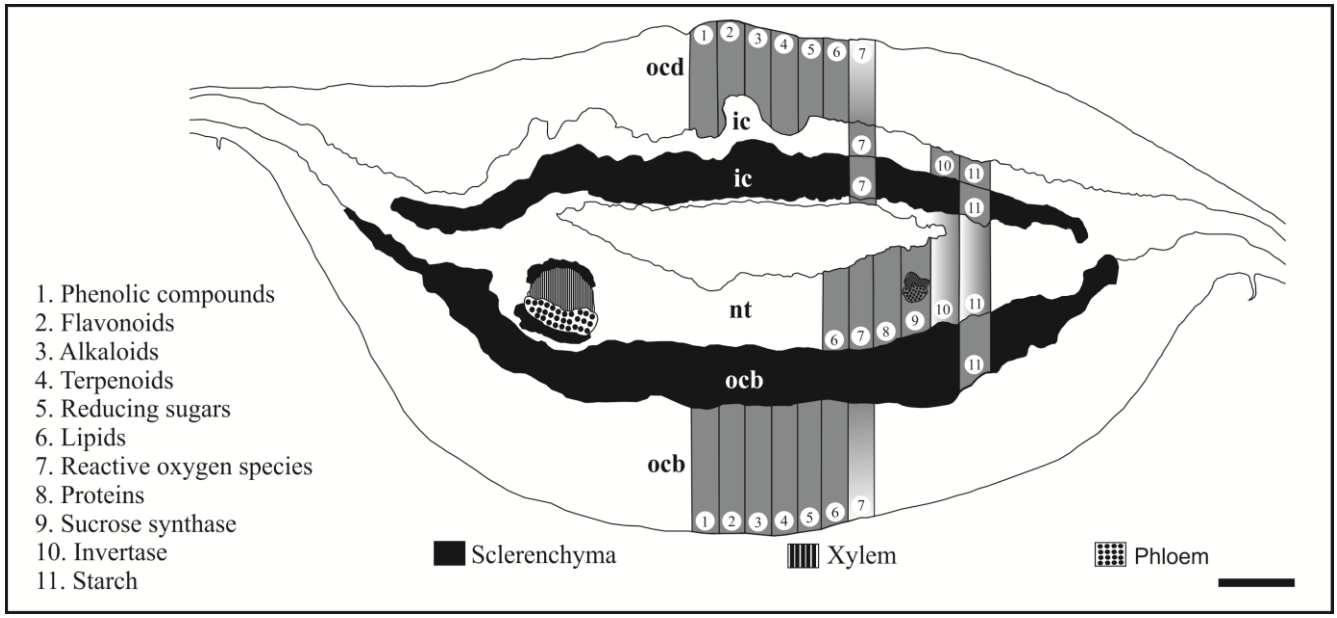


Fig. 3 Diagram of histochemical profile of mature galls (MG) of Cecidomyiidae in *Piper arboreum* Aubl. (Piperaceae). **ocd** adaxial outer cortex; **ic** inner cortex; **nt** nutritive tissue; **ocb** abaxial outer cortex. Fixed coloring is the detection of the compound evenly. Gradient of different color shows intensities of the detection. Bar = 30 µm

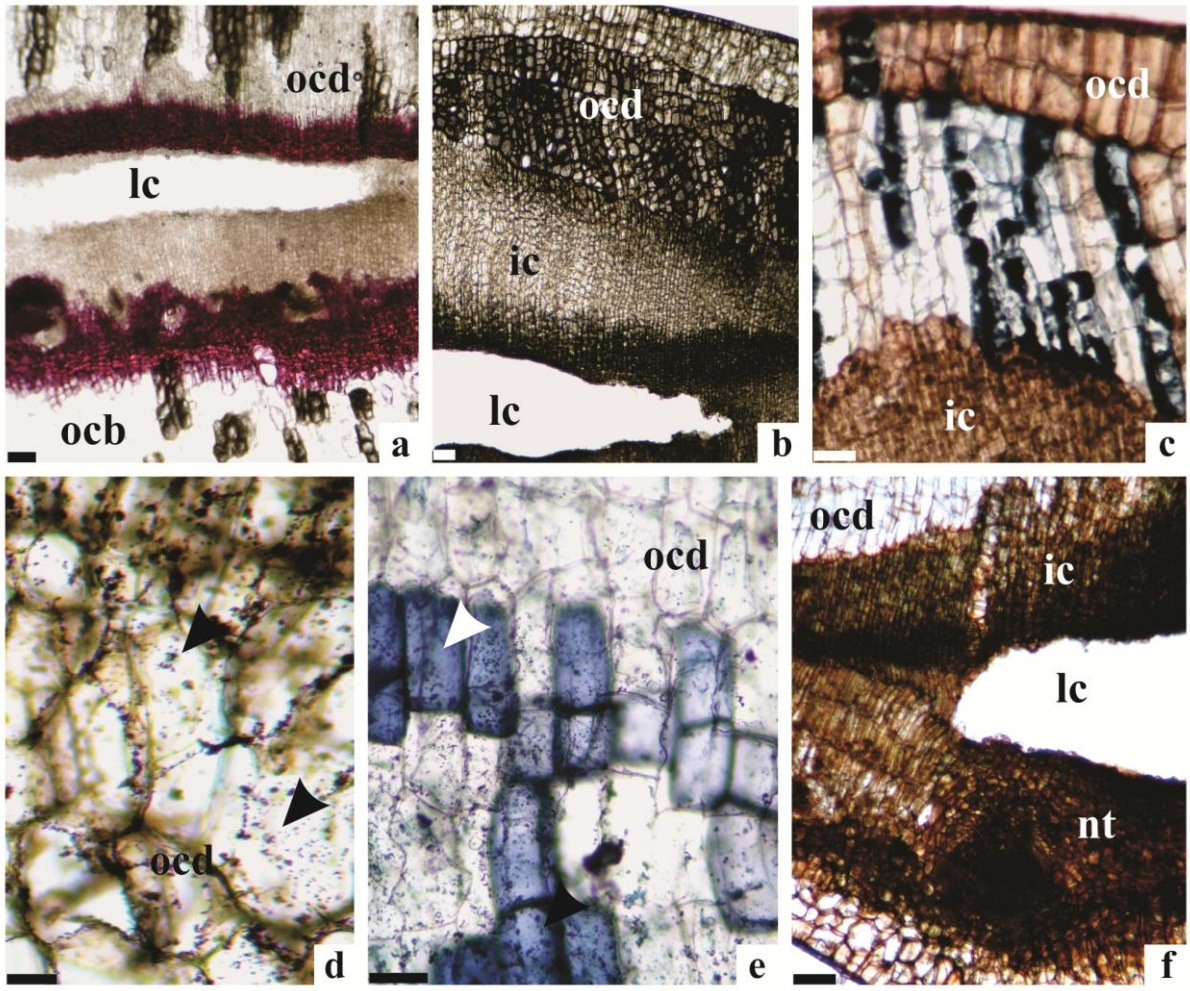
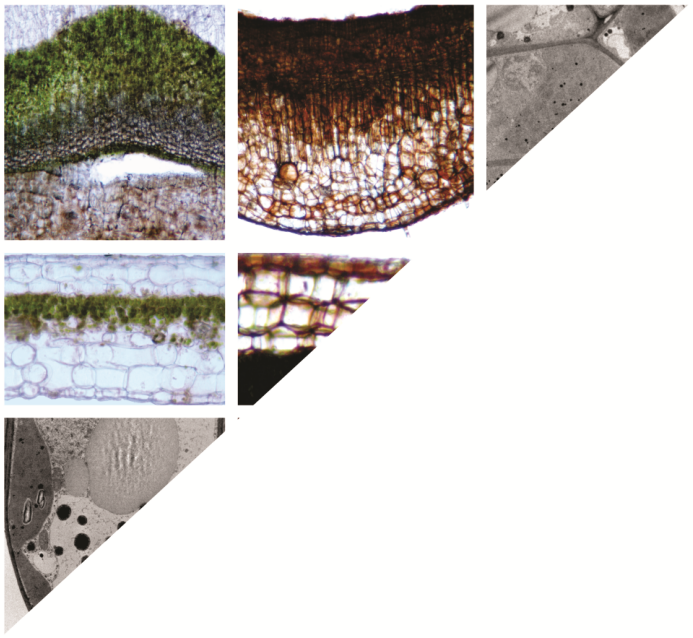
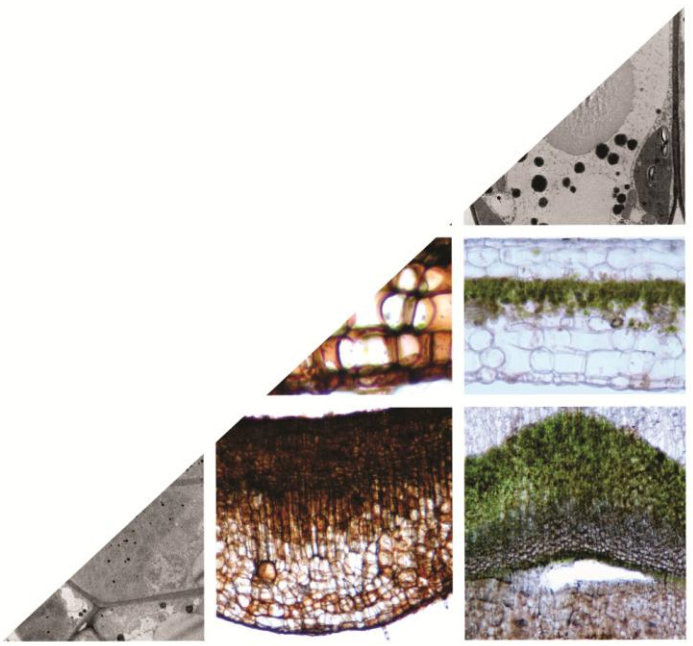


