UNIVERSIDADE FEDERAL DE MINAS GERAIS
INSTITUTO DE CIÊNCIAS BIOLÓGICAS
Departamento de Botânica
Programa de Pós-Graduação em Biologia Vegetal
PPGBV UFMG
UFMG

**GRACIELLE PEREIRA PIMENTA BRAGANÇA**

# COMPLEXIDADE ESTRUTURAL NOS SISTEMAS *INGA INGOIDES* - CECIDOMYIIDAE

**Tese apresentada ao Programa de Pós-Graduação em Biologia Vegetal do Departamento de Botânica do Instituto de Ciências Biológicas da Universidade Federal de Minas Gerais, como requisito parcial à obtenção do título de Doutor em Biologia Vegetal.**

**Área de concentração: Morfologia, sistemática e diversidade vegetal**

**BELO HORIZONTE – MG**
**2020**

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**Orientadora: Profa. Dra. Rosy Mary dos Santos Isaias**
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**RESUMO**

Galhas são resultantes de interações envolvendo geralmente dois organismos, a planta hospedeira e o galhador, com níveis de complexidade estrutural variados, definidos como simples, intermediário ou complexo. Tais níveis têm como determinantes o hábito alimentar do galhador e as respostas dos sistemas de tecidos vegetais. Pressupomos que a origem-destino dos tecidos das galhas, os atributos vasculares, a citologia do tecido nutritivo, e a presença de um terceiro organismo no sistema pode influenciar fortemente as respostas da planta hospedeira e consequentemente o nível de complexidade das galhas. Galhas globoides, lenticulares e fusiformes induzidas por Cecidomyiidae em *Inga ingoides* (Rich.) Willd. (Fabaceae: Caesalpinioideae) foram usadas como modelo de estudo para testar esta hipótese, e verificar a amplitude dos níveis de complexidade estrutural de cada morfotipo sob as restrições morfogenéticas de uma mesma planta hospedeira. Na perspectiva da dinâmica do desenvolvimento das galhas, percebemos que o aporte hídrico e a manutenção da pressão de turgor necessários para a expansão celular, os novos padrões de alongamento e a dinâmica das fibrilas de celulose-hemiceluloses são peculiares a cada morfotipo ora estudado. Nas galhas fusiformes intralaminares, o padrão de diferenciação vascular no sentido ápice-base é interrompido levando a maior proporção de tecido vascular e maior diâmetro dos vasos na galha e nas regiões abaixo dela, enquanto as galhas globóides e lenticulares extralaminares, análogas a órgãos adventícios, não apresentam grande investimento em tecidos vasculares e o diâmetro dos vasos é menor quando comparado às outras regiões do órgão hospedeiro. Em termos nutricionais, as galhas globóides, caracterizadas como galhas de ambrosia, apresentam o fungo como intermediador de processos de morte celular facilitando o acesso do galhador aos nutrientes acumulados no citoplasma e aos xiloglucanos na parede celular. Galhas fusiformes e lenticulares apresentam apenas dois organismos associados e um perfil citológico distinto nas células nutritivas, sendo de morte e manutenção da maquinaria celular. Nas galhas fusiformes, os processos de morte celular para liberação dos nutrientes ocorrem sem intermediação fúngica, enquanto que nas galhas lenticulares, os aspectos citológicos indicam a manutenção do metabolismo celular sob estímulo do galhador. Concluímos que não somente o hábito alimentar do galhador influencia a complexidade estrutural dos morfotipos de galhas em *I. ingoides*, mas também a origem-destino dos tecidos e a presença peculiar de um terceiro ocupante na galha. O impacto destes fatores determina a complexidade estrutural e define mecanismos compensatórios vasculares peculiares a cada morfotipo de galhas.

**Palavras-chave:** anatomia de galhas, citologia, galhas de ambrosia, histoquímica, imunocitoquímica de parede celular, morte celular, sistema vascular

**ABSTRACT**

Galls generally result from interactions involving two organisms, the host plant and the gall inducing organism, and may be simple, intermediate or complex in terms of structural complexity. These levels of structural complexity are commonly related to the gall inducer feeding habit and by the responses of plant tissue systems. We assume that the origin-fate of the gall tissues, the vascular attributes, the cytology of the nutritive tissue, and the presence of a third organism can strongly influence the responses of the host plant, and consequently the level of gall structural complexity. The globoid, lenticular and fusiform galls induced by Cecidomyiidae on *Inga ingoides* (Rich.) Willd. (Fabaceae: Caesalpinioideae) were used as study model to test this hypothesis, and to verify the amplitude of the levels of structural complexity of each gall morphotype under the same host plant morphogenetical constraints. From the perspective of gall developmental dynamics, the water supply and maintenance of the turgor pressure necessary for cell expansion, the cell elongation patterns, and the dynamics of cellulose fibrils-hemicelluloses are peculiar to each gall morphotype studied here. In the fusiform intralaminar galls, the pattern of vascular differentiation in the apex-base direction is interrupted, and greater proportion of vascular tissues and larger diameter of the vessels in the gall and in the leaflet portions below it develop. Contrastingly, the globoid and lenticular extralaminar galls, analogous to adventitious organs, do not have a large investment in vascular tissues and the diameter of the vessels is smaller when compared to the vessels in host leaflet portions above and below the gall. In nutritional terms, the globoid ambrosia galls count on the fungus to intermediate cell death processes, facilitating the access of the gall inducer to the nutrients accumulated in the cytoplasm and cell wall xyloglucans. The fusiform and the lenticular galls have only two organisms and distinct cytological profiles in their nutritive tissues. In the fusiform galls, the processes of nutritive cell death release nutrients without fungal intermediation, whereas in the lenticular galls, the cytological aspects indicate the maintenance of cell metabolism under the gall inducer stimuli. We conclude that not only the feeding habits influence the structural complexity of gall morphotypes on *I. ingoides*, but also the origin-fate of the tissues and the peculiar presence of a third organism. The impact of these factors determines the structural complexity and defines vascular compensatory mechanisms peculiar to each gall morphotype.

**Key words:** ambrosia galls, cell wall immunocytochemistry, cytology, gall anatomy, histochemistry, cell death, vascular system

## INTRODUÇÃO GERAL

*Inga ingoides* (Rich.) Willd. (Fabaceae: Caesalpinioideae) é uma super hospedeira que apresenta pelo menos três Cecidomyiidae (Diptera) associados responsáveis pela formação de três morfotipos de galhas distintos em seus folíolos. As galhas fusiformes são induzidas na nervura e têm dois organismos associados (planta hospedeira - indutor), assim como as galhas lenticulares que, contudo, são induzidas no mesofilo. O mesofilo é também o sítio de indução das galhas globóides que, por sua vez, apresentam três organismos associados (planta hospedeira – indutor – fungo), caracterizando-se como galhas de ambrosia. A análise das implicações anatômicas, citológicas e histoquímicas em uma super hospedeira de galhadores, como *I. ingoides*, revela de forma comparada o estúmulo de cada galhador e as potencialidades de respostas de uma mesma planta hospedeira a estímulos distintos. A super hospedeira *I. ingoides* e os seus três morfotipos de galhas associados foram aqui estudados comparativamente em seus aspectos de desenvolvimento, na influência de caracteres vasculares, na dinâmica de fibrilas de celulose-hemiceluloses nos diferentes compartimentos teciduais e nos atributos citológicos do tecido nutritivo sobre a complexidade estrutural das galhas. Pressupomos também que a origem-destino dos tecidos e os níveis tróficos associados em cada sistema contribuem para os níveis de complexidade estrutural estabelecidos. Mesmo atuando sobre o mesmo potencial morfogenético de *I. ingoides*, mecanismos compensatórios distintos são ativados para garantir o aporte hídrico, a reestruturação celular e a nutrição dos três galhadores associados, incrementando a proposta do perfil de complexidade das galhas baseado em atributos anatômicos e funcionais.

A complexidade estrutural das galhas categorizada nos níveis simples, intermediária e complexa, se baseia em parâmetros anatômicos, especialmente dos sistemas de revestimento e fundamental (Ferreira et al. 2019). No entanto, o potencial meristemático de grupos celulares

distintos pode interferir nos processos de diferenciação, inibição ou rediferenciação celular e consequentemente influenciar neste nível de complexidade. Uma vez que o potencial meristemático de grande parte das células vegetais (Lev Yadun 2003) pode ser ativado pelos galhadores durante o desenvolvimento das galhas (Ferreira e Isaias, 2013; Carneiro et al. 2017), galhas desenvolvidas mediante rediferenciação de células parenquimáticas são mais plásticas e podem apresentar um conjunto maior de tipos celulares quando comparadas às galhas provenientes de meristemas secundários (Isaias et al. 2013; Ferreira et al. 2019; Bragança et al. 2020a), que apresentam a maior proporção de tecido vascular (Ferreira e Isaias, 2014). A relação entre a origem e o destino dos tipos celulares nos sítios de desenvolvimento das galhas é aqui abordada com especial atenção ao sistema vascular, revelando mecanismos compensatórios variados para garantir o aporte hídrico ao novo órgão (Bragança et al. 2020a).

A manutenção da condutividade hidráulica é necessária para a hidratação dos tecidos e para gerar a pressão de turgor necessária a intensa hipertrofia celular (Crowel et al. 2010), um dos processos determinantes para o desenvolvimento das galhas (Mani 1964; Rohfritsch 1992; Isaias et al. 2014; Oliveira et al. 2016). Os novos padrões de alongamento celular estabelecidos em função da atividade alimentar dos galhadores necessitam não somente do aumento na pressão de turgor, mas também do rearranjo dos componentes da parede celular (Magalhães et al. 2014; Suzuki et al. 2015; Teixeira et al. 2018), via atuação hormonal e o desacoplamento entre as fibrilas de celulose e as hemiceluloses (Cosgrove 2016, 2018; Bragança et al. 2020b). Este rearranjo pode estar relacionado aos estágios de desenvolvimento das galhas e ainda determinar o mosaico de tecidos especializados que compõe cada morfotipo de galha (Bragança et al. 2020b). A variação dos tecidos neoformados no sítio de desenvolvimento das galhas pode ainda estar sob influência de outros organimos adicionais que podem interferir nos padrões de

reestruturação celular (Arduin e Kraus 2001; Dias et al. 2013; Rezende et al. 2019). Este terceiro organismo adicional pode ser representado por um parasitóide, um inquilino ou um organismo simbionte (Luz et al. 2019), como ocorre por exemplo em galhas de ambrosia, nas quais a fêmea do indutor transporta e inocula um fungo nos tecidos vegetais no momento da oviposição (Yukawa and Rohfritsch 2005; Rohfritsch 2008). A presença deste fungo em contato com os tecidos vegetais pode alterar os padrões celulares, e consequentemente, impactar a complexidade e os atributos químicos e citológicos das galhas. Tais perfis, especialmente nos compartimentos teciduais internos, ligados a nutrição do galhador, estão relacionados ao hábito alimentar deste (Rohfritsch 1992; Stone e Schonrögge 2003) ou da guilda associada (Rezende et al. 2019). Neste aspecto, o aparelho bucal das larvas e o comportamento do terceiro nível trófico determina o modo de aquisição dos nutrientes (Bronner 1992, Rohfritsch 2008, Chao e Liao 2013, Ferreira et al. 2019), que se dá via morte celular para liberação dos metabólitos energéticos compartimentalizados no citoplasma (Harris et al. 2006; Oliveira et al. 2010, 2011), ou ainda por consumo direto das células nutritivas (Bronner 1992).

Esta Tese tem como modelo de estudo *Inga ingoides* e três morfotipos de galhas associados com o objetivo de avaliar comparativamente (1) os atributos anatômicos determinantes da complexidade dos três morfotipos de galhas (Bragança et al. submetido); (2) a influência do sítio de indução e do posicionamento das galhas extralaminares (globoide e lenticular) e intralaminares (fusiforme) nas características do xilema e nos mecanismos compensatórios para manutenção da condutividade hidráulica (Bragança et al. 2020a); (3) o padrão das fibrilas de celulose e das hemiceluloses durante a reestruturação celular para formação dos três morfotipos de galhas (Bragança et al. 2020b) e (4) os atributos citológicos relacionados à disponibilização de recursos nutricionais em sistemas formados por dois (galhas

fusiformes e lenticulares) e três organismos (galhas de ambrosia globoides) (Bragança et al. submetido). A Tese foi dividida em quatro capítulos que abordam os tópicos mencionados acima e buscam categorizar os níveis de complexidade estrutural (simples, intermediário e complexo) das galhas (Ferreira et al. 2019), bem como associar a origem-destino dos tecidos, a influência de um terceiro nível trófico no sistema e os novos atributos vasculares e citológicos do tecidos nutritivos induzidos pelos cecidomiídeos galhadores na super hospedeira *I. ingoides* que podem contribuir para o entendimento dos mecanismos que definem a complexidade estrutural das galhas.

**Referências bibliográficas**


Arduin M, Kraus JE (2001) Anatomia de galhas de ambrosia em folhas de *Baccharis concinna* e *Baccharis dracunculifolia* (Asteraceae). Brazilian Journal of Botany 24: 63–72

Bragança GPP, Costa MSC, Isaias, RMS (2020a) The influence of gall position over xylem features in leaflets of *Inga ingoides* (Rich.) Willd. (Fabaceae: Caesalpinioideae). Trees. https://doi.org/10.1007/s00468-020-02027-1

Bragança GP, Alencar CF, Freitas, MSC, Isaias RMS (2020b) The dynamics of hemicelluloses-cellulose microfibrils and cell elongation axes determine gall morphotypes on *Inga ingoides* (Fabaceae). Plant Biology 22 (6): 981:991

Bronner R (1992) The role of nutritive cells in the nutrition of cynipids and cecidomyiids. In J. D Shorthouse JD, Rohfritsch O (Eds), Biology of insect-induced galls, Oxford University Press, Oxford, pp 118–140

Carneiro RGS, Isaias, RMS, Moreira ASFP, Oliveira DC (2017) Reacquisition of new meristematic sites determines the development of a new organ, the Cecidomyiidae gall on *Copaifera langsdorffii* Desf. (Fabaceae). Frontiers in plant Science 8: 1622

Chao JF, Liao GI (2013) Histocytological aspects of four types of ambrosia galls on *Machilus zuihoensis* Hayata (Lauraceae). Flora 208: 157–164

Cosgrove DJ (2016) Plant cell wall extensibility: connecting plant cell growth with cell wall structure, mechanics, and the action of wall modifying enzymes. Journal of Experimental Botany 67: 463–476

Cosgrove DJ (2018) Diffuse growth of plant cell walls. Plant Physiology 176: 16–27

Crowell EF, Gonneau M, Vernhettes S, Höfte H (2010) Regulation of anisotropic cell expansion in higher plants. Comptes Rendus Biologies 333: 320–324

Dias GG, Moreira GRP, Ferreira BG, Isaias RMS (2013) Why do the galls induced by *Calophya duvauae* Scott on *Schinus polygamus* (Cav.) Cabrera (Anacardiaceae) change color? Biochemical Systematics and Ecology 48: 111–122

Ferreira BG, Isaias RMS (2013) Developmental stem anatomy and tissue redifferentiation induced by a galling Lepidoptera on *Marcetia taxifolia* (Melastomataceae). Botany 91:752–760

Ferreira BG, Álvarez R, Bragança GP, Alvarenga DR, Hildago NP, Isaias RM (2019) Feeding and other gall facets: patterns and determinants in gall structure. The Botanical review 85:78–106

Fernandes GW, Fagundes M, Woodman RL, Price PW (1999) Ant effects on three-trophic level interactions: plant, galls, and parasitoids. Ecological Entomology 24 (4): 411-415

Harris MO, Freeman TP, Rohfritsch O, Anderson KG, Payne SA (2006) Virulent Hessian Fly (Diptera: Cecidomyiidae) larvae induce a nutritive tissue during compatible interaction with wheat. Annual Entomological Society of America 99:305–316

Isaias RMS, RGS Carneiro, DC Oliveira, JC Santos (2013) Illustrated and annotated checklist of Brazilian gall morphotypes. Neotropical Entomology 42:230–239

Isaias RMS, Oliveira DC, Carneiro RGS, Kraus JE (2014) Developmental anatomy of galls in the neotropics: arthropods stimuli versus host plant constraints. In: Fernandes GW, Santos JC (Eds), Neotropical Insect Galls, Springer-Verlag, Berlin, Germany, pp 15–34

Lev-Yadun S (2003) Stem cells in plants are differentiated too. Current Opinion in Plant Biology 4: 93–102

Luz FA, Mendonça Júnior MS (2019) Guilds in Insect Galls: Who is Who. BioOne (1): 207-210

Magalhães TA, Oliveira DC, Suzuki AYM, Isaias RMS (2014) Patterns of cell elongation in the determination of the final shape of *Baccharopelma dracunculifoliae* (Psyllidae) on *Baccharis dracunculifolia* DC. Protoplasma 251: 747–753.