Fig. 4 Histochemical tests in Cecidomyiidae galls on *Piper arboreum* (Piperaceae). Secondary metabolites and Reactive Oxygen Species (ROS). **a** Presence of lignin in the cell walls of the sclerenchymatic sheath. **b** Reaction with ferric chloride evidencing the presence of phenolics in the outer cortex. **c** DMACA revealing flavonoids in outer cortex. **d** Alkaloids in the outer cortex (arrow). **e** NADI detecting terpenoids in outer cortex (arrow). **f** Accumulation of ROS in the outer and inner cortices of the gall. **ocd** adaxial outer cortex; **ic** inner cortex; **nt** nutritive tissue; **lc** larval chamber; **ocb** abaxial outer cortex. Bars **a, b, f** = 200µm; **c, d, e** = 100µm



Capítulo III

Como as células vegetais se protegem dos danos oxidativos no sistema *Piper arboreum* - Cecidomyiidae?



Como as células vegetais se protegem dos danos oxidativos no sistema *Piper arboreum* - Cecidomyiidae?

Resumo

Herbívoros galhadores são capazes de impactar de diferentes formas as células vegetais hospedeiras, alterando seu destino de modo a formar uma nova estrutura, a galha. Recentemente, espécies reativas de oxigênio (EROs) foram propostas como sendo o gatilho para a morfogênese das galhas de insetos desencadeando uma cascata de sinalizações celulares. Por outro lado, as EROs também estão associadas ao estresse oxidativo responsável por danos celulares. O equilíbrio entre estes dois papeis desempenhados pelas EROs é vital para o sucesso do estabelecimento do galhador em meio as células vegetais. No presente estudo, analisa-se comparativamente, as respostas estruturais e ultraestruturais de folhas não galhadas e galhas maduras de Diptera: Cecidomyiidae em *Piper arboreum* Aubl. (Piperaceae) e seu desempenho fotossintético, visando relacioná-las a localização de EROs. O acúmulo de EROs foi estabelecido de modo centrífugo alterando o perfil citológico nas diferentes camadas celulares da galha. Plastoglóbulos e corpos multivesiculares são caracteres conservativos nas galhas, denotando que características da planta hospedeira juntamente com as alterações estruturais foram essenciais para garantir a similaridade entre taxas fotossintéticas de folhas não galhadas e galhas maduras sob estresse oxidativo.

Palavras chave: Estresse, rendimento fotossintético, galhas, perfil citológico

Abstract

Galling herbivores are able to differently impact their host plant cells, changing their destination to form a new structure, the gall. Recently, reactive oxygen species (ROS) have been proposed as the trigger for the morphogenesis of insect gall triggering a cascade of cellular signals. Moreover, ROS are also associated with oxidative stress responsible for cellular damage. The balance between these two roles of ROS is vital for the success of gall establishment in plant cells. Currently, we comparatively analyze the structural and ultrastructural responses of non-galled leaves and mature galls of Diptera: Cecidomyiidae on *Piper arboreum* Aubl. (Piperaceae), and its photosynthetic performance, in order to relate them to the ROS location. ROS accumulation established a centrifugal gradient, and changed the cytological profile in the different gall cell layers. Plastoglobules and multivesicular bodies are conservative character maintained in galls, which indicates that host plant characteristics together with structural changes were essential to ensure the similarity between the photosynthetic profile of non galled leaves and mature galls under oxidative stress.

Key words: Stress, photosynthetic performance, galls, cytological profile

Introdução

Ao induzir alterações nos tecidos vegetais durante a formação do novo órgão, a galha, os galhadores interferem em diversos mecanismos da planta hospedeira (Fay et al 1993; Larson 1998; Oliveira et al 2011a; Isaias et al 2011; Castro et al 2012; Samsone et al 2012). No nível fisiológico, a interação inseto-planta pode influenciar o desempenho fotossintético (Fernandes et al 2010; Oliveira et al 2011a; Castro et al 2012; Oliveira et al 2014). Tal influência pode ser neutra, como nas galhas de Cecidomyiidae em *Aspidosperma* spp. (Oliveira et al 2011a), positiva, como no sistema *Silphium integrifolium*–Cynipidae (Fay et al 1993) ou negativa, como nas galhas de Cecidomyiidae em *Copaifera langsdorffi* (Castro et al 2012) e de *Colopha compressa* em *Ulmus laevis* (Samsone et al 2012). Nos casos em que há influência negativa, as alterações fotossintéticas observadas nas galhas podem ser devido a demanda energética necessária para manter a nova estrutura e assegurar a alimentação do indutor (Castro et al 2012). Estudos recentes nos Neotrópicos têm demonstrado que o padrão morfogenético das galhas e sua forma final influencia o rendimento fotossintético, sendo este mantido em galhas intralaminares que formam um contínuo com a folha não galhada (Oliveira et al 2011a), enquanto que em galhas extralaminares, há redução das taxas fotossintéticas quando comparadas às folhas hospedeiras (Castro et al 2012).

No nível bioquímico e citológico, a atividade alimentar e a alta atividade metabólica em galhas intensifica a produção de espécies reativas de oxigênio (EROs) (Carneiro et al 2014; Oliveira et al 2014; Bragança et al 2015a submetido), especialmente ao redor da câmara larval (Bragança et al 2015a submetido). Estas EROs são produzidas durante diferentes processos celulares, especialmente em organelas como cloroplastos, peroxissomos e mitocôndrias(Bell et al 2009; Liu et al 2013; Møller et al 2007), e são acumuladas tanto como resposta de defesa (Karpinski et al 1999; Liu et al 2013) ou de morte celular (Bell et al 2009; Shetty et al 2008), quanto como gatilho formação de gradientes citológicos e histoquímicos nas galhas (Oliveira et al 2010; Oliveira e Isaias 2010; Bragança et al 2015a, submetido). As EROs podem ainda interferir no próprio desenho estrutural da galha (Oliveira et al 2014). Em conseqüência deste espectro funcional, o acúmulo excessivo das EROs precisa, eventualmente, ser controlado por mecanismos celulares (Oliveira et al 2010; Oliveira e Isaias 2010; Liu et al 2013), tais como a produção de enzimas antioxidantes (Møller et al 2007) e de compostos fenólicos e flavonoides (Blokhina et al 2003), a reciclagem do sistema de endomembranas (Levine 2002; An et al 2006a; An et al 2006b; Caneiro et al 2014b) e a formação de plastoglóbulos (Austin et al 2006, Oliveira et al 2011a; Oliveira et al 2011b).

Baseado nestas premissas, a galha intralaminar lenticular de *Piper arboreum* Aubl. (Piperaceae) (Bragança et al 2015b, submetido) foi utilizada como modelo para investigar se o acúmulo de EROs está ligado às alterações fotossintéticas induzidas pelo galhador e se há caracteres citológicos que possam reduzir os possíveis danos desencadeados pelo estresse oxidativo. O estudo objetiva responder as seguintes questões: (I) O estresse oxidativo, detectado pelo acúmulo de EROs, está ligado a formação de um gradiente citológico nas galhas em *P. arboreum*? (II) As células de *P. arboreum* têm potencial para reduzir o impacto do estresse oxidativo gerado pela presença do galhador? (III) A posição intralaminar da galha favorece a manutenção de um perfil fotossintético similar entre as galhas e as folhas não galhadas?

Materiais e métodos

Área de estudo. O presente estudo foi realizado no Parque Estadual Serra Verde (PESV), com 142,02 hectares de áreas dos biomas Cerrado e Mata atlântica, localizado na região norte de Belo Horizonte. O clima é categorizado como semi-úmido, com precipitações significativas nos meses de outubro-março. Anualmente, as taxas pluviométricas são de 190 mm a 1.515 mm, com temperatura anual variando entre 15°C - 21,1° (IEF, 2010). As análises foram realizadas em indivíduos localizados na Trilha da Mata (Portugal-Santana e Isaias 2014) no período de 2013 – 2014.

Análise histométrica. Medidas da área do parênquima clorofiliano em folhas não galhadas (n=30) e galhas maduras (n=30) foram tomadas utilizando o programa Axion Vision 4.9.1®, em seções transversais obtidas a mão livre com auxílio de lâmina de barbear.

Análises histoquímicas. Folhas não galhadas (FNG) e galhas maduras (GM) foram seccionadas transversalmente e submetidas a reação com 3,3'-diaminobenzidina (Sigma®-D4293) 0,5% durante 15-60 minutos (Rosseti e Bonatti, 2001) no escuro, posteriormente lavadas em água, montadas entre lâmina e lamínula e fotografadas em microsópio óptico (Zeiss Primo Star ®) com câmera digital (Canon Power Shot A 630®).

Análises Fotossintéticas

Conteúdos de pigmentos. Para determinação dos pigmentos fotossintéticos, discos de 1cm^2 de folhas não galhadas (n=15) e galhas maduras (n=15) foram cortados e pesados em balança analítica (Bioprecisa® FA2104N) para obtenção da massa fresca. Os pigmentos foram

extraídos em 5mL de dimetilsulfóxido (DMSO) (Sigma®-472301) mantidos no escuro durante 24 horas (Porra 2002) e posteriormente quantificados por espectrofotometria (Bel Photonics SP 2000 UV) a 480 nm, 649 nm e 665 nm. O conteúdo de carotenóides e clorofilas *a* e *b* foi quantificado utilizando as equações propostas por Lichtenthaler e Wellburn (1983).

Fluorescência da clorofila a. Medidas de fluorêscência da clorofila a em FNG e GM foram obtidas *in situ* em maio de 2014, em ambiente sombreado, utilizando um medidor de fluorêscencia modulada (MINI PAM, Walz, Elffelthich, Alemanha). As análises foram realizadas em 2 folhas e 2 galhas de 3 indivíduos diferentes. A determinação do rendimento quântico potencial do fotossistema II (Fv/Fm) foi realizada no pre-daum e ao meio-dia em amostras previamente adaptadas a 30 minutos de escuro (Genty et al. 1989). A performance da fotossíntese em função de níveis gradativos de luz foi obtida a partir de curvas de luz com duração de 4 minutos em oito fases de 30 segundos cada. Foi aplicado um pulso de saturação ao término de cada nível de luz. Por meio destas medidas, foi obtida a taxa máxima de transporte de elétrons (ETR_{MAX}) sob saturação da radiação fotossinteticamente ativa (PARSAT), e PAR em ½ de ERT_{MAX} e PAR 0.9 ETR_{MAX} (Rascher et al 2000). A taxa aparente de transporte de elétrons (ETR) foi mensurada através da seguinte equação: $ETR= 0{,}5.\ (\Delta F/F_m)$ x PPF x 0,84, onde 0,5 é considerado a energia de excitação entre os dois fotossistemas, PPF é o fluxo incidente de fótons e 0,84 é a taxa de luz incidente absorvida (Krall e Edwards, 1992; Schreiber et al. 1985).

Para obtenção dos valores de temperatura (°C), luminosidade ($\mu mol.m^{-2}\ s^{-1)}$ e umidade, sensores foram acoplados ao Data Logger modelo LI 1400 - LI - COR, durante o período da análise dos parâmetros fotossintéticos (Araújo et al 2010).

Análises estatísticas. Para dados que apresentaram normalidade e homocedasticidade foi realizado o teste T ($\alpha \leq 0{,}05$) para verificar diferenças entre os valores obtidos para FNG e GM. Dados que não atendiam os pressupostos para análises paramétricas foram transformados em escala logarítmica ou comparados por meio do teste não paramétrico Mann Whitney, com auxílio dos programas Sigma Stat® e Graphprism 5.0®.

Análises citológicas. Fragmentos de folhas não galhadas e galhas maduras foram fixados em Karnovsky (5% glutaraldehyde, 4% paraformaldehyde in phosphate buffer 0.1M, pH 7.0) (Karnovsky, 1965), pós-fixados em tetróxido de ósmio 1%, desidratados em série etanólica

(Johansen 1940) e infiltrados em Spurr® (Lufth 1961). As seções foram realizadas utilizando o ultramicrotomo Leica® UC6, contrastadas em acetato de uranila e citrato de chumbo (Reynolds 1963) e analisados através de microscopia eletrônica de transmissão (Tecnai G2-20 - SuperTwin FEI - 200 kV).

Resultados

As galhas intralaminares de *P. arboreum* são induzidas por uma espécie não identificada de Diptera: Cecidomyiidae em regiões próximas as nervuras secundária e terciária (Fig.1A). As galhas apresentam somente uma câmara larval que abriga apenas um indutor (Fig. 1B). Em GM, a região fotossintetizante forma o córtex interno da face adaxial, em contínuo com a região não galhada da folha (Fig. 1C). Nas FNG, o tecido clorofiliano é dividido em parênquima paliçádico e lacunoso (Fig. 1D). A porcentagem de tecido clorofiliano diminui 17% em relação à folha não galhada (Fig. 2).

Detecção de espécies reativas de oxigênio (ROS) . A concentração de EROs foi visualmente alta nas células epidérmicas, e nos parênquimas paliçádico e lacunoso das FNG. Nas células hipodérmicas, a concentração de EROs foi aparentemente moderada (Fig.3A), enquanto que na região da nervura central, o acúmulo alto foi observado no colênquima e feixes vasculares (Fig. 3B). Em GM, a maior concentração de EROs foi detectada nas células do tecido nutritivo diminuindo em direção as camadas externas da galha (Fig. 3C,D).

Conteúdo de pigmentos. A razão de clorofilas *a*/*b* foi maior em GM do que em FNG, enquanto o conteúdo de carotenoides não diferiu estatisticamente entre os grupos. O conteúdo de clorofilas totais e a razão entre clorofilas totais/carotenoides foi maior em FNG do que GM (Tabela 1).