Mani MS (1964) Ecology of plant galls Dr. W. Junk Publishers, The Hague, Netherlands

Oliveira DC, Magalhães TA, Carneiro RGS, Alvin MN, Isaias RMS (2010) Do Cecidomyiidae galls of *Aspidosperma spruceanum* (Apocynaceae) fit the pre-established cytological and histochemical patterns? Protoplasma 242:81–93

Oliveira DC, Carneiro RGS, Magalhães TA, Isaias RMS (2011) Cytological and histochemical gradients on two *Copaifera langsdorffii* Desf (Fabaceae) Cecidomyiidae gall systems. Protoplasma 248(4): 829-837

Oliveira DC, Isaias RMS, Fernandes GW, Ferreira BG, Carneiro RGS, Fuzaro L (2016) Manipulation of host plant cells and tissues by gall inducing insects and adaptive strategies used by different feeding guilds. Journal Insect Physiology 84:103–113

Rohfritsch O (1992) Patterns in gall development. In: Shorthouse JD, Rohfritsch O (Eds), Biology of insect-induced galls. Oxford University Press, New York, pp 60-86

Rohfritsch O (2008) Plants, gall midges, and fungi: A three-component system. Entomologia Experimentalis et Applicata 128: 208–216

Rezende UC, Custódio JF, Kuster VC, Gonçalves LA, Oliveira DC (2019) How the activity of natural enemies changes the structure and metabolism of the nutritive tissue in galls? Evidence from the *Palaeomystella oligophaga* (Lepidoptera)-*Macairea radula* (Metastomataceae) system. Protoplasma 256: 669-667

Stone GN, Schonrögge K (2003) The adaptive significance of insect gall morphology. Trends Ecology and Evolution 18: 512–522

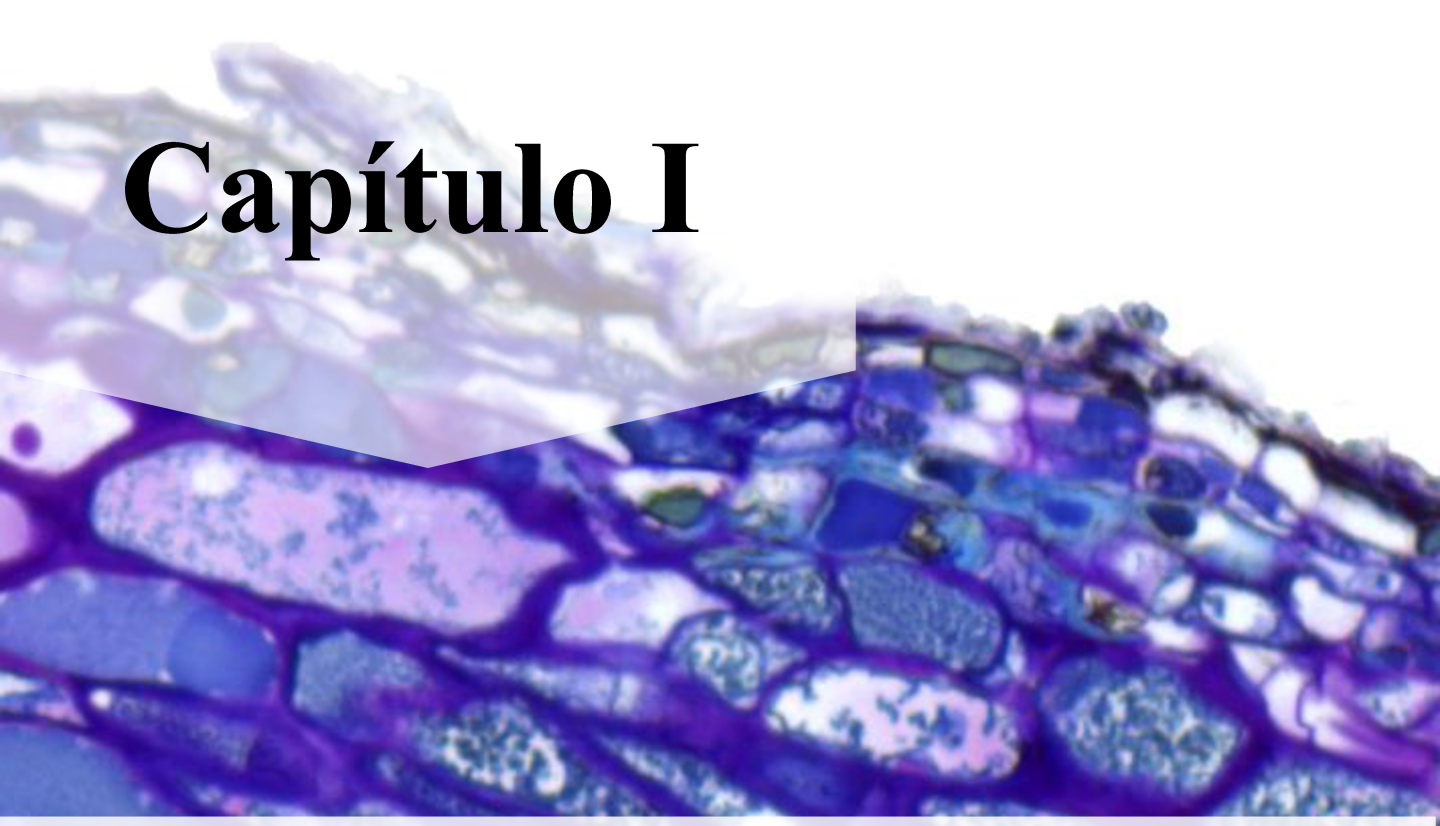
Suzuki AYM, Bedetti CS, Isaias RMS (2015) Detection and distribution of cell growth regulators and cellulose microfibrils during the development of *Lopesia* sp. galls on *Lonchocarpus cultratus* (Fabaceae). Botany 93: 435–444

Teixeira CT, Oliveira DC, Kuster VC, Isaias RMS (2018) Immunocytochemical demonstration of cell wall components related to tissue compartments in the globoid galls induced

by *Clinodiplosis* sp. (Cecidomyiidae) on *Croton floribundus* Spreng. (Euphorbiaceae). *Botany* 96: 9–18

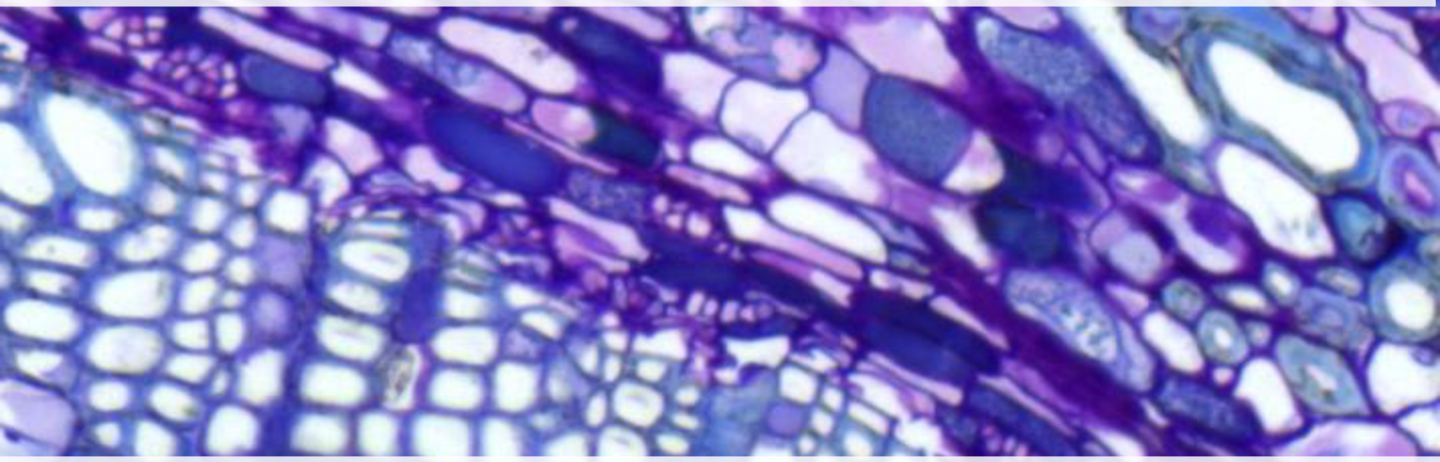
Yukawa J, Rohfritsch O (2005) Biology and ecology of gall inducing Cecidomyiidae (Diptera). In: Raman A, Schaefer CW, Withers TW (Eds), Biology, ecology, and evolution of gall-inducing Arthropods. Science Publishers, Enfield, pp 273–304

Weis AE (1982) Resource utilization patterns in a community of gall-attacking parasitoids. Environmental Entomology 11:809–815



# Capítulo I

# Depicting ontogenetical divergences related to trophic levels in leaf galls on *Inga ingoides* (Rich.) Willd. (Fabaceae: Caesalpinioideae)



**Manuscrito submetido ao periódico científico Arthropod-Plant Interactions**

Depicting ontogenetical divergences related to associated organisms in leaf galls on *Inga ingoides* (Rich.) Willd. (Fabaceae: Caesalpinioideae)

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**Declarations**

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***Code availability:*** Not applicable

***Authors' contributions:*** GPPB, RMSI, and BGF designed experiments. GPB collected material and performed experiments. All authors analyzed the results, discussed data, wrote and reviewed the manuscript. RMSI coordinated the project.

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**Abstract**

The complexity of a gall is associated with the anatomical traits established by the feeding habit of each galling taxa. Herein, we propose that the induction site, the origin of gall tissues, and the gall occupation by more than two components also influence the level of gall complexity. To test this hypothesis, we evaluated three gall morphotypes (globoid, lenticular and fusiform) induced by cecidomyiids on *Inga ingoides* (Fabaceae) using anatomical and histochemical techniques. The globoid and the lenticular galls share the same induction site, the mesophyll, but the globoid galls are ambrosia galls and have a fungus as the associated organism. The lenticular and the fusiform galls have two associated organism: the host plant and the Cecidomyiidae, but the fusiform galls are induced in the veins. The anatomical features of the galls are related to the feeding habits of the cecidomyiids. In addition, the induction site influences gall structure and the formation of the mechanical zone of each morphotype. The intense lignification process in globoid and fusiform galls reinforces the interpretation of lignification as a mechanism of the dissipation of oxidative stress. The levels of oxidative stress are associated with the associated groups or with the protective mechanisms against natural enemies. The symbiotic association with the fungi culminates in the development of a nutritive-like tissue, a peculiar anatomical feature that define the globoid morphotype as the most complex of the three galls on *I. ingoides*.

**Keywords:** ambrosia gall, gall anatomy, histochemistry, induction site

**Introduction**

Galling herbivores are parasites that induce alterations in the morphogenetic patterns of their host plants, generating specific structures, the galls (Mani 1964). The interaction between a galling herbivore and its host plant is highly specific and culminates in a unique structure (Carneiro et al. 2015). Despite the species-specific relationships, the galls may provide food for different organisms (Arduin and Kraus 2001; Rohfritsch, 2008; Luz et al. 2019; Harris and Pitzschke 2020), and also protection and a favorable microenvironment for the development of different guilds (Stone and Schonrögge 2003; Miller and Raman 2018, Luz et al. 2019).

Galls are characterized by the redifferentiation of plant tissues, whose histological complexity is classified according to the structural traits of the new organ (Formiga et al. 2015; Ferreira et al. 2017, 2019). In such perspective, simple galls may be characterized by processes of hyperplasia, cell hypertrophy and epidermal alterations; intermediate galls have the redifferentiation of specialized cells forming storage and

nutritive tissues, while complex galls have additional ontogenetic changes, such as the redifferentiation of nutritive-like tissues, neoformed sclerenchymatic zones, with a variety of cell types in the ground system, and reacquisition of promeristematic activity (*sensu* Ferreira et al. 2019).

The three levels of gall structural complexity were based on traits of the dermal and ground system developed by the association of the host plant potentialities and the feeding habit of the taxa of gall inducer (Ferreira et al. 2019). The induction site, the ontogenetical origin of gall tissues, and the presence of a third component in the system mediating the interaction may also influence the level of gall complexity through chemical and structural alterations. To test this hypothesis, we choose a superhost of galling herbivores, *Inga ingoides* (Rich.) Willd. (Fabaceae: Caesalpinioideae), which has three associated gall morphotypes (globoid, lenticular and fusiform) on different leaflet regions. The globoid and the lenticular galls have the mesophyll as the induction site, while the fusiform galls are induced in veins of primary and secondary order. The globoid galls are ambrosia galls with host plant-fungi-Cecidomyiidae interaction, while the lenticular and the fusiform galls are the product of host plant-Cecidomyiidae interactions. The following questions guide the discussion: a) how do the different induction sites in the leaflets influence gall tissue ontogenesis and level of complexity? And b) is the gall that consisting of three components structurally more complex than gall consisting of two components in the system?