Fluorescência da clorofila. A temperatura no local e no momento analisado variou de 13°C a 23°C. A umidade relativa do ar variou de 76% a 89% e, a luminosidade teve média de 15,11 ± 11,51 $\mu mol.m^{-2}.s^{-1}$. No mês de maio de 2014, a pluviosidade em Belo Horizonte foi de aproximadamente 20mm (INMET). Os valores de rendimento quântico potencial (Fv/Fm) ao amanhecer foram de 0,79 ± 0,01 e de 0,79 ± 0,03 para FNG e GM, respectivamente. Ao meio dia, estes valores foram de 0,83 ± 0,05 para FNG e 0,76 ± 0,07 para GM, não diferindo estatisticamente entre os dois grupos e em ambos horários. Não houve diferença estatística entre valores de ETR_{MAX}, PAR½ ETR_{MAX} e PAR 0.9 ETR_{MAX} tanto para FNG quanto para GM (Tabela 2).

Análise citológica. Células epidérmicas das FNG apresentam núcleo e nucléolo conspícuos, grandes vacúolos ocupam a parte central da célula e o citoplasma adjacente à parede celular, com a presença de corpos multivesiculares (Fig. 4A,B). As células hipodérmicas apresentam paredes celulares polilameladas, grandes vacúolos com compostos fenólicos, citoplasma periférico com mitocôndrias e cloroplastos contendo plastoglóbulos, e lamelação organizada dos tilacóides (Fig. 4C). Celulas clorofilianas são compostas por núcleo e nucléolo evidentes, citoplasma denso, peroxissomos, mitocôndrias associadas a plastídios, grandes vacúolos com inclusões osmiofílicas e compostos fenólicos, numerosos cloroplastos com lamelação organizada de tilacóides e plastogóbulos associados (Fig. 4D-F). Em todas as regiões da FNG, ocorre a formação de corpos multivesiculares (Fig. 4A e G).

Nas GM, a região do córtex externo é composto por células de paredes polilamelares, plastídios contendo amido e grandes vacúolos com compostos fenólicos (Fig. 5A). A região fotossintetizante do córtex interno apresenta vacúolos com inclusões osmiofílicas, citoplasma denso com numerosos cloroplastos com lamelação e grana evidentes, plastoglóbulos e grãos de amido. Espaços intercelulares foram observados (Fig. 5B-C). No córtex interno lignificado, as células têm vacúolos pequenos e numerosos, contendo fenólicos. No citoplasma adjacente a parede celular, plastídios apresentam desorganização do sistema de tilacoides e acúmulo de grãos de amido (Fig. 5D-E). As células nutritivas apresentam paredes finas, núcleo e nucléolo evidentes, citoplasma denso, complexo de Golgi e maior número de plastídios e mitocôndrias (Fig. 6A-C). Cloroplastos acumulando amido e plastoglóbulos parecem estar em processo de divisão binária e não apresentam organização do sistema tilacoides (Fig. 6D). Na região perivascular, as células apresentam núcleo evidente, numerosas mitocôndrias e complexo de Golgi (Fig. 6E-F). As células nutritivas ao redor da câmara larval apresentam paredes celulares finas e sinuosas (Fig. 6G). Em todo tecido nutritivo ocorre a formação de corpos multivesiculares e lamelares associados a membrana plasmática (Fig. 6H-I).

Discussão

O acúmulo de espécies reativas de oxigênio (EROs) evidenciado nas galhas de *P. arboreum* foi reportado anteriormente em diversas galhas de insetos (Oliveira et al 2010; Oliveira e Isaias 2010; Isaias et al 2011; Ferreira e Isaias 2013). Este acúmulo parece estar relacionado ao estresse oxidativo gerado pela atividade metabólica e cecidogênica (Oliveira et al 2014), sendo uma das primeiras respostas de hipersensibilidade (Isaias e Oliveira 2012; Isaias et al 2014). Mais do que uma tentativa de bloquear o estabelecimento da galha, o

acúmulo de EROs desencadeia uma cascata de respostas celulares, tais como a formação de gradientes citológicos e histoquímicos em galhas induzidas por diferentes taxa (Oliveira e Isaias 2010) e a determinação do design final da galha (Oliveira et al 2014). A formação dos gradientes citológicos e histoquímicos ocorre concomitantemente ao gradiente centrífugo de acúmulo de EROs como observado nas galhas de *Aspidosperma spruceanum* (Oliveira et al 2010), *Aspidosperma australe* (Oliveira e Isaias 2010) e *Piper arboreum*.

Nas FNG de *P. arboreum*, o maior acúmulo de EROs nas células da epiderme, do colênquima e do parênquima clorofiliano pode estar relacionado ao metabolismo celular e fotossintético, conforme proposto por Møller et al (2007). Caracteres referentes a minimização de impactos oxidativos presentes nas folhas não galhadas de *Piper arboreum* foram conservativos nas galhas, sendo fundamentais para o equilíbrio entre os danos causados pelo acúmulo excessivo de EROs e a alta atividade metabólica nos tecidos da galha. A minimização dos impactos pode se dar pelo acúmulo de compostos fenólicos evidenciados nas células da hipoderme das FNG e no córtex externo de GM, uma vez que tais compostos podem agir como potentes antioxidantes diminuindo e restringindo danos oxidativos (Detoni et al 2010; Detoni et al 2011), corpos multivesiculares e lamelares atuam juntamente com o complexo de Golgi e o retículo endoplasmático reestruturando o sistema de endomembranas (Staehelin 1997; Tanaka et al 2000; Levine 2002; Evert 2006; Carneiro e Isaias 2014) e prevenindo a morte celular, especialmente durante o estresse oxidativo (Levine et al 2002; An et al 2006a; An et al 2006b). No tecido nutritivo das galhas em *P. arboreum*, local de intenso acúmulo de EROs devido à alta atividade celular e alimentação do galhador, os caracteres citológicos indicam a reciclagem do sistema de membranas. Esta reciclagem pode ter minimizado os impactos gerados pela ação de EROs, assim como observado em galhas de *Northotrioza myrtoidis* em *Psidium myrtoides* (Carneiro e Isaias 2014).

Um dos sintomas do estresse oxidativo é a alteração na estrutura e consequentemente na funcionalidade dos cloroplastos (Liu et al 2013), que aparecem em divisão binária em células nutritivas da galha de *P. arboreum*. Cloroplastos em divisão são sintomas da hiperplasia desencadeada pelo estímulo do galhador, uma vez que o processo de sua fissão binária acompanha o crescimento dos tecidos e órgãos (Glynn et al 2007). Ademais, alterações estruturais, podem refletir prejuízos ao rendimento fotossintético (Austin et al 2006). A desorganização das membranas dos tilacoides observada nas células clorofilianasdas folhas não galhadas e no tecido nutritivo e nas células lignificadas das galhas indica o intenso estresse oxidativo desta região, assim como evidenciado nos cloroplastos do tecido de reserva

nas galhas em *Copaifera langsdorfii* (Oliveira et al 2011b). Uma estratégia citológica que minimiza o dano oxidativo é a diferenciação de plastoglóbulos, os quais atuam na síntese de carotenoides, tocoferol (Vitamina E), terpenoides e plastoglobulinas que estão associadas a reestruturação das membranas dos tilacoides sob estresse (Austin et al 2006; Ytteberg et al 2006; Oliveira et al 2011a). Nas galhas de *Aspidosperma* spp., a presença de plastoglóbulos representou uma defesa ao estresse oxidativo causado pelo galhador (Oliveira et al 2011a). No entanto, nas galhas em *P. arboreum*, assim como em C. *langsdorfii* (Oliveira et al 2011b), a presença dos plastoglóbulos constitui um caráter conservativo. A manutenção destes corpúsculos nas galhas pode ter favorecido a integridade do sistema de membranas dos tilacoides, influenciando positivamente na manutenção do rendimento fotossintético similar entre FNG e GM, avaliado por meio das medições de fluorescência da clorofila. Diversas características foram fundamentais para manter as similaridades deste rendimento. Os cloroplastos localizados nas células da região fotossintetizante da galha, do córtex externo e da epiderme não apresentaram alterações nos granas dos tilacoides, evidenciando que a distância entre o sítio de alimentação, local de intenso estresse e a região fotossinteticamente ativa, foi importante para diminuir os impactos ultraestruturais causados pelo acúmulo de EROs (Oliveira et al 2011a). Nas galhas em *C. langsforffi* (Castro et al 2012) e *Psidium myrtoides* (Carneiro et al 2014), a redução no conteúdo de pingmentos parece influenciar o desempenho fotossintético. Face a redução da área fotossinteticamente ativa nas galhas em *P. arboreum*, a conservação similar dos carotenoides, representa mecanismo de proteção que culmina por garantir a similaridade no rendimento fotossintético entre FNG e GM. Embora tenha como função primária ser acessório à fotossíntese, os carotenoides também atuam na eliminação dos radicais livres (Adams-Demig et al 1996) devido a sua função fotoprotetora, contribuindo para a integridade do fotossistema II (Adams-Demig et al 1996).

Além dos caracteres subcelulares, a forma das galhas, também pode influenciar significativamente o perfil fotossintético (Oliveira et al 2011a; Castro et al 2012; Carneiro et al 2014). O design lenticular intralaminar das galhas de *P. arboreum* permite a continuidade da região fotossintetizante do córtex interno das galhas com as regiões não galhadas da folha. Esta continuidade parece ser essencial para conservar as taxas fotossintéticas similares entre FNG e GM, mesmo diante da redução na porcentagem de tecido fotossintetizante nas galhas, tal como observado por Oliveira et al (2011a) em galhas de *Aspidosperma australe* e *Aspidosperma spruceanum*.

Considerações finais

Em diferentes sistemas planta hospedeira-herbívoro galhador, assim como em *Piper arboreum*-Cecidomyiidae, o estímulo do indutor gerou um gradiente centrífugo de espécies reativas de oxigênio, concomitante ao campo cecidogenético, que induziu respostas citológicas nas diferentes camadas de tecidos. Estas respostas foram fundamentais para garantir o metabolismo celular e o desenvolvimento da galha. A presença de plastoglóbulos e de corpos multivesiculares configuram caracteres foliares conservativos nas galhas denotando que características da planta hospedeira foram essenciais para garantir a homeostase entre FNG e GM mesmo sob condições de acúmulo de EROs e consequente estresse oxidativo. O design estrutural da galha assim como a distância entre o local de maior acúmulo de EROs e a região fotossintéticamente ativa foram também determinantes na manutenção do perfil fotossintético similar entre FNG e GM.

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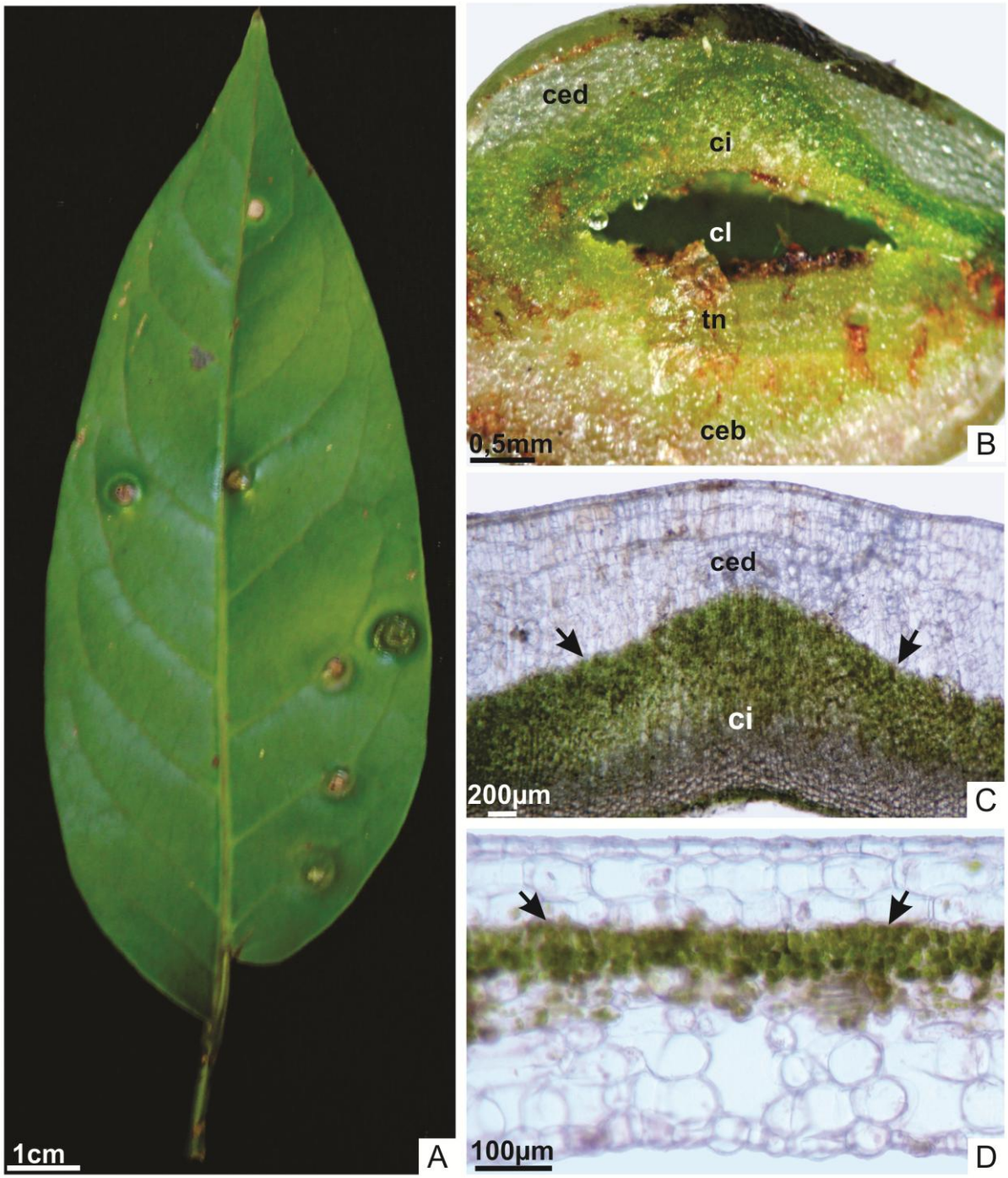

Figura 1: Características morfológica e anatômica de folha não galhada (FNG) e galha madura (GM) de Cecidomyiidae em *Piper arboreum* Aubl. (Piperaceae). **A:** Face adaxial da folha com galhas. **B**: Seção transversal da GM evienciando córtex externo adaxial, córtex interno, câmara larval, e córtex externo abaxial **C:** Seção transversal da GM evidenciando tecido clorofiliano localizado no córtex interno (setas). **D:** Seção transversal da FNG evidenciando a posição central do tecido clorofiliano (setas). **ci:** córtex interno; **ced:** córtex externo adaxial.

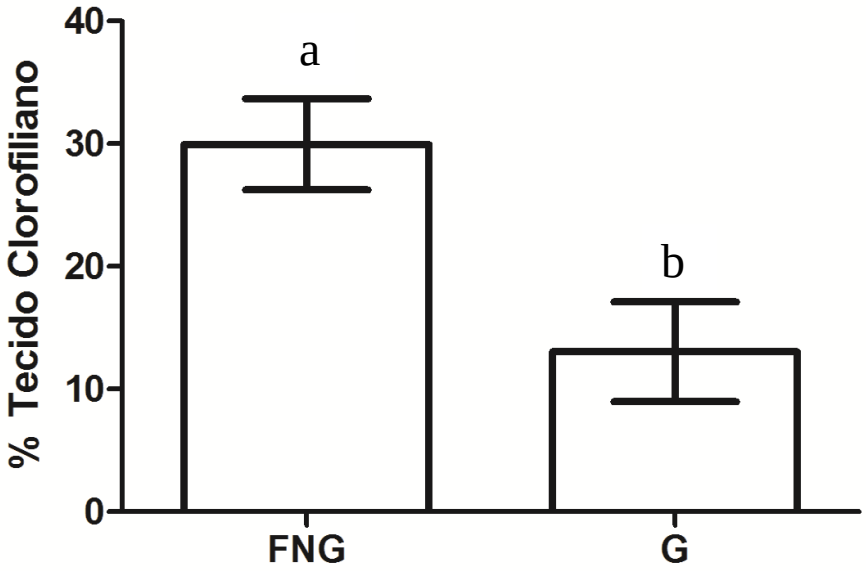

Figura 2: Área ocupada por tecido clorofiliano em folhas não galhadas (FNG) e em galhas maduras (GM) de Cecidomyiidae em *Piper arboreum* (Piperaceae). Barras seguidas por letras diferentes diferem significativamente entre si pelo Teste T ($\alpha \leq 0,05$). (n = 30)