**Material and methods**

*Sampling.* The population of *I. ingoides* with globoid, lenticular and fusiform galls induced by Cecidomyiidae is located at the Parque Estadual Serra Verde (PESV) (19°47, 22.47"S, 43°57'32.35"W), Belo Horizonte, Minas Gerais, Brazil. Samples of mature non-galled leaflets (n=5) and globoid, lenticular and fusiform galls in young (n=5), mature (n=5) and senescent (n=5) stages were collected from seven individuals. The developmental stages of the galls were distinguished by color and anatomical features. Globoid galls are green during all stages of development. Lenticular galls are green in the young stage and red in the mature and senescent stage and the fusiform galls are green in the young stage and brown in the mature and senescent stage. Anatomically the gall stages are differentiated by the differentiation of lignified cells in the mature and senescent stage and the number of the nutritive cell layers in the mature and senescent stage. The samples (fragments of $5mm^2$) were fixed in Karnovsky's solution (2.5% glutaraldehyde and 4.5% formaldehyde) (Karnovsky 1965, modified to 0.1 mol $L^{-1}$ phosphate buffer, pH 7.2) for 24-48h.

*Structural analyses.* For anatomical analyses, a set of fixed samples were dehydrated in ethanol series, followed by isoamyl acetate, and embedded in Paraplast X-TRA® (Kraus and Arduin 1997; Álvarez et al.

2009). The sections (12-14µm) were obtained in a rotary microtome (Leica® 2035 BIOCUT) and affixed to the slides with Bissing's adhesive (Kraus and Arduin 1997). The sections were deparaffinized at 40°C using butyl acetate, rehydrated in ethanol series, and stained with 0.5% Astra blue and 0.5% safranin (9:1, v/v) (Bukatsch 1972). After staining, the sections were dehydrated in ethanol series followed by butyl acetate, and the slides were mounted with colorless varnish Acrilex® (Paiva et al. 2006) and covered with coverslips. A second set of sample fragments ($2mm^2$) were dehydrated in ethanol series and embedded in glycolmethacrylate Leica® Historesin (Kraus and Arduin 1997), sectioned in rotary microtome (5-7 µm), and stained with 0.1% toluidine blue O (O'Brien et al. 1964), modified to dilution in 0.1 mol $L^{-1}$ acetate buffer, pH 4.7. The sections were analyzed, and the images were captured with a Leica ICC50 HP® digital camera coupled to a Leica DM 500 light microscope.

*Histochemical analyses.* For the staining of fungi, globoid galls embedded in Leica® historesin were stained with 5% cotton blue in lactophenol for 20 min, and counter-stained in 1% safranin for 10s. In such a test, the fungi hyphae were stained in blue (Marques et al. 2013). For lignin detection, fixed samples of the three gall morphotypes were free-hand sectioned and submitted to Wiesner's reagent (2% phloroglucinol in acidified solution for 5 min) (Johansen 1940), with lignins stained in red. Sections of fixed samples of the fusiform galls were stained with a saturated solution of ruthenium red (Jensen 1962) for detection of pectins, and with a saturated solution of Sudan Red B in 70ºGL ethanol during 5 min (Brundett et al. 1991) for detection of suberin. Pectins were stained in pink and suberin in red. Blank sections, without staining, were used as controls. The sections were analyzed, and the images were captured with a Leica ICC50 HP® digital camera coupled to a Leica DM500 light microscope.

**Results**

*Non-galled leaflet*

*Inga ingoides* has compound leaves, with extrafloral nectaries in the rachis between the leaflet pairs. Leaf petioles and rachis have lateral alate expansions (Figure 1a). The epidermis of non-galled leaflets is one-layered with stomata on the abaxial surface. The mesophyll has a one-layered palisade parenchyma, and a 3-5 layered spongy parenchyma (Figure 1b). Vascular bundles have collateral arrangement and are surrounded by one layer of pericyclic fibers. The adaxial cortical parenchyma has 5-7 cell layers and vascular system has a concentric arrangement around a homogeneous parenchymatic pith. The vascular system consists of a collateral unit close the abaxial surface and a bicollateral unit close the adaxial surface. The vascular system is surrounded by 1-4 layers of pericyclic fibers (Figure 1c).

*Globoid gall*

Globoid galls are extralaminar, induced in the mesophyll of mature leaflets, in the adaxial or abaxial surface, and are isolated and green during all stages of development (Figure 2a). The globoid structure is originated by redifferentiation of epidermis, palisade, and spongy parenchyma, and vascular tissues (Figure 2b).

During gall growth (Figure 2c-f), the leaflet abaxial epidermal cells divide anticlinally, accompanying gall expansion. In the young stage, the outer tissue compartment (OC) has 11-13 layers of homogeneous parenchymatic cells, with phenolic inclusions in vacuoles, which occupy most of cell volume. Neoformed vascular bundles are immersed in the outermost cells of the OC parenchyma (Figure 2c). The vascular bundles of the gall peduncle have hypertrophied vascular parenchyma cells (Figure 2d-e). The IC has 4-8 layers of nutritive cells with dense and granulose cytoplasm and evident nucleus (Figure 2f), lining the larval chamber.

Mature globoid galls (figure 2g-l) have lignified epidermal cell walls (Figure 2h). Under the epidermis, the OC is divided into three distinct regions. The subepidermal region is formed by 5-8-layered outer parenchyma, with periclinally elongated cells with thin and sinuous primary walls (Figure 2h), followed by 4-9 median parenchyma cell layers. The median parenchyma cells have unevenly thickened and lignified cell walls (Figure 2i). Subjacent to the median parenchyma, there are 3-5 layers of sclereids, with homogeneously thickened and lignified secondary walls (Figure 2j). Collateral vascular bundles are immersed in the OC. Near the peduncle, i.e., the point of insertion of the gall in the leaflet lamina, there are 2-4 nutritive cell layers with diminutive intercellular spaces, associated with fungal hyphae, forming a nutritive-like tissue (Figure 2k-l). In the senescence stage, the nutritive-like tissue may be absent or collapsed.

*Lenticular gall*

The lenticular gall is extralaminar and induced on the abaxial surface of mature leaflets. The galls are isolated and green during young stage (Figure 3a), and red-colored in mature and senescent stages. The tissue compartments develop by redifferenatiation of epidermis, spongy and vascular parenchyma cells (Figure 3b). In the young stage, the epidermis is one-layered and the cells divide anticlinally, accompanying gall expansion. The gall connects to the leaflet lamina by 10-12 cell layers of an upper OC opposite to 6-10 layers of anticlinally elongated cells constituting the lower OC (Figure 3c). The cells of the OC accumulate phenolic compounds. The IC is located between the upper and lower OC regions, and has 5-8 cell layers of

typical nutritive tissue (Figure 3d). The vascular bundles of the gall peduncle are originated from NGL and have hypertrophied vascular parenchyma cells (Figure 3e). The lateral extension of the lower OC has 6-9 cell layers, which confer an umbrella-shape to the young gall (Figure 3f).

During the maturation stage (Figure 3g-l), the upper OC has 10-14 cell layers. Periclinal cell divisions and anticlinal elongation determine the gall lenticular shape. At this stage (Figure 3g), the 10-16 cell layers of the upper and 6-15 layers of the lower OC are lignified (Figure 3h-i). The vascular bundles extend toward the extremities of the lateral extensions (Figure 3j, k). The nutritive cells organized in 10-16 layers have dense cytoplasm and evident nuclei (Figure 3l). In the senescence stage (Figure 3m), the 7-12 layers of the typical nutritive tissue have lignified cell walls.

*Fusiform gall*

The fusiform galls are intralaminar and induced on the midrib or the veins of primary or secondary order of young leaflets. The galls occur isolated (Figure 4a) or grouped and are green in the young stage, and brown in mature and senescent stages The tissue compartments develop by the vascular cambium and phellogen activities (Figure 4b). During the young stage (Figure 4c-e), the gall is covered by a single-layered epidermis, with cells that divide anticlinally to accompany the gall superficial increment in area. The OC parenchyma is formed by 5-6 cell layers and the vascular system is surrounded by 2-4 layers of pericyclic fibers (Figure 4c-d). At this stage, the vascular cambium produces a radial series of isodiametric non-lignified fibers with thickened cell walls. These cells constitute the 10-15-layered typical nutritive tissue (Figure 4e).

At the stage of maturation (Figure 4f-j), the vascular cambium produces secondary xylem inwards, and secondary phloem outwards (Figure 4f). The dermal system of the gall is gradually replaced by a periderm, with 3-4 layers of suberized cells, one-layered phellogen, and 2-3 layered phelloderm (Figure 4g-h). The cortical parenchyma consists of 8-14 cell layers and the vascular system is surrounded by pericyclic fibers (Figure 4i). The secondary phloem is less conspicuous than the secondary xylem, which is composed of 18-35 cell layers. The typical nutritive tissue is divided into two regions (Figure 4j-l): 3-15 cell layers of parenchyma with thick and pectic cell walls (Figure 4j-k) and 6-8 cell layers with thin and sinuous cell walls (Figure 4l), which are in contact with the gall chamber. The senescent stage is marked by the absence of the nutritive tissue. The periderm, cortical parenchyma, pericyclic fibers, vascular cambium, and secondary vascular tissues remain.

**Complexity level of galls**

The three gall morphotypes share all the anatomical traits of simple galls (Figure 5). Comparatively, the lenticular galls are anatomically simpler than the globoid and the fusiform galls, with the development of a mechanical zone as the only trait of complexity. Globoid galls have epidermis alterations, and the development of a mechanical zone and nutritive-like tissue, which are traits of anatomical complexity. The level of complexity of the globoid and the fusiform galls differs by the type of nutritive tissue, the absence of mechanical zone, and the activation of a secondary meristem in the fusiform galls (Figure 5).

**Discussion**

The three gall morphotypes induced by scraping-sucking larvae follow the patterns proposed for the Cecidomyiidae-induced galls (Rohfritsch 1992). Cecidomyiidae galls usually have specialized tissues involving the larval chamber, organized in a nutritive zone, surrounded by layers of lignified cells, and a parenchymatic outer cortex (Rohfritsch 1992), which provide nutrition and protection to the galling herbivore (Stone and Schonrögge 2003). Despite the histological patterns with similar functions, distinct traits influence the development and structural complexity of each gall morphotype.

The globoid and the lenticular galls share the same induction site, i.e. the mesophyll, and involve the reorganization of the three plant tissue systems. Despite the similarity between the induction site and the origin of the tissues, the globoid and the lenticular galls are occupied by distinct organisms, which influence the alterations in mesophyll histological patterns. The globoid galls have three associated organisms (host plant - fungi - Cecidomyiidae), while the lenticular galls have two associated organisms (host plant - Cecidomyiidae), similarly to the fusiform galls. Taking the between two organisms for granted, a higher similarity between the lenticular and the fusiform galls should be expected, however the fusiform galls are induced in leaflet veins. The differences associated with the site of induction, tissue origin and associated organisms generate distinct anatomical traits.

**Induction site, the origin of tissues and the complexity of galls**

Along gall development, the dermal system alterations confer a favorable microenvironment to the galling herbivore (Kraus et al. 2002; Oliveira and Isaias 2010; Formiga et al. 2011; Ferreira et al. 2019). On *I. ingoides*, the globoid and the fusiform galls have the greatest epidermal variations in relation to the original pattern of their host organs. In the globoid galls, epidermal cell walls are lignified, while in the fusiform galls, the epidermis is substituted by a periderm with suberin cell wall deposition. Both the lignification and suberization of the dermal system improve the physical barrier against the attack of parasitoids (Kraus et al.

2002), and confer impermeabilization, which protect the galling herbivores against their natural enemies (Stone and Schonrögge 2003).

Galls induced in the leaf lamina, like the globoid and lenticular galls on *I. ingoides*, with distinct final shapes have different axes of cell expansion and different patterns of redifferentiation due to the meristematic potential of the ground system (Lev-Yadun 2013; Ferreira et al. 2019). Parenchymatic cells redifferentiate into new cell types and tissues, and may originate the common storage tissue, the nutritive-like tissue, the typical nutritive tissue, and the mechanical zone, common to most of the Cecidomyiidae galls (Suzuki et al. 2015; Bedetti et al. 2017; Nogueira et al. 2018; Costa et al. 2018), as is true for the globoid and lenticular galls on *I. ingoides*. The combination between the site of origin of the new tissues and the possibility of new cell expansion axes set the ground system, especially the parenchyma cells, as a flexible resource for the formation of the various gall morphotypes observed in nature (Isaias et al. 2013; Ferreira et al. 2019). The predominance of the periclinal axis of cell expansion is a common pattern observed both in the globoid galls on *Piptadenia gonoacantha* (Mart.) J.F.Macbr. (Bedetti et al. 2017) and on *I. ingoides* (Bragança et al. 2020a). The lenticular galls on *I. ingoides*, on the other hand, exhibit different patterns of cell expansion in the same tissue compartment (Bragança et al. 2020a). The establishment of different patterns of cell expansion to the development of the circular halo and the radial expansions, characteristic of this lenticular morphotype. Concerning the fusiform galls, which are induced in veins, the cells tend to follow the axis of elongation of the host organ (Ferreira and Isaias 2013, Corró-Molas et al. 2014; Singh et al. 2017). The vascular cambium activity generated the high of hyperplasia in the gall inner tissue compartment, which end up in gall increasing in diameter (Bragança et al. 2020a).

In galls, the main alterations in the vascular system are associated with neovascularization (Formiga et al. 2011; Carneiro et al. 2015) and/or hypertrophy of the vascular bundles (Álvarez et al. 2013; Ferreira et al. 2019). The new vascular cells and tissues can originate from the procambium, the vascular cambium, or from the redifferentiation of parenchyma cells (Ferreira and Isaias 2013; Fleury et al. 2015; Costa et al. 2018; Bragança et al. 2020b). In *I. ingoides*, the vascular bundles originated from the procambium and/or from the parenchyma are located in the outer tissue compartment of the globoid and the lenticular galls, and can assist in the transport of water in galled tissues (Bragança et al. 2020b). The fusiform galls are induced in primary and secondary veins and originate from the activity of the secondary meristems (vascular cambium and phellogen), which results in a vascular configuration distinct from those of the globoid and the lenticular galls. The induction of the fusiform galls on major veins may involve constraints of the meristematic

potential of the vascular cambium and phellogen cells, when compared to the totipotency of the parenchymatic cells and the primary meristems. Conservatively, both secondary meristems maintain their predictable fates in galls, the phellogen activity replace the epidermis, while the vascular cambium produces secondary xylem and phloem (Guedes et al. 2016; Guedes et al. 2018). However, peculiar, and significant deviations of the common pattern can be observed in gall developmental sites (Ferreira and Isaias 2013; Corró-Molas et al. 2014; Guedes et al. 2016; Singh et al. 2017; Guedes et al. 2018). The stimuli of the galling herbivore associated to the fusiform gall on *I. ingoides* redirect the activity of the vascular cambium at the beginning of gall growth and stimulate the production of a typical nutritive tissue with non-lignified fibers. The cell walls of these fibers are composed of cellulose, xyloglucans (Bragança et al. 2020a) and pectins, which, when metabolized, provide free sugars that may work out as an additional nutritional resource for the Cecidomyiidae (Bronner 1992; Barnes and Anderson 2018). The absence of lignification in fibers is an intriguing ontogenetic factor, observed in different host plant-galling herbivore systems (Arduin et al. 1991, 1995, 2001; Souza et al. 2000; Sá et al. 2009). In the fusiform galls on *I. ingoides*, the lignin precursors seems to be redirected to the formation of the lignified walls of the secondary xylem cells, which are functionally analogous to the sclerenchymatic zone of the globoid and the lenticular galls developed by parenchyma redifferentiation.