Figura 3: Detecção de espécies reativas de oxigênio (EROs) em folhas não galhadas e galhas de Cecidomyiidae em *Piper arboreum* Aubl. (Piperaceae). A-D**:** Seções transversais. **A:** Folha não galhada evidenciando intenso acúmulo no parênquima paliçádico (pp) e parênquima lacunoso (pl) **B:** Nervura mediana revelando acúmulo intenso de EROs em células do colênquima (col) e dos feixes vasculares (círculo pontilhado) C - D: Galha madura. **C:** Elevada concentração de ROS em células nutritivas (tn), decrescendo em direção ao córtex externo abaxial da galha (ceb). **D:** Alta concentração de ROS na região fotossintetizante da galha madura (ci) , diminuindo em direção ao córtex externo adaxial (ceb). **cl:** câmara larval; pl: parênquima paliçadico; pp: parênquima lacunoso; **hp:** hipoderme; **tr:** tricoma;

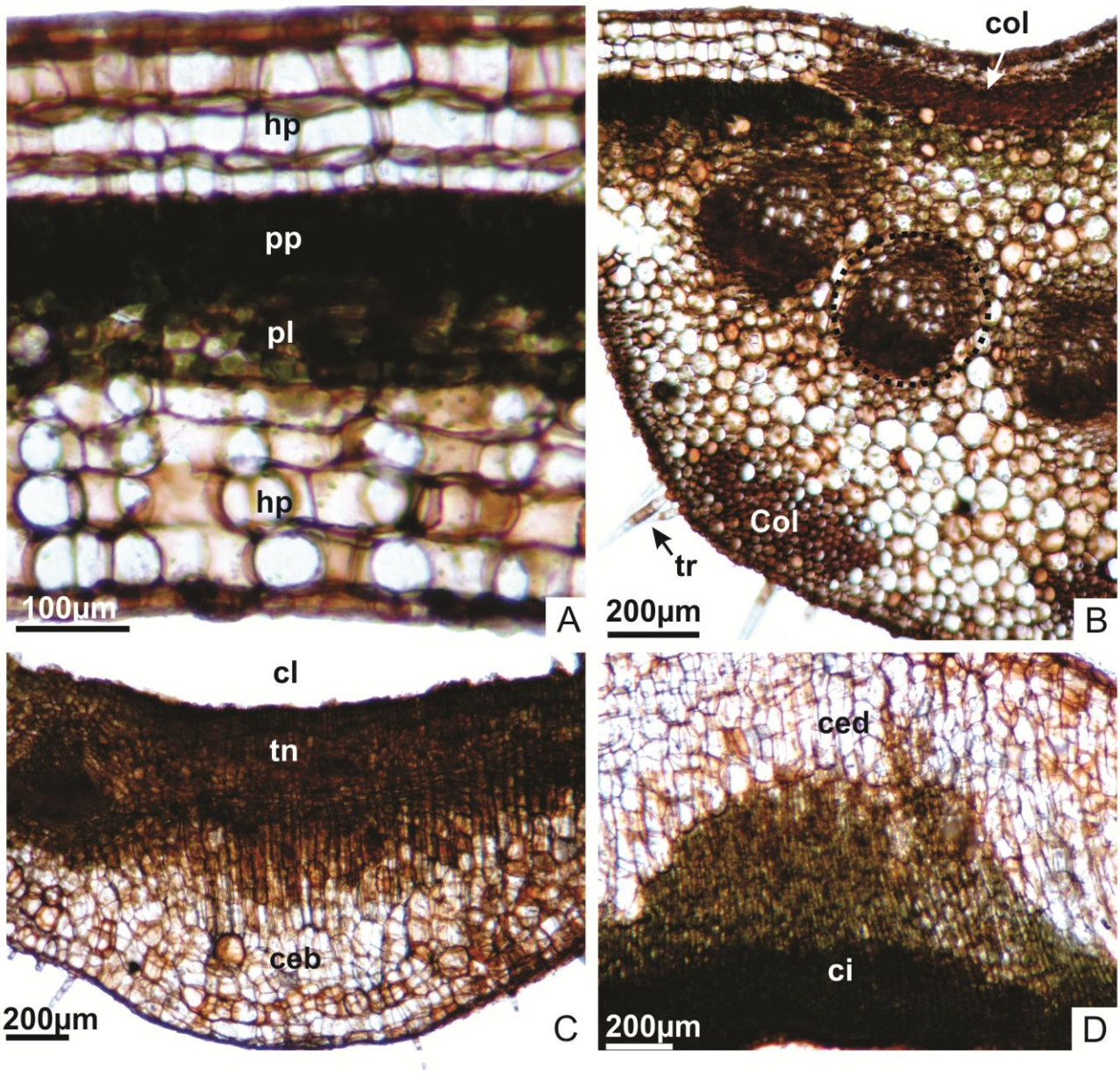
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Tabela 1: Conteúdo de clorofilas *a,b* e carotenoides de folhas não galhadas (FNG) e galhas maduras (GM) de Cecidomyiidae em *Piper arboreum* (Piperaceae).

	Clorofilas totais (mg g^{-1} MF)	Carotenoides (mg g^{-1} MF)	Clorofila *a/b*	Total cl/carot
FNG	9,67±1,82a	4,89±1,28a	2,45±0,16a	2,01±0,21a
GM	5,19±1,82b	4,93±0,50a	2,49±1,03b	1,17±0,09b
p	p<0,0001	p= 0,313	p= 0,020	p<0,0001

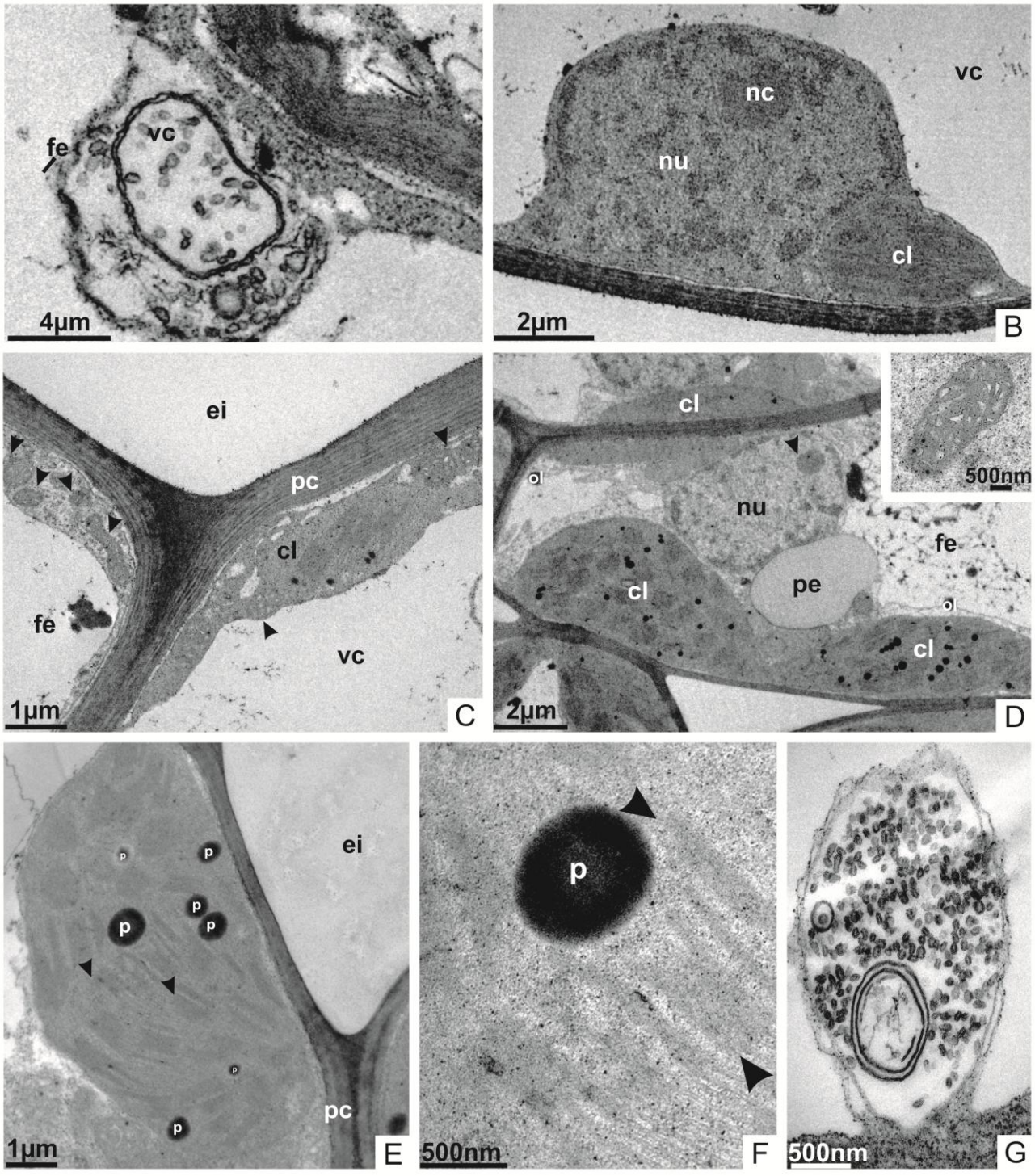
Médias seguidas por mesmas letras não são estatisticamente diferentes entre si pelo teste T ou Mann Whitney ($\alpha \leq 0,05$). (n = 15)

Tabela 2: Taxa relativa de transporte de elétrons (ETR) calculado através de (ETR_{MAX}), PAR em ½ de ERT_{MAX} e PAR 0.9 ETR_{MAX} nas folhas não galhadas (FNG) e galhas maduras (GM) de Cecidomyiidae em *Piper arboreum* (Piperaceae).

	$\frac{ETR_{MAX}}{(\mu mol\ m^{-2}\ s^{-1})}$	$\frac{PAR\ 1/2\ ETR_{MAX}}{(\mu mol\ m^{-2}\ s^{-1})}$	$\frac{PAR\ 0.9\ ETR_{MAX}}{(\mu mol\ m^{-2}\ s^{-1})}$
FNG	26,7±12,0a	77,3±40,8a	284,6±150,4a
GM	37,9±12,9a	75,7±13,9a	284,6±42,3a
p	p=0,353	p=0,954	p=0,999

Médias seguidas por letras iguais não diferem entre si pelo teste T ou Mann Whitney ($\alpha \leq 0,05$). (n=3)

Figura 4: Células da folha não galhada de *Piper arboreum* (Piperaceae) Aubl. (Piperaceae) em seção transversal. **A:** Células epidérmicas contendo corpo multivesicular **B:** Núcleo (nu), nucléolo (nc) e cloroplasto (cl) de célula epidérmica. **C:** Célula hipodérmica com parece celular polilamelar (pc), grandes vacúolos (vc) com presença de compostos fenólicos (fe). Citoplasma periférico com mitocôndrias (setas) e cloroplastos (cl) D-G: Células do mesofilo. **D:** Células com núcleo (nu) e nucléolo (seta) evidentes, acúmulo de fenólicos, peroxissomos (pe), inclusões osmiofílicas, cloroplastos com lamelação organizada e plastoglóbulos (p). Detalhe de uma mitocôndria. **E:** Célula com cloroplasto com plastoglóbulos (p) e espaço intercelular (ei). **F:** Detalhe da lamelação de tilacoides do cloroplasto (seta) e plastoglóbulo (p). **G:** Corpo multivesicular associado a membrana plasmática.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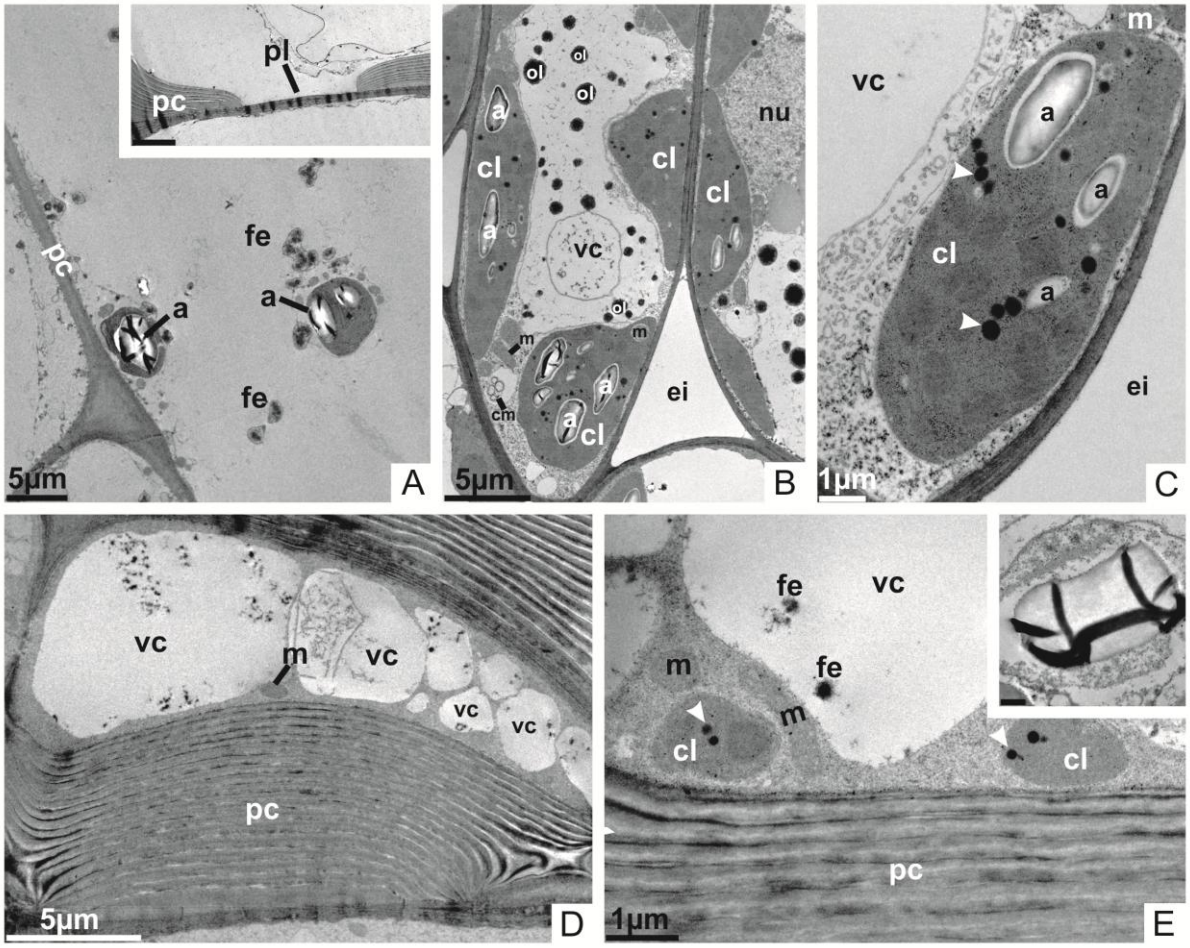
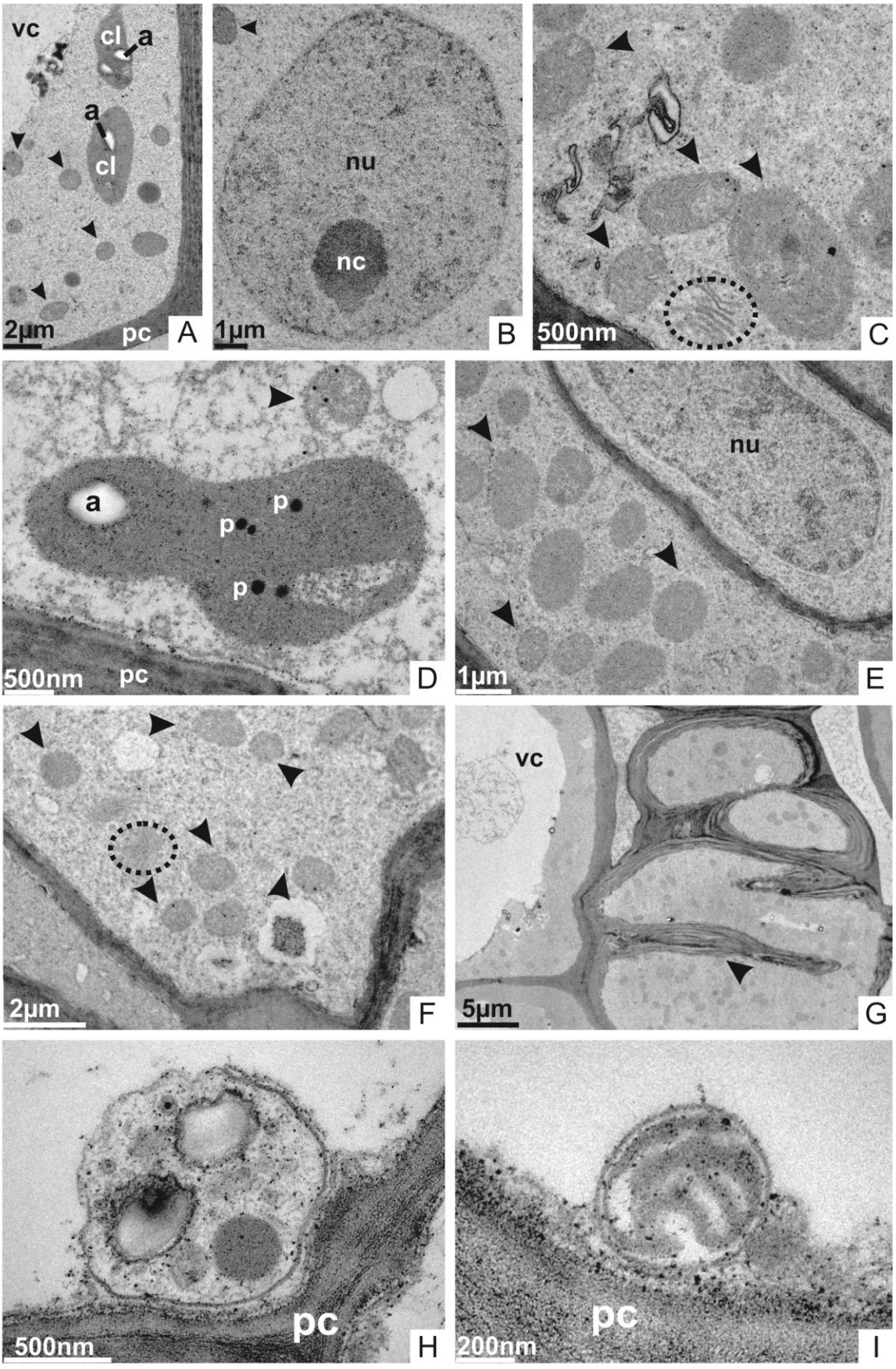