Comparatively, the three gall morphotypes are structurally complex (*sensu* Ferreira et al. 2019; see figure 5), and such complexity is related to the induction site and origin of the specialized tissues. In the globoid and the lenticular galls induced in the mesophyll, parenchyma reorganization lead to the formation of the mechanical zones, while in the fusiform galls, the secondary xylem originated from the vascular cambium forms a protective layer analogous to the mechanical zone surrounding the larval chamber. In the fusiform galls induced in the veins, the structural complexity is defined by the activity of the secondary meristems, which provides an increase in gall volume. In addition to ontogenetical origins and related peculiar traits of each gall, we consider the involvement of a third organism associated to the globoid galls as an influencer toward gall complexity.

**The presence of third organims and the structural complexity of ambrosia galls**

The globoid gall morphotype on *I. ingoides* is a typical ambrosia gall, characterized by a interaction involving the host plant, the fungi, and the Cecidomyiidae. The ambrosia galls on *I. ingoides* are considered complex for the fungi occupy the intercellular spaces forming a nutritive-like tissue, with fungi hyphae interspersed to plant cells limited outwards by a lignified zone. We consider the formation of the lignified

zone involving the nutritive-like tissue with fungi hyphae as the main divergence between simple and complex ambrosia galls. Such variation in complexity may be associated with the feeding site of each gall-inducing species, and the level of oxidative stress generated over the galled tissues. In complex galls, as in the globoid morphotype on *I. ingoides*, the galling herbivores can feed from the fungi or from the cells of the nutritive-like tissue, which may be indicative of the induction of a high oxidative stress, a hypothesis to be investigated. The control of the high levels of oxidative stress lead to tissue zonation, and mechanical zone establishment (Isaias et al. 2015; Oliveira et al. 2016). Therefore, the intense lignification in complex ambrosia galls can be a mechanism for the maintenance of redox homeostasis in galled tissues.

**Final considerations**

Current analyses on gall anatomy lead us to propose that in addition to the feeding habits, the site of induction, the origin of the galled tissues and the associated organisms determine the anatomical complexity of galls. The distinct anatomical features of the three gall morphotypes are associated with specific traits developed due to the cellular potential of the original host plant tissue sites, as well as to the development of the nutritive-like tissue and of the lignified zone due to the interference of the fungi in ambrosia galls.

**References**


Álvarez R, Encina A, Pérez-Hidalgo N (2009) Histological aspects of three *Pistacia terebinthus* galls induced by three different aphids: *Paracletus cimiciformis*, *Forda marginata* and *Forda formicaria*. Plant Sci 176:303–314

Álvarez R, González-Sierra S, Candelas A, Martinez JJI (2013) Histological study of galls induced by aphids on leaves of *Ulmus minor*: *Tetraneura ulmi* induces globose galls and *Eriosoma ulmi* induces pseudogalls. Arthropod Plant Interact 7: 643–650

Arduin M, Kraus JE, Venturelli M (1991) Estudo morfológico de galha achatada em folha de *Struthanthus vulgaris* Mart. (Loranthaceae). Rev Bras Bot 14:147-156

Arduin M, Kraus JE (1995) Anatomia e ontogenia de galhas foliares de *Piptadenia gonoacantha* (Fabales, Mimosaceae). Boletim de Botânica da Universidade de São Paulo 14:109–130

Arduin M, Kraus JE (2001) Anatomia de galhas de ambrosia em folhas de *Baccharis concinna* e *Baccharis dracunculifolia* (Asteraceae). Rev Bras Bot 24:63–72

Barnes WJ, Anderson CT (2018) Release, recycle, rebuild: cell-wall remodeling, autodegradation, and sugar salvage for new wall biosynthesis during plant development. Mol Plant 8:31–46

Bedetti CS, Bragança GP, Isaias RMS (2017) Influence of auxin and phenolic accumulation on the patterns of cell differentiation in distinct gall morphotypes on *Piptadenia gonoacantha* (Fabaceae) Aust J Bot 65:411–420

Bragança GP, Alencar CF, Freitas MSC, Isaias RMS (2020a) The dynamics of hemicelluloses-cellulose microfibrils and cell elongation axes determine gall morphotypes on *Inga ingoides* (Fabaceae). Plant Biol. https://doi.org/10.1111/plb.13151

Bragança GP, Freitas MSC, Isaias RMS (2020b) The influence of gall position over xylem features in leaflets of *Inga ingoides* (Rich.) Willd. (Fabaceae: Caesalpinioideae). Trees. https://doi.org/10.1007/s00468-020-02027-1

Bronner R (1992) The role of nutritive cells in the nutrition of cynipids and cecidomyiids. In: Shorthouse JD, Rohfritsch O (eds) Biology of insect-induced galls, Oxford University Press, New York, pp 118–140

Brundett MC, Kendrick B, Peterson CA (1991). Efficient lipid staining in plant material with Sudan Red 7B or Fluorol Yellow 088 in Polyethylene Glycol-glycerol. Biotech Histochem 66:111–116

Bukatsch F (1972) Bermerkungenzur Doppelfärbung Astrablau-Safranin. Mikrokosmos 61:255

Carneiro RG, Pacheco P, Isaias RMS (2015) Could the extended phenotype extend to the cellular and subcellular levels in insect-induced galls? PLoS One 8:01–20

Chao JF, Liao GI (2013) Histocytological aspects of four types of ambrosia galls on *Machilus zuihoensis* Hayata (Lauraceae). Flora 208:157–164

Corró-Molas B, Martínez JJ, Carbonell S., Silleta LC, Gallia MC, Alfonso GL (2014) Modificaciones morfológicas inducidas por *Tetradiplosis panghitruz* Martínez (Diptera: Cecidomyiidae) en tallos de *Prosopis caldenia* Burkart (Fabaceae). Rev Mus Argentino Cienc Nat 16:1–12

Costa EC, Carneiro RGS, Silva JS, Isaias, RMS (2018) Biology and development of galls induced by *Lopesia* sp. (Diptera: Cecidomyiidae) on leaves of *Mimosa gemmulata* (Leguminosae: Caesalpinioideae) Aust J Bot 66:161–172

Dawkins R. (1982) The extended phenotype: the gene as the unit of selection. Oxfeord: Oxford University Press

Dorchin N, Freidberg A, Aloni R (2002) Morphogenesis of stem gall tissues induced by larvae of two cecidomyiid species (Diptera: Cecidomyiidae) on *Suaeda monoica* (Chenopodiaceae). Can J Bot 80: 1141–1150

Ferreira BG, Isaias RMS (2013) Developmental stem anatomy and tissue redifferentiation induced by a galling Lepidoptera on *Marcetia taxifolia* (Melastomataceae). Botany 91:752–760

Ferreira BG, Avritzer SC, Isaias RMS (2017) Totipotent nutritive cells and indeterminate growth in galls of *Ditylenchus gallaeformans* (Nematoda) on reproductive apices of *Miconia*. Flora 227:36–45

Ferreira BG, Álvarez R, Bragança GP, Alvarenga DR, Hildago NP, Isaias RM (2019) Feeding and other gall facets: patterns and determinants in gall structure. Bot Rev 85:78–106

Fleury G, Ferreira BG, Oliveira DC, Soares GLG, Isaias RMS (2015) Elucidating the determination of the rosette galls induced by *Pisphondylia brasiliensis* Couri & Maia 1992 (Cecidomyiidae) on *Guapira opposita* (Vell.) Reitz (Nyctaginaceae). Aust J Bot 63:608–617

Formiga AT, Silveira FAO, Fernandes GW, Isaias RMS (2015) Phenotypic plasticity and similarity among gall morphotypes on a superhost, *Baccharis reticularia* (Asteraceae). Plant Biol 17:512–521

Formiga AT, Soares GLG, Isaias RMS (2011) Responses of the host plant tissues to gall induction in *Aspidosperma spruceanum* Müell. Arg. (Apocynaceae). Am J Plant Sci 2:823–834

Guedes LM, Aguilera N, Becerra J, Hernandez V, Isaias, RMS (2016) Leaf and stem galls of *Schinus polygamus* (Cav.) Cabr (Anacardiaceae): anatomical and chemical implications. Biochem Syst Ecol 69:266–273

Guedes LM, Aguilera N, Ferreira BG, Becerra José, Saez K, Péres C, Hernandez V, Isaias RMS (2018) Factors influencing the morphogenesis of galls induced by *Calophya mammifex* (Calophyidae) on *Schinus polygama* (Anacardiaceae) leaves. Botany 96:589–599

Harris MO, Pitzschke A (2020) Plants make galls to accommodate foreigners: some are friends, most are foes. New Phytol 222:1852–1872

Isaias RMS, RGS Carneiro, DC Oliveira, JC Santos (2013) Illustrated and annotated checklist of Brazilian gall morphotypes. Neotrop Entomol 42:230–239

Isaias RMS, Oliveira DC, Moreira ASFP, Soares GLG, Carneiro RGS (2015) The imbalance of redox homeostasis in arthropod-induced plant galls: mechanisms of stress generation and dissipation. Biochim Biophys Acta 1850:1509–1517

Jensen WA (1962) Botanical histochemistry: principles and practice. W.H. Freeman, San Francisco

Johansen DA (1940) Plant microtechnique. McGraw-Hill Book Company, New York, London

Karnovsky MJ (1965) A formaldehyde-glutaraldehyde fixative of high osmolarity for use in electron microscopy. J Cell Sci 27:137–138

Kraus JE, Arduin M (1997) Manual básico de métodos em morfologia vegetal. Editora da Universidade Federal Rural do Rio de Janeiro, Seropédica.

Kraus JE, Arduin M, Venturelli M (2002) Anatomy and ontogenesis of hymenopteran leaf galls of *Struthanthus vulgaris* Mart. (Loranthaceae). Rev Bras Bot 25:449–458

Lev-Yadun S (2003) Stem cells in plants are differentiated too. Curr Top Plant Biol 4:93–102

Luz FA, Mendonça Júnior MS (2019) Guilds in Insect Galls: Who is Who. BioOne 102:207–210

Mani MS (1964) Ecology of plant galls. Dr. W. Junk Publishers, The Hague, Netherlands

Marques JPR, Soares MKM, Appezzato-da-Glória B (2013) New staining technique for fungal-infected plant tissues. Turk J Bot 37:784–787

Miller DG, Raman A (2018) Host–plant relations of gall-inducing insects. Ann Entomol Soc Am112:1–19

Nogueira RM, Costa EC, Silva, JS, Isaias, RMS (2018) Structural and histochemical profile of *Lopesia* sp. Rübsaamen 1908 pinnula galls on *Mimosa tenuiflora* (Willd.) Poir. in a Caatinga environment. (Willd.) Hoehnea 45: 314–322

O'Brien TP, Feder N, McCully ME (1964) Polychromatic staining of plant cell walls by toluidine blue O. Protoplasma 59:368–373

Oliveira DC, Isaias RMS (2010) Cytological and histochemical gradients induced by a sucking insect in galls of *Aspidosperma australe* Arg. Muell (Apocynaceae). Plant Sci 178:350–358

Oliveira DC, Isaias RMS, Fernandes GW, Ferreira BG, Carneiro RGS, Fuzaro L (2016) Manipulation of host plant cells and tissues by gall inducing insects and adaptive strategies used by different feeding guilds. J Insect Physiol 84:103–113

Paiva JGA, Fank-de-Carvalho SM, Magalhães MP, Graciano-Ribeiro D (2006) Verniz vitral incolor 500®: uma alternativa de meio de montagem economicamente viável. Acta Bot Bras 20:257–264

Rohfritsch O (1992) Patterns in gall development. In: Shorthouse JD, Rohfritsch O (eds) Biology of insect-induced galls. Oxford University Press, New York, pp 60–86

Rohfritsch O (2008) Plants, gall midges, and fungi: A three-component system. Entomol Exp Appl 128:208–216

Sá CEM, Silveira AO, Santos JC, Isaias, RMS, Fernandes GW (2009) Anatomical and developmental aspects

of leaf galls induced by *Schizomyia macrocapillata* Maia (Diptera: Cecidomyiidae) on *Bauhinia brevipes* Vogel (Fabaceae). Rev Bras Bot 32: 319–327

Sing S, Patni V, Arora DK. (2017) Histological studies on stem gall of *Acacia leucophloea* Willd. induced by insect. Indian J Plant Science 6:45–49

Suzuki AYM, Bedetti CS, Isaias RMS (2015) Detection and distribution of cell growth regulators and cellulose microfibrils during the development of *Lopesia* sp. galls on *Lonchocarpus cultratus* (Fabaceae). Botany 93:435–444

Souza SCPM, Kraus JE, Isaias RMS, Neves LJ (2000) Anatomical and ultrastructural aspects of leaf galls in *Ficus microcarpa* L.f. (Moraceae) induced by *Gynaikothrips ficorum* Marchal (Thysanoptera). Acta Bot Bras 14:57-69

Stone GN, Schonrögge K (2003) The adaptive significance of insect gall morphology. Trends Ecology and Evolution 18:512–522

**Figure captions**

**Fig. 1 Anatomy of non-galled leaflets of *Inga ingoides* (Fabaceae: Caesalpinioideae). a)** Non-galled leaflets. Transverse section of lamina (b) and midrib vein (c). Abbreviations: Ep, epidermis; Fi: Fiber; Cp, cortical parenchyma; Ph, phloem; Pa: parenchyma; PP, palisade parenchyma; SP, spongy parenchyma; Xy, xylem

**Fig. 2 Anatomy of globoid galls on *I. ingoides* (Fabaceae: Caesalpinioideae). a)** Macroscopic aspect in young stage. **b)** Diagram of the origin and fates of gall tissues. **c-f)** Young stage. **c)** The outer tissue compartment (OC) with neoformed vascular tissues (arrow) and accumulation of phenolics. Nutritive tissue with dense cytoplasm. **d-e)** Detail of gall peduncle with hypertrophy of vascular cells (arrow). **f)** Detail of nutritive tissue with dense-cytoplasm cells and prominent nuclei (arrows). **g-l)** Mature stage. **g)** Three regions of the outer tissue compartment (OC). Parenchyma cells with unevenly thickened secondary walls. The numbers (1-4) indicate the cells of the correspondent regions in (h-l). **h-l)** Detail of the 1-4 regions: **h)** Region 1 with lignified epidermal cell (arrow) and vascular bundles (dotted circle). **i)** Region 2 with cells with unevenly thickened secondary walls. **j)** Region 3 with lignified cell walls (arrows). **k-l)** Region 4 with nutritive-like tissue. **k)** Presence of fungi in blue between cells of nutritive-like tissue (arrows). **l)** Anatomical features of nutritive tissue with fungi (arrows). Staining: c-i, l: Astra blue and safranin; j: Wisner's test; k: cotton blue and safranin

**Fig. 3 Anatomy of lenticular galls on *Inga ingoides* (Fabaceae: Caesalpinioideae). a)** Macroscopic aspect. **b)** Diagram of the origin and fates of gall tissues. **c-f)** Young stage. **c)** Gall compartments: lower outer tissue compartment (LOC), typical nutritive tissue (red dotted), upper outer tissue compartment (UOC). **d)** Detail of tissue compartment and typical nutritive tissue. **e)** Detail of hyperplasia and hypertrophy of vascular tissues (red arrows). **f)** Extremity of lower outer tissue compartment. **g-l)** Mature stage. **g)** Diagram of a transverse section, showing the tissue organization of gall in mature stage. **h-i)** The lignification of the LOC. **j-k)** Vascular tissues (red arrows) around the nutritive tissue (j) and toward the extremity of the outer tissue compartment (k). **l)** Dense cytoplasm and prominent nucleus (arrow) in nutritive cells. **m)** Lignification of nutritive tissue in senescent stage. Staining: a-f: toluidine O blue; h: Wisner's test; i-m: Astra blue and safranin

**Fig. 4 Anatomy of fusiform galls on *Inga ingoides* (Fabaceae: Caesalpinioideae). a)** Macroscopic aspect. **b)** Diagram of the origin and fates of gall tissues. **c-e)** Young stage. **c)** The hyperplasia and hypertrophy of cortical parenchyma (CP) and atypical activity of the vascular cambium toward the larval chamber (LC). The number 1 indicates cells of the correspondent region in (d). **d-e)** Detail of cambium region (arrows) and nutritive tissue (NT). The number 2 indicates cells of the correspondent region in (e). **f-l)** Mature stage. **f)**

Overview of gall with periderm (pe), cortical parenchyma (CP), fibers (Fi), phloem (Pho), cambium zone (CZ), xylem (Xy) and nutritive tissue (NT). The red border square and the number 3 indicate cells of the correspondent region in (j). **g)** Detail of periderm with suber (Sb), phellogen (Ph) and phelloderm (Phe). **h)** Suberin in the periderm. **i)** Lignins in the xylem and fibers. **j-l)** Inner tissue compartment. **j)** Cells with thick walls. **k)** Pectins in cell walls. **l)** Cells with irregular thickened walls and cells with thin and sinuous walls. Staining: c-e: Astra blue and safranin; f-g, j,l: toluidine O Blue; h: Sudan red B; i: Wisner's test, k: ruthenium red

**Fig. 5 Network of complexity levels of galls on *I. ingoides* (Fabaceae: Caesalpinioideae).** The color gradient (light to dark) indicates the anatomical traits associated with the complexity level of the galls (*sensu* Ferreira et al. 2019). Dashed, uninterrupted and gray lines indicate anatomical traits present in globoid, lenticular and fusiform galls, respectively. The direction of the arrow indicates the level of complexity of the three gall morphotypes

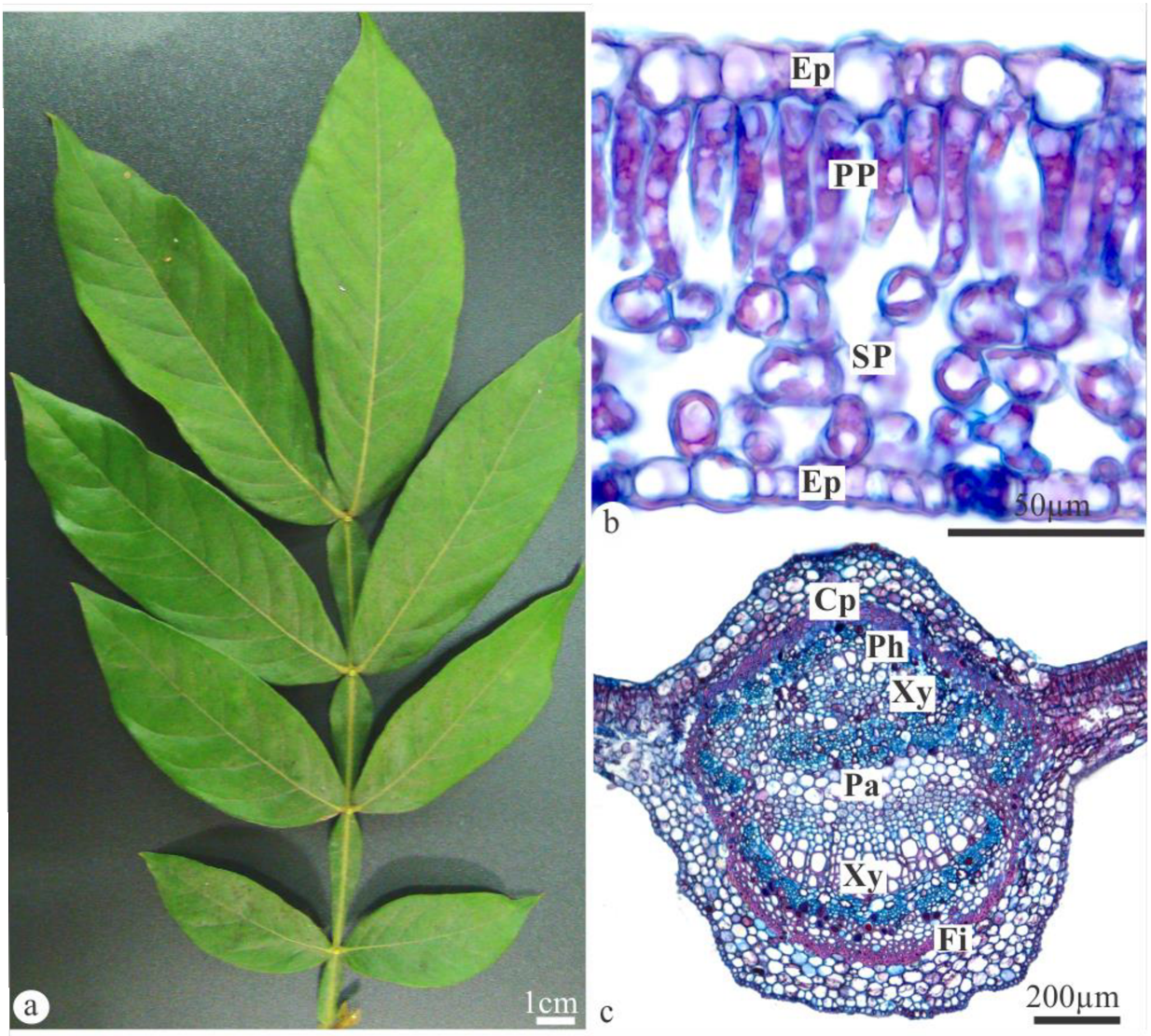



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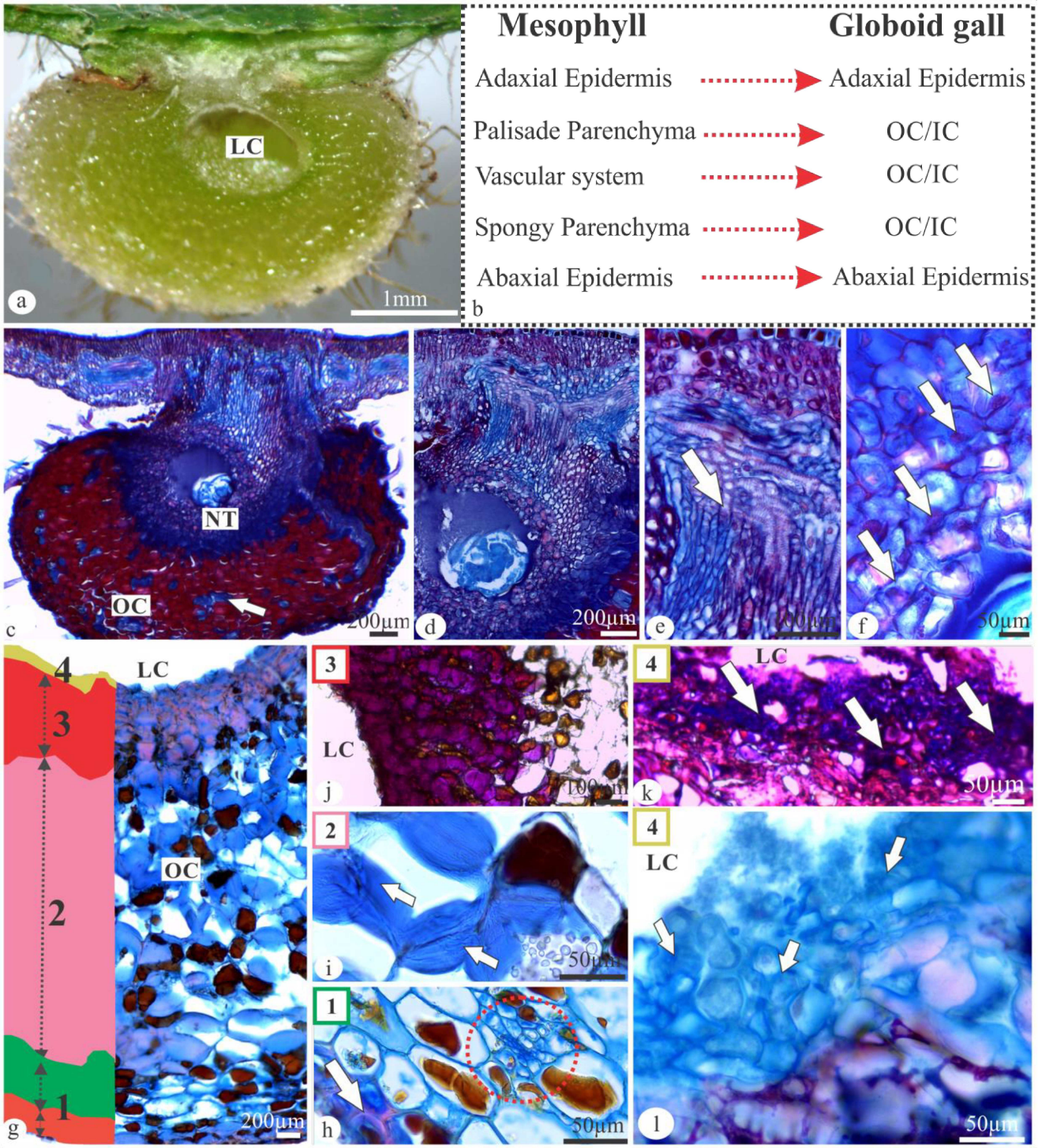

LC
a
1mm
Mesophyll
Globoid gall
Adaxial Epidermis
Adaxial Epidermis
Palisade Parenchyma
OC/IC
Vascular system
OC/IC
Spongy Parenchyma
OC/IC
Abaxial Epidermis
Abaxial Epidermis
b
NT
OC
c
200µm
d
200µm
e
100µm
f
50µm
4
3
2
1
LC
OC
g
200µm
3
LC
j
100µm
4
LC
k
50µm
2
i
50µm
1
h
50µm
4
LC
l
50µm

**Fig. 3 Anatomy of lenticular galls on *Inga ingoides* (Fabaceae: Caesalpinioideae). a)** Macroscopic aspect. **b)** Diagram of the origin and fates of gall tissues. **c-f)** Young stage. **c)** Gall compartments: lower outer tissue compartment (LOC), typical nutritive tissue (red dotted), upper outer tissue compartment (UOC). **d)** Detail of tissue compartment and typical nutritive tissue. **e)** Detail of hyperplasia and hypertrophy of vascular tissues (red arrows). **f)** Extremity of lower outer tissue compartment. **g-l)** Mature stage. **g)** Diagram of a transverse section, showing the tissue organization of gall in mature stage. **h-i)** The lignification of the LOC. **j-k)** Vascular tissues (red arrows) around the nutritive tissue (j) and toward the extremity of the outer tissue compartment (k). **l)** Dense cytoplasm and prominent nucleus (arrow) in nutritive cells. **m)** Lignification of nutritive tissue in senescent stage. Staining: a-f: toluidine O blue; h: Wisner's test; i-m: Astra blue and safranin

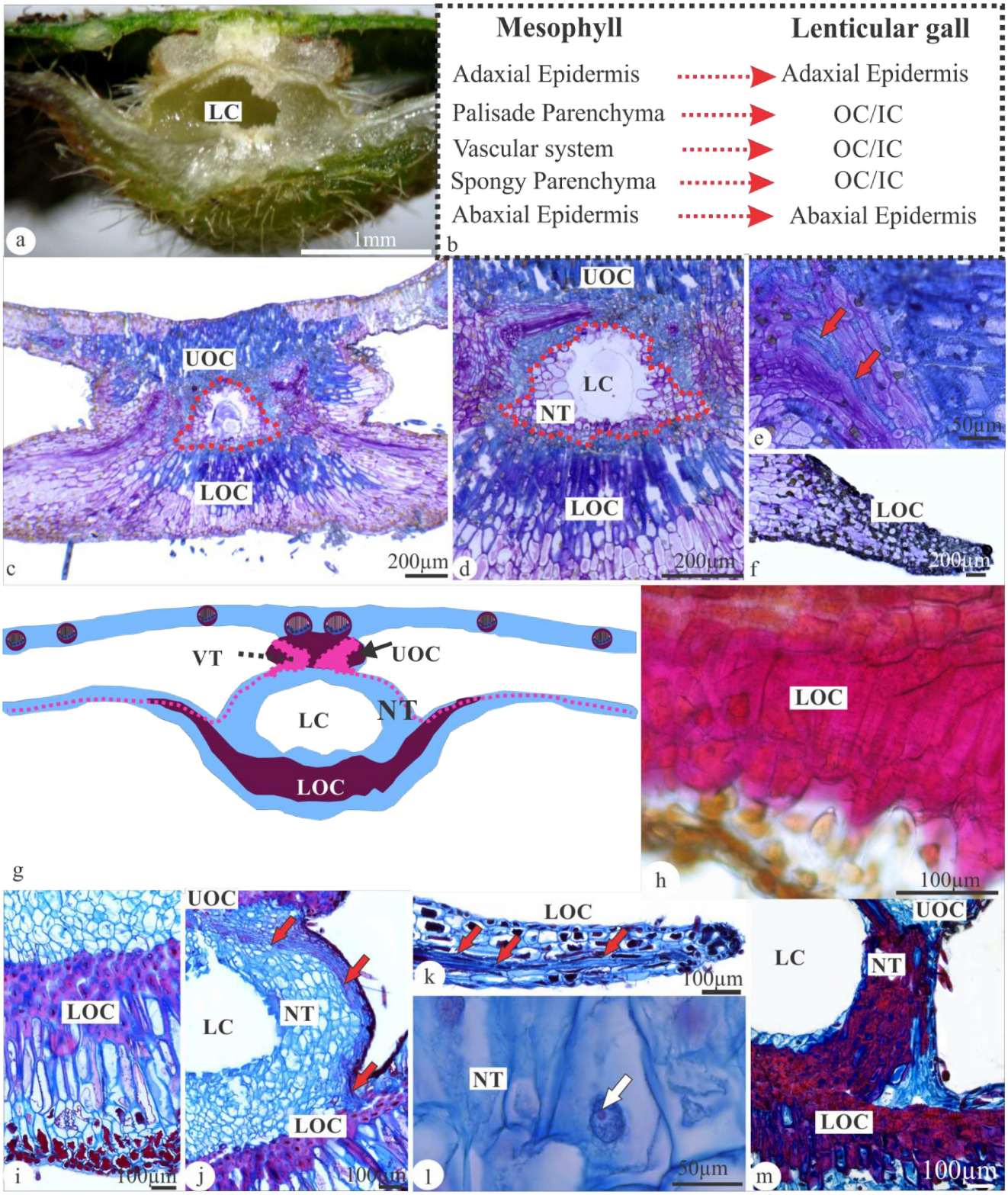
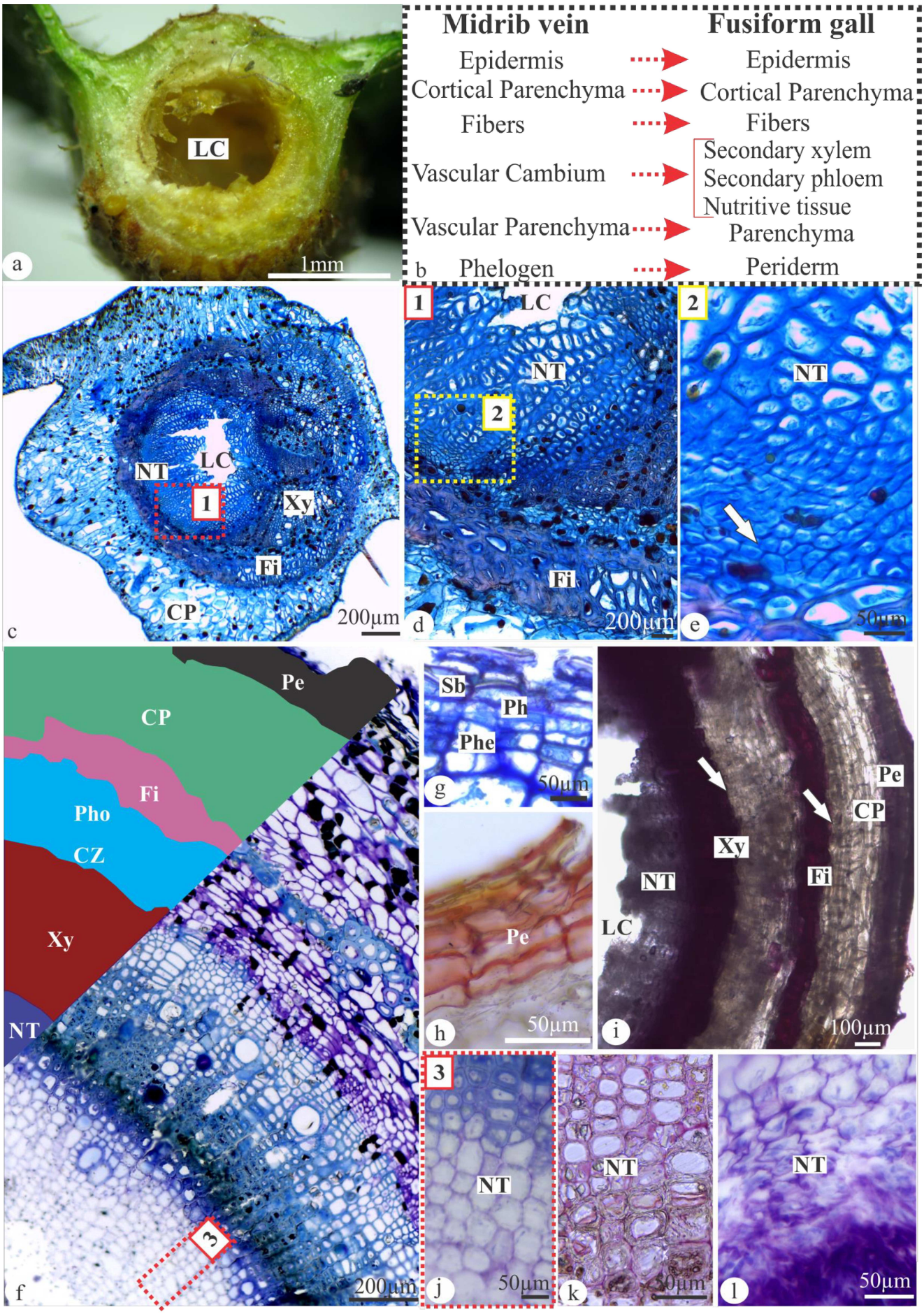

a
LC
1mm
b
Mesophyll
Lenticular gall
Adaxial Epidermis
Adaxial Epidermis
Palisade Parenchyma
OC/IC
Vascular system
OC/IC
Spongy Parenchyma
OC/IC
Abaxial Epidermis
Abaxial Epidermis
c
UOC
LOC
200µm
d
UOC
LC
NT
LOC
200µm
e
50µm
f
LOC
200µm
g
VT
UOC
LC
NT
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h
LOC
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i
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j
UOC
LC
NT
LOC
100µm
k
LOC
100µm
l
NT
50µm
m
UOC
LC
NT
LOC
100µm

**Fig. 4 Anatomy of fusiform galls on *Inga ingoides* (Fabaceae: Caesalpinioideae). a)** Macroscopic aspect. **b)** Diagram of the origin and fates of gall tissues. **c-e)** Young stage. **c)** The hyperplasia and hypertrophy of cortical parenchyma (CP) and atypical activity of the vascular cambium toward the larval chamber (LC). The number 1 indicates cells of the correspondent region in (d). **d-e)** Detail of cambium region (arrows) and nutritive tissue (NT). The number 2 indicates cells of the correspondent region in (e). **f-l)** Mature stage. **f)** Overview of gall with periderm (pe), cortical parenchyma (CP), fibers (Fi), phloem (Pho), cambium zone (CZ), xylem (Xy) and nutritive tissue (NT). The red border square and the number 3 indicate cells of the correspondent region in (j). **g)** Detail of periderm with suber (Sb), phellogen (Ph) and phelloderm (Phe). **h)** Suberin in the periderm. **i)** Lignins in the xylem and fibers. **j-l)** Inner tissue compartment. **j)** Cells with thick walls. **k)** Pectins in cell walls. **l)** Cells with irregular thickened walls and cells with thin and sinuous walls. Staining: c-e: Astra blue and safranin; f-g, j,l: toluidine O Blue; h: Sudan red B; i: Wisner's test, k: ruthenium red


a
LC
1mm
Midrib vein
Epidermis
Cortical Parenchyma
Fibers
Vascular Cambium
Vascular Parenchyma
Phelogen
b
Fusiform gall
Epidermis
Cortical Parenchyma
Fibers
Secondary xylem
Secondary phloem
Nutritive tissue
Parenchyma
Periderm
c
NT
LC
1
Xy
Fi
CP
200µm
d
1
LC
NT
2
Fi
200µm
e
2
NT
50µm
f
Pe
CP
Fi
Pho
CZ
Xy
NT
3
200µm
g
Sb
Ph
Phe
50µm
h
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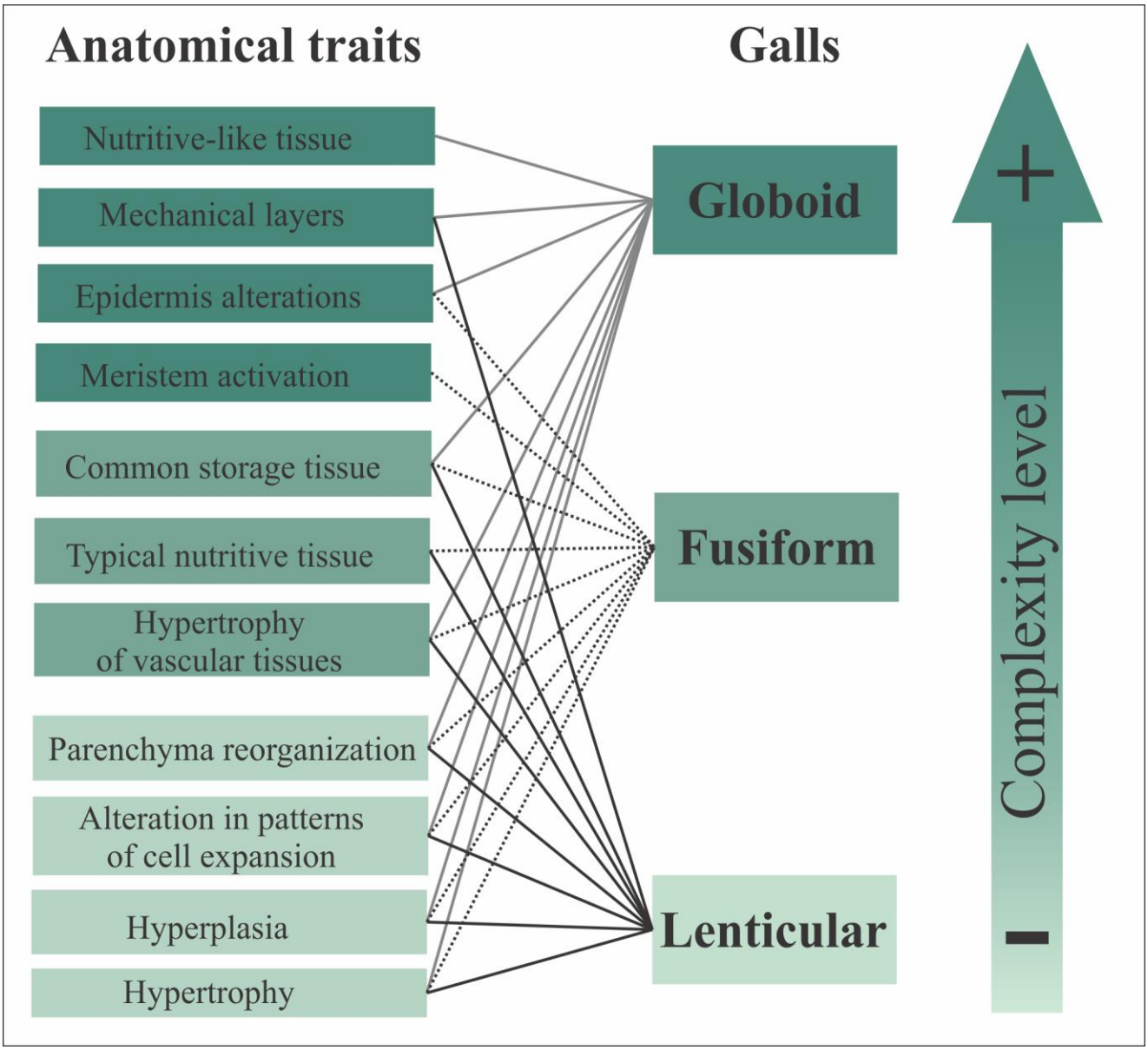
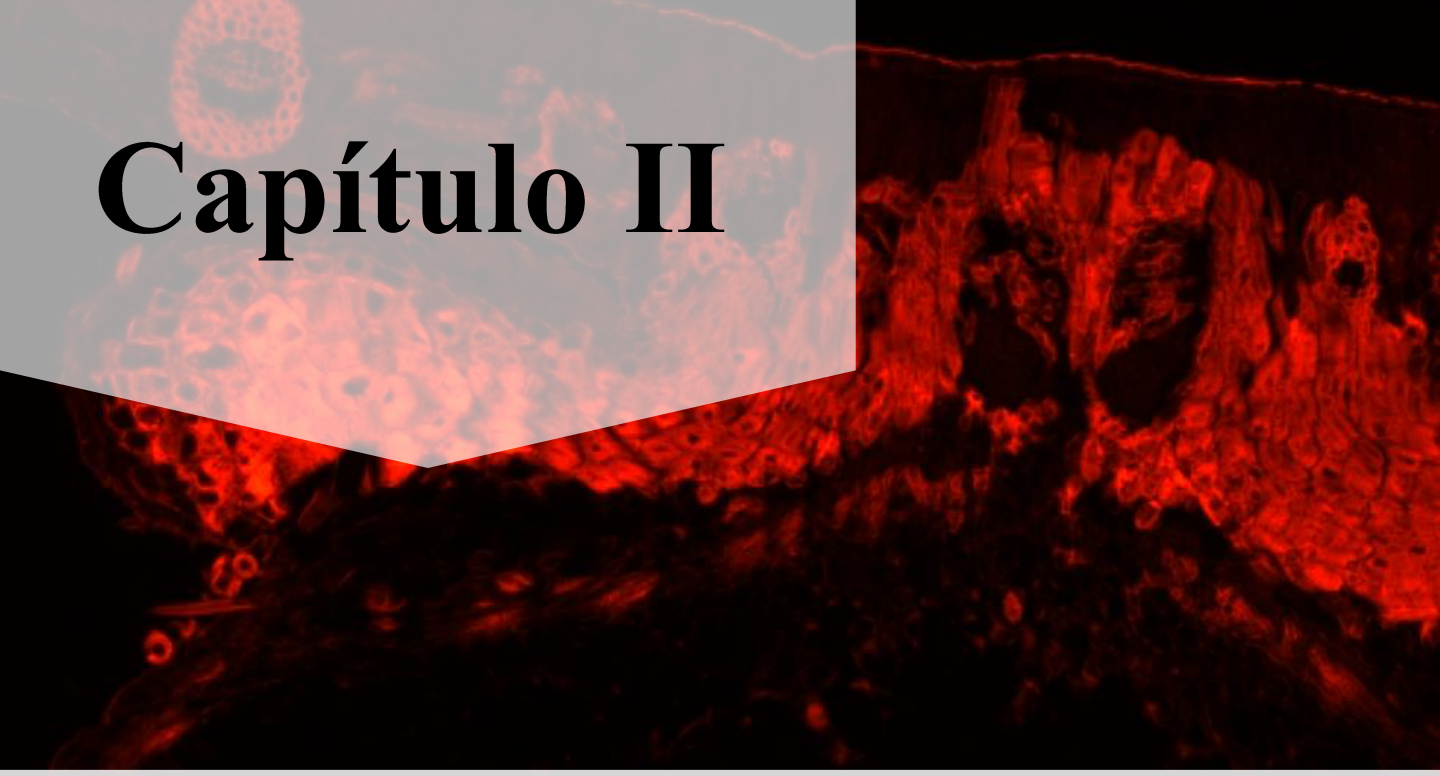
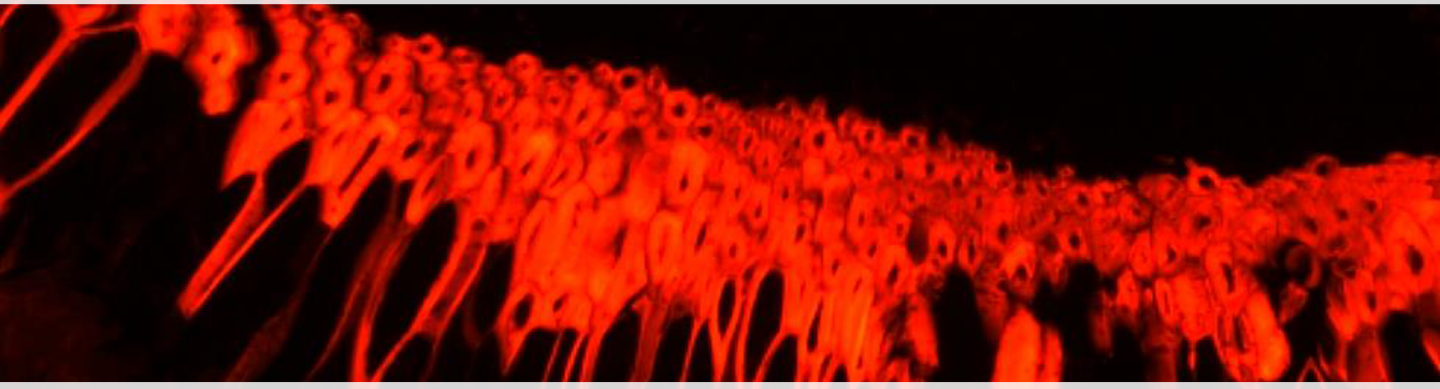



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# Capítulo II

# The influence of gall position over xylem features in leaflets of *Inga ingoides* (Rich.) Willd. (Fabaceae: Caesalpinioideae)



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ORIGINAL ARTICLE

# The influence of gall position over xylem features in leaflets of *Inga ingoides* (Rich.) Willd. (Fabaceae: Caesalpinioideae)

**Gracielle Pereira Pimenta Bragança[1] · Mariana de Sousa Costa Freitas[1] · Rosy Mary dos Santos Isaias[1]**

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## Abstract

***Key message*** **The origin of xylem cells, intracellular variation, and gall position relative to the host plant organ are related to mechanisms that positively compensate hydraulic conductivity in galls.**

**Abstract** Gall position in its host organ may be determinant for the new patterns of xylem differentiation. Leaf galls may be attached to the host leaf by a peduncle, as the extralaminar galls, or may develop in a morphological continuum with their host organ, as the intralaminar galls. Due to these two distinct positions and the related patterns of cell differentiation, gall vascular cells and tissues may vary in area and size, which were investigated in the structural features of the vessel elements in galls and in their connections with their host leaflets in three gall morphotypes associated to *Inga ingoides*. Also, as phytohormones orchestrate the differentiation of vascular cells, the sites of accumulation of auxins and cytokinins were detected by histochemical techniques. Besides aspects of cell differentiation, cytometric and histometric xylem features, as well as the relative water content were evaluated looking for compensatory mechanisms related to hydraulic status of the galls and the gall constriction hypothesis. The detection of auxins was related with the differentiation of new vascular cells. The origin of xylem cells, intracellular variation, and gall position relative to the host plant organ are related to mechanisms that positively compensate the hydraulic conductivity in galls. Extralaminar galls develop as appendages of adventitious origin, which theoretically imply in rupture with the host leaf ontogenetical pattern. Based on the fact that gall position influences xylem cell features, the gall-constriction hypothesis was revisited with leaflet galls as models of study, but was corroborated just for the intralaminar galls.

**Keywords** Auxins · Compensatory hydraulic mechanism · Cytokinins · Histochemistry · Insect gall · Vessel-constriction hypothesis

## Introduction

The redifferentiation of new vascular cells in gall developmental sites, and their connection to the pre-existing vascular cells of the host plant organ are fundamental for gall development (Dolzblasz et al. 2018). The gall vascular tissues may have similarities in terms of dimensions and proportions of cells with the host organs (Aloni et al. 1995; Arduin and Kraus 2001; Dias et al. 2013; Ferreira and Isaias 2013), but dissimilarities may occur. Accordingly, gall position relates to peculiar features regarding the neo-vascular patterns (Carneiro et al. 2014), as well as the vascular connections of the galls with their host organs. Vascular connections may demand cytological peculiarities linked to the origins of the new vascular tissues: the procambium (Kraus et al. 2002), the vascular cambium (Ferreira and Isaias 2013), or the parenchyma cells (Aranda-Rickert et al. 2017). The activation of cytological programs toward the differentiation of the vascular cells demands the intermediation of auxins and cytokinins (Aloni 1987, 2013; Ye 2002; Matsumoto-Kitano et al. 2008; Schuetz et al. 2013). The neo-vascularization mediated by these hormones (Aloni et al. 1995; Ulrich and Aloni 2000) connects the vascular system of the gall with that of the host plant, and supply the hydraulic conductivity of the new organ.

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We assume that the needs for high rates of cell hypertrophy and cell turgidity require adequate water supply, which may demand alterations for gall vascularization, regarding xylem cells. The xylem system in regions bellow to the gall can be more developed, which limits the transport of water to the aerial portion above the gall, as postulated by the gall-constriction hypothesis (Aloni et al. 1995; Ulrich and Aloni 2000). In this case, the development of compensatory mechanisms may guarantee hydraulic functioning, crucial for gall development and host organ survivorship. Among these compensatory mechanisms, the origin and proportion between gall parenchyma and vascular tissues, as well as the intracellular variation in the gall vascular system may be associated. The neo-differentiation of vascular cells seems more evident in extralaminar galls than in intralaminar galls (Isaias et al. 2013, 2014), where a "*continuum*" of tissues is clearly established. The implications of gall position to vascularization will be addressed here regarding insect galls on *Inga ingoides* (Rich.) Willd. (Fabaceae: Caesalpinioideae).

Current study focuses on mapping xylem area and cell features in the three gall morphotypes, and the relationship with the sites of accumulation of auxins and cytokinins. Additionally, gall vascular patterns are analyzed on the premise that the vessel elements in the extralaminar leaflet galls will be longer and wider than in the intralaminar galls, on the light of the "gall-constriction hypothesis", firstly proposed for stem galls (Aloni et al. 1995). The following questions are addressed: (1) do the sites of xylem neoformation in gall developmental sites relate to the accumulation of auxins and cytokinins? (2) How does the vascular pattern relate to compensatory hydraulic mechanisms in extralaminar and intralaminar galls? And (3) does xylem vessel constriction occurs similarly in extralaminar and intralaminar leaflet galls?

## Materials and methods

### Sampling

The study involves the leaflets of the superhost *I. ingoides* and the three associated Cecidomyiidae-induced gall morphotypes. Two of these morphotypes are extralaminar, but diverge in shape (globoid and lenticular), and one morphotype is intralaminar (fusiform). Mature leaflets with globoid, lenticular and fusiform galls in the young and mature stages, induced by three unidentified species of Cecidomyiidae on *I. ingoides*, were collected at Parque Estadual Serra Verde (PESV-MG) (19° 47′ 22.47″ S, 43° 57′ 32.35″ W), Belo Horizonte, Minas Gerais, Brazil. Fragments (2 $mm^2$) of galled leaflets in three portions: distal, gall and proximal portions (Fig. 1) were used for the preparation of semi-permanent and permanent slides for histochemical and structural analyzes.

### Histochemistry

To detect the sites of accumulation of phytohormones, in young and mature galls, hand-made transverse sections of fresh samples ($n = 3$ per gall) were used. For auxin detection, Ehrlich reagent (1% *p*-dimethylaminobenzaldehyde in 1 mol $L^{-1}$ HCl; w/v) for 5 min at room temperature was used (Leopold and Plummer 1961; Bedetti et al. 2018); the positive

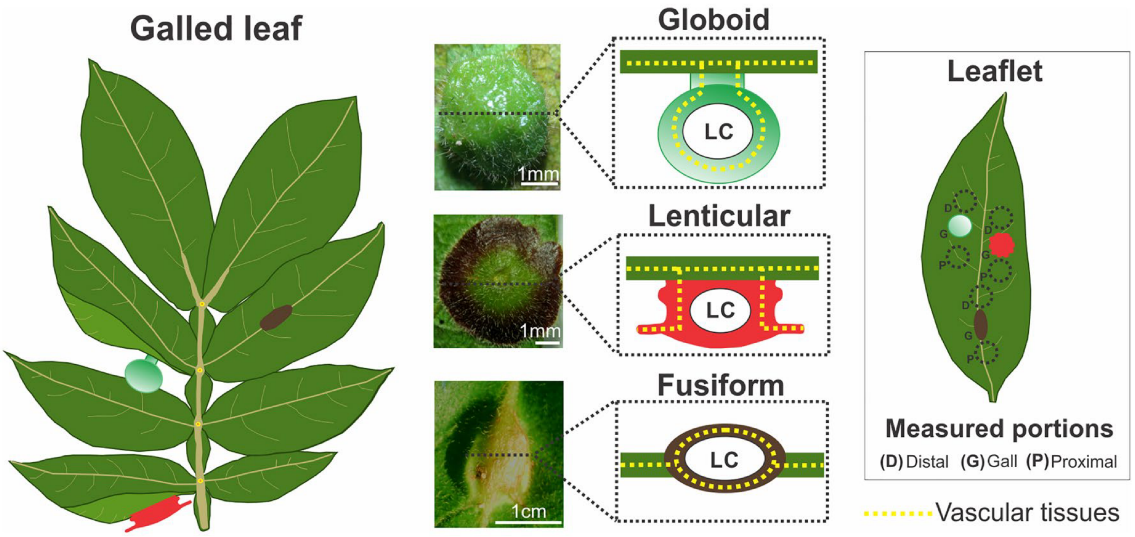



**Fig. 1** Schematic representation of a leaf hosting globoid, lenticular and fusiform galls induced by Cecidomyiidae (Diptera) on *Inga ingoides* (Fabaceae). Globoid galls are represented in green, lenticular galls in red and fusiform galls in brown. The leaflet in the box illustrates the distal (D), gall (G) and proximal (P) portions used to measure the height and width of the vessel elements, and to calculate the relative water content. *LC* larval chamber

reaction is pink. To detect *trans*-zeatin riboside (cytokinin) accumulation, 0.5% silver nitrate in absolute ethanol was used for 5 min, followed by incubation in 0.2% bromophenol blue and 0.15% silver nitrate in ethanol PA-ethyl acetate for 5 min at room temperature. The sections were washed three times in ethanol PA and mounted on slides with ethanol PA (Touchstone 1992; Bedetti et al. 2018); the positive reaction is blue. Blank sections were used as control.

## Structural analyses

### Embedding

Fragments of young and mature galls ($n = 5$) fixed in 2.5% glutaraldehyde and 4.5% formaldehyde in phosphate buffer (Karnovsky 1965, modified to 0.1 mol $L^{-1}$; pH 7.2) were processed for embedding in Paraplast X-TRA® or Leica® Historesin (O'Brien and McCully 1981; Kraus and Arduin 1997). For Paraplast X-TRA® embedding, the fragments were dehydrated in an *n*-butyl series (Johansen 1940). Transverse and longitudinal anatomical sections (12–14 µm) were obtained in a rotatory microtome (Leica® BIOCUT 2035), and affixed to the slides with Bissing's adhesive (Kraus and Arduin 1997). The slides were deparaffinized with butyl acetate and re-hydrated in an ethanol series (Bukatsch 1972). The sections embedded in Paraplast X-TRA® were stained with Astra blue and safranin (9:1 *v/v*) (Bukatsch 1972, modified to 0.5%), dehydrated in an ethanol series and butyl acetate, and mounted in colorless varnish Acrilex® (Paiva et al. 2006). For historesin inclusion, the sections were dehydrated in an ethanol series (Johansen 1940), embedded in Leica® Historesin (Leica, Wetzlar, Germany), and sectioned (5–10 µm) with a rotatory microtome (Leica® BIOCUT 2035). The sections were stained with 0.05% toluidine blue O in 0.1 mol $L^{-1}$ acetate buffer, pH 4.7 (O'Brien and McCully 1981).

### Maceration

For individualization of vessel elements, fixed fragments (2 $mm^2$) of distal portions, galls and proximal portions (n = 5 of each portion) of the leaflets with mature galls were macerated with Jeffrey mixture (10% nitric acid and 10% chromic acid) for 48 h (Jeffrey 1919). Subsequently, the samples were washed with distilled water, and stained with 0.5% safranin for 24 h. The slides were mounted with Kaiser's jelly glycerin (Kraus and Arduin 1997).

## Image capture

For anatomical, cytometrical and histometric analysis, images were obtained with a digital camera Leica ICC50 HP® coupled to a Leica DM500® light microscope (Leica, Wetzlar, Germany). For the visualization of lignified cell walls and analysis of the continuity of the vascular tissues, sections of young and mature galls stained with Astra blue and safranin were analyzed and photographed with a digital camera (Leica® DFC 7000 T) coupled to a fluorescence microscope (Leica® DM 2500 LED), with green emission light (515 nm), with rodamine filter (515–560 nm excitation and 590 nm emission filter) (Ferreira et al. 2017a).

### Cytometric and histometric analyses

The height and width of 25 vessel elements in distal portions, galls and proximal portions were measured ($n = 5$ of each portion, and five vessel elements per portion). The percentage of area occupied by the ground and vascular tissues in the globoid, lenticular and fusiform galls ($n = 5$ of each gall morphotype, three areas per gall) was calculated with photomicrographs using the Zeiss AxionVision® software.

## Relative water content (RWC)

Discs of mature globoid galls and of the relative non-galled leaflet distal and proximal portions (26.75 $mm^2$) were isolated ($n = 10$). These disks were weighted in three conditions: (1) immediately after cutting for the determination of the fresh weight (FW), (2) after immersion in distilled water for 24 h at 4 °C, and drying with a towel paper for the determination of the turgid weight (TW), and (3) after drying in a 60 °C oven during 48 h, for determination of the dry weight (DW). The relative water content was obtained following the equation: (FW–DW)/(TW–DW) (França et al. 2012). Due to the low availability in the field, the lenticular and fusiform galls were not used for this analysis.

## Statistical analysis

Parametric data were compared using the Student's *T* test (for two categories) or one-way ANOVA (for three or more categories) followed by the Tukey's test. Non-parametric data were tested with the Mann–Whitney test (for two categories) and the Kruskal–Wallis test (for three or more categories) followed by the Dunn's test. The tests were performed with the SigmaStat® (Systat Software, Inc., Chicago, Illinois) and the GraphPad prism 5.0® softwares. All tests used $\alpha = 0.05$.

# Results

## Gall general features

The extralaminar galls (globoid and lenticular) are connected to the host organ through a peduncle. The globoid

galls may be induced both on the abaxial and adaxial leaflet surface; and the lenticular galls are induced only on the abaxial surface. The intralaminar fusiform galls may be induced both in the primary and secondary veins, and maintain the continuity with the midrib of the host leaflet (Fig. 1).

## Gall anatomy and histochemical detection of phytohormones

### Globoid gall

Extralaminar globoid galls result from the redifferentiation of the epidermis, parenchyma, and vascular cells. In young and mature stages, the gall has an outer tissue compartment with uniseriate epidermis and parenchymatic cells, which involve the inner tissue compartment constituted by the nutritive tissue (Fig. 2a). The vascular system differentiates from the procambium and parenchymatic cells, and connects with the vascular system of the host leaflet by a peduncle (Fig. 2b). In mature stage, the vascular system is constituted by diminutive vascular bundles immersed in the parenchyma of the outer tissue compartment (Fig. 2c). Auxins are detected in the cytoplasm of the parenchyma cells of the outer tissue compartment (Fig. 2d), while cytokinins are detected in the cytoplasm of nutritive cells and vascular cells located in the parenchyma of the outer tissue compartment (Fig. 2e).

### Lenticular gall

Extralaminar lenticular galls result from the redifferentiation of epidermis, parenchyma, and vascular cells. In the young and mature stages, the galls may be divided in three tissue zones: the upper, the lower tissue compartment and the nutritive tissue. In young stage, the vessel elements are poorly or non-lignified (Fig. 3a, b). The mature stage is characterized by the lignification of parenchyma cell walls in the upper and lower tissue compartments, and by the development of vascular bundles and nutritive tissue (Fig. 3c, d). The gall vascular bundles are directed to the gall peduncle and connect to the non-galled vascular portion (Fig. 3e). Auxins are detected in the cytoplasm of parenchymatic cells of the outer tissue compartment (Fig. 3f), while cytokinins are detected in the cytoplasm of cells of the outer tissue compartment and of the nutritive tissue (Fig. 3g).

### Fusiform gall

Intralaminar fusiform galls are induced both in the primary and secondary veins, and result from vascular cambium and phellogen activities. In the young stage, the gall has uniseriate epidermis and cortical parenchyma with hypertrophied cells. The vascular system is in secondary growth, and the vascular cambium produces the nutritive tissue in an atypical pattern (Fig. 4a, b). In the mature stage, the gall has an outer tissue compartment constituted by periderm, cortical parenchyma, fibers, secondary

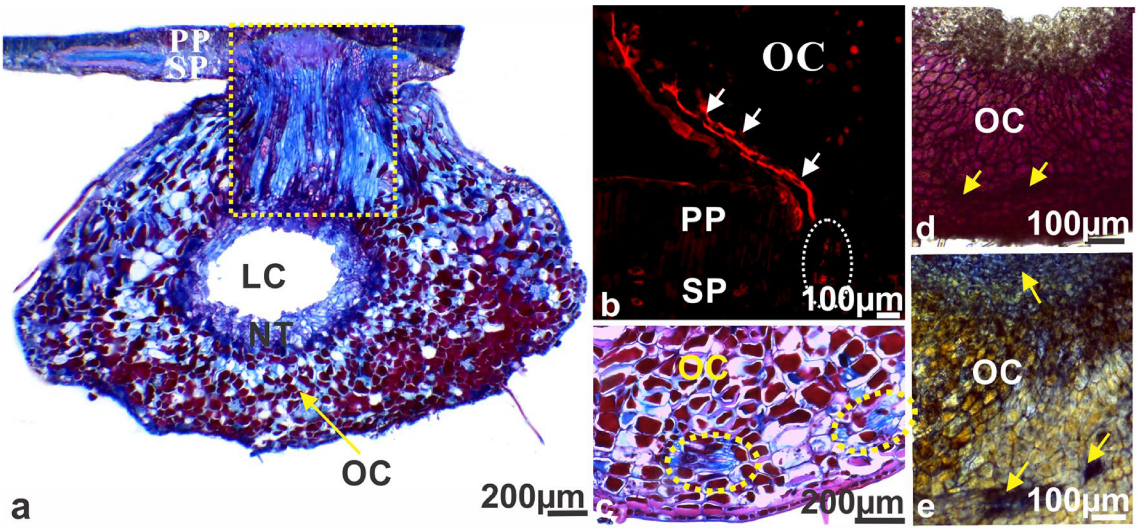



**Fig. 2** Globoid galls induced by Cecidomyiidae (Diptera) on *Inga ingoides* (Fabaceae). **a** Transverse anatomical section of a young gall, evidencing the peduncle and the continuum with the leaflet (yellow square). **b** Epifluorescence microscopy evidencing the continuity of vascular tissues (white arrows and dotted circle). **c** Small vascular bundles in the outer tissue compartment in a mature gall (yellow arrows). **d**, **e** Histochemical analyses. **d** Indole acetic acid (IAA) detected in the cells of the outer tissue compartment (yellow arrows). **e** Transzeatin riboside (cytokinin) detected in the nutritive cells and vascular bundles (yellow arrows). *LC* larval chamber, *OC* outer tissue compartment, *NT* nutritive tissue, *PP* palisade parenchyma, *SP* spongy parenchyma

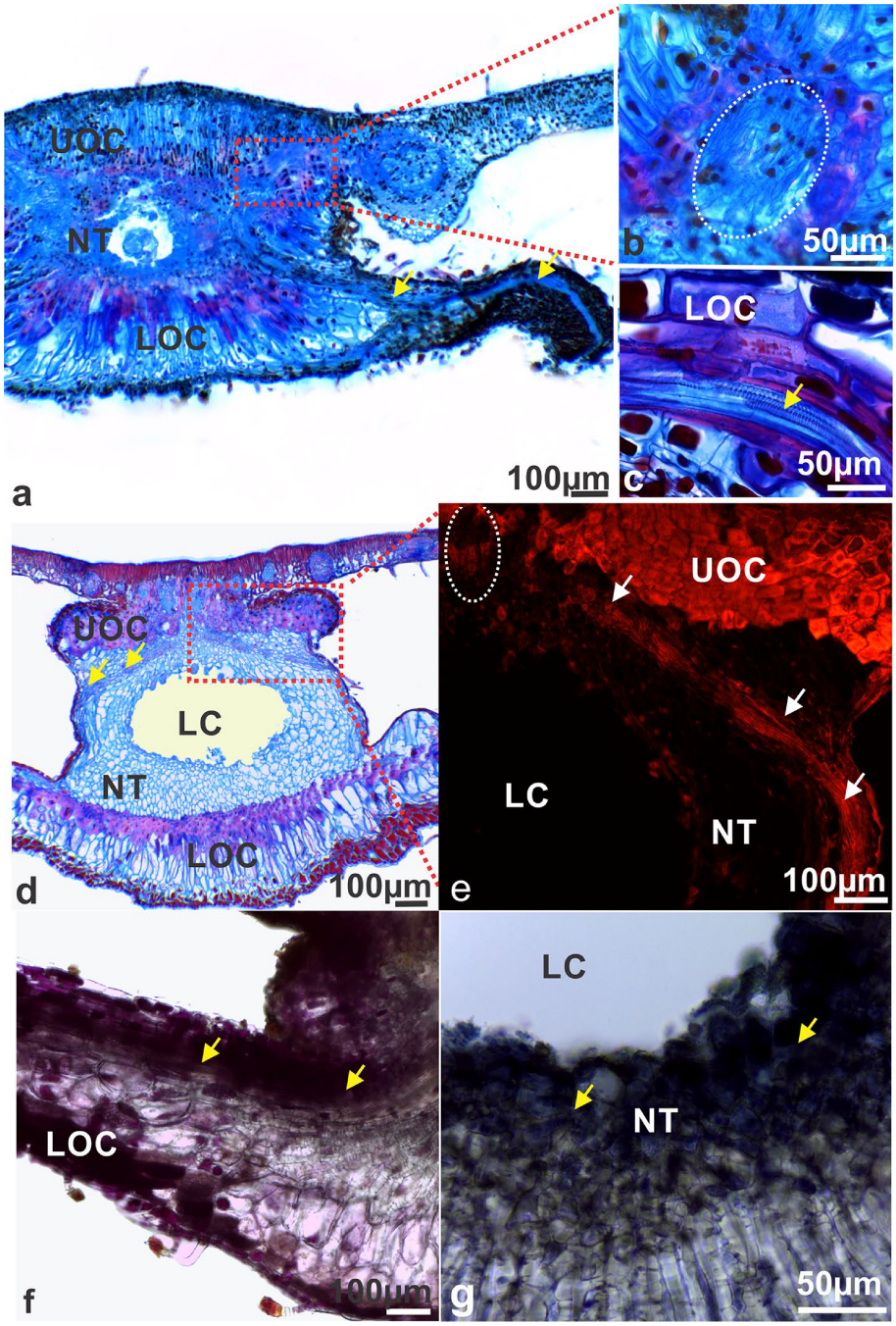
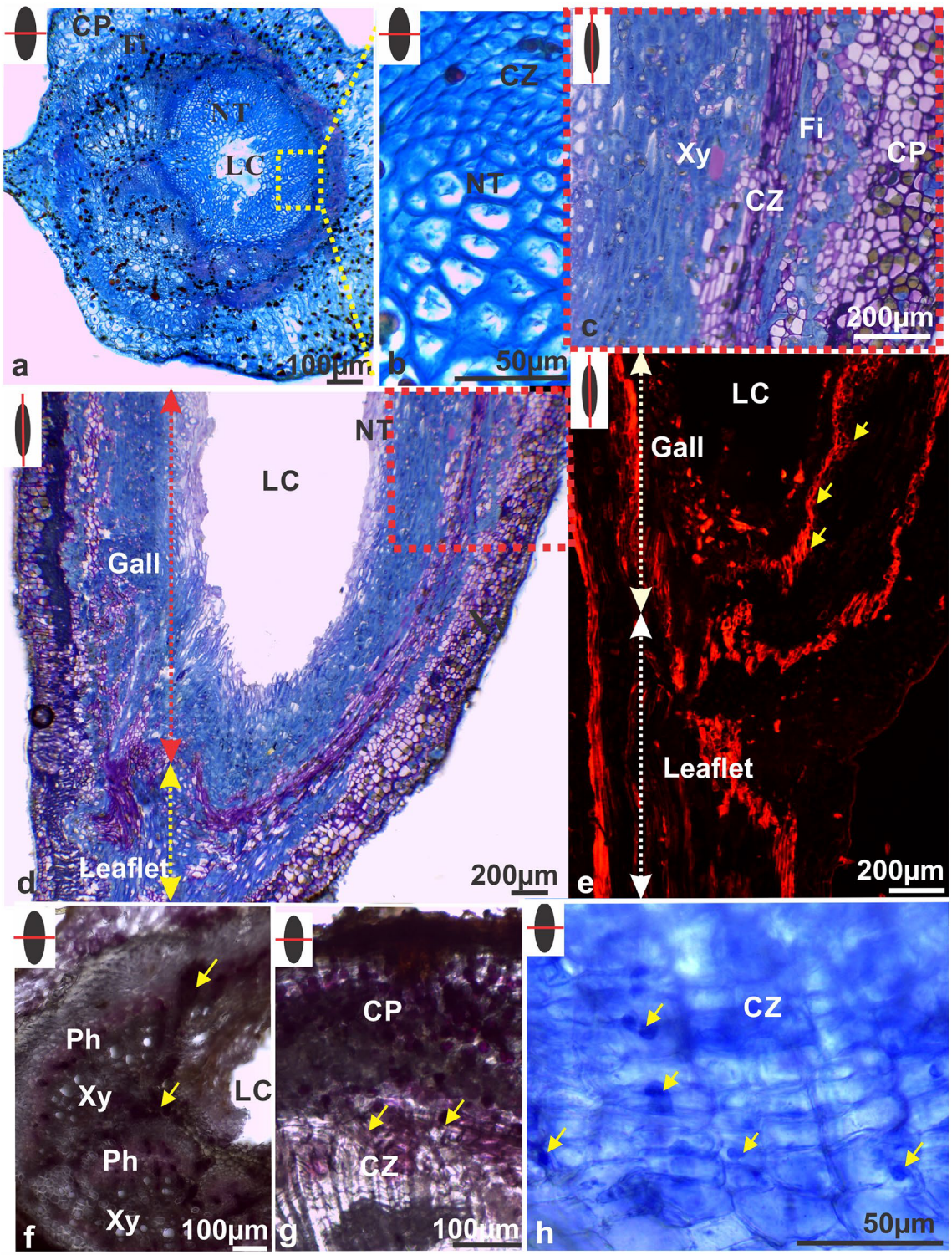



**Fig. 3** Lenticular galls induced by Cecidomyiidae (Diptera) on *Inga ingoides* (Fabaceae). **a** Transverse anatomical section of a young gall, evidencing the peduncle with vascular bundle (yellow square). **b** Detail of non-differentiation of vascular cells in a young gall. **c**, **e** Detail of vascular tissues (yellow arrows) in a mature gall. **c** Lateral tissue compartment. **d** Nutritive tissue. **e** Epifluorescence microscopy evidencing the continuity of vascular tissues (white arrows). **f**, **g** Histochemical analyses. **f** Detection of indole acetic acid (IAA) in the cells of outer tissue compartment (yellow arrows). **g** Detection of transzeatin riboside (cytokinin) in nutritive tissue and outer tissue compartment (yellow arrows). *LC* larval chamber, *LOC* lower outer tissue compartment, *NT* nutritive tissue, *OC* outer tissue compartment, *UOC* upper outer tissue compartment

phloem, cambial region, and secondary xylem (Fig. 4c); the vascular cambium reassumes its typical activity. The inner compartment is constituted by the nutritive tissue (Fig. 4d). The gall vascular tissues are connected to those of the non-galled leaflet portion in a continuum (Fig. 4d, e). Auxins are detected in the cytoplasm of vascular parenchyma cells, phloem and cambial zone of the outer tissue compartment during the young stage (Fig. 4f), and in the cytoplasm of cortical parenchyma and cambial zone in mature stage (Fig. 4g). Cytokinins are detected in the nuclei of the cambial zone cells (Fig. 4h).

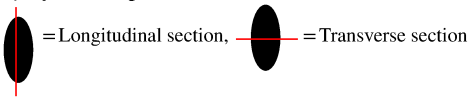
## Cytometry and histometry

The vessel elements in non-galled distal portions are 82% ($p = 0.01$) longer than those of the globoid galls, and 72% ($p = 0.01$) longer than those of the non-galled proximal portions. However, there is no significant difference in the width of the vessel elements among the three regions ($p > 0.07$). In lenticular galls, the length of the vessel elements is statistically similar between the three regions ($p = 0.36$), but the vessel elements are 35% narrower ($p = 0.02$) in galls than in the non-galled distal portions, and 45% ($p = 0.005$)




narrower than that of the non-galled proximal portions. In fusiform galls, the length of the vessel elements in the non-galled distal portions is statistically similar to that of the galls