Figura 5: Células do córtex externo e interno das galhas de Cecidomyiidae em *Piper arboreum* Aubl. (Piperaceae). **A:** Células do córtex externo com acúmulo de fenólicos (fe) e amiloplastos (a). Detalhe da parede celular (pc) e de plasmodesmos (pl). **B:** Célula do córtex interno da região fotossintetizante da galha madura. Grande vacúolo com inclusões osmiofílicas (ol), núcleo evidente (nu), cloroplastos localizados na região periférica da célula contendo amido (a) de assimilação e plastoglóbulos, mitocôndrias (m) e corpos multivesiculares (cm) associados a membrana plasmática. **C:** Detalhe de cloroplasto (cl) contendo grãos de amido (a), lamelação organizada de tilacoides e plastoglóbulos (setas), mitocôndria (m), retículo endoplasmático (re) e vacúolo central (vc). D-E: Células do córtex interno lignificado. **D:** Célula com pequenos vacúolos (vc) contendo fenólicos, mitocôndrias (m) e lignificação desigual da parede celular secundária (pc). **E:** Detalhe da célula do córtex interno lignificado contendo mitocôndria (m), cloroplastos (cl) com plastoglóbulos (setas) e lamelação, desorganizada de tilacoides. Detalhe de um grão de amido.

Figura 6: Células do tecido nutritivo das galhas de Cecidomyiidae em *Piper arboreum* (Piperaceae). **A:** Célula contendo cloroplastos acumulando grãos de amido (a), numerosas mitocôndrias (seta), vacúolo central (vc). **B:** Núcleo (nu), núcleolo evidentes (nc) e mitocôndria (seta). **C:** Citoplasma denso com numerosas mitocôndrias (seta) e corpo de Golgi (círculo pontilhado). **D:** Cloroplasto em divisão contendo grão de amido e plastoglóbulos (p). E-F: Células da região perivascular. **E:** Núcleo (nu) evidente e diversas mitocôndrias. **F:** Numerosas mitocôndrias (seta), vacúolo e aparelho de Golgi (círculo pontilhado). **G:** Células com paredes sinuosas na região adjacente a câmara larval (seta). **H - I:** Corpos multivesiculares e lamelares associados a membrana plasmática.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Considerações finais

O estudo das galhas em *Piper arboreum* Aubl. (Piperaceae) permitiu avaliar alterações estruturais, metabólicas, citológicas e fisiológicas induzidas por um Diptera: Cecidomyiidae. Tais alterações revelaram caracteres únicos e relevantes dentro da perspectiva de estudo em galhas de insetos.

O desenvolvimento do novo design funcional foi resultante de modificações no padrão morfogenético das folhas jovens de *P. arboreum.* Processos de rediferenciação celular estabeleceram funções distintas nos tecidos vegetais, especialmente nas células do sistema fundamental. Funções primárias deste sistema, foram redirecionadas em virtude do estabelecimento de um microambiente favorável e o fornecimento de recursos alimentares e proteção contra inimigos naturais. Estes novos destinos celulares também corroboraram o padrão de desenvolvimento descrito para este inseto galhador, ou seja, a família Cecidomyiidae. O desenvolvimento do design intralaminar lenticular foi decorrente de hiperplasia especialmente no córtex interno, seguido pela hipertrofia do córtex externo durante as fases de crescimento e desenvolvimento até a fase de maturação. Mudanças no padrão de expansão celular assim como os sítios de alimentação foram cruciais para assegurar o desenvolvimento deste novo design.

A aquisição de novas funcionalidades também foi determinada pela relação específica entre a planta-hospedeira e o estímulo do galhador. A compartimentalização espacial de compostos dos metabolismos primário e secundário e o dreno de açúcares para o local de desenvolvimento da galha reforçaram a proteção, fornecimento de recursos nutricionais para o galhador, além da manutenção do intenso metabolismo celular, presente na região galhada. O estímulo do indutor também gerou um gradiente centrífugo de espécies reativas de oxigênio, concomitante ao campo cecidogenético. O aumento no acúmulo destas moléculas nas galhas não alterou o desempenho fotossintético quando comparado a folha não galhada. Para manter esta homeostase, características estruturais foram determinantes, assim como respostas primárias da planta hospedeira que foram conservativas nas galhas.

O estudo das galhas de Cecidomyiidae em *Piper arboreum* evidencia que a especificidade de cada sistema impõe características únicas. O metabolismo de açúcares e de lipídios via atividade enzimática apresenta como uma nova perspectiva de estudo capaz de elucidar etapas metabólicas em galhas de insetos, e mecanismos de biologia celular em geral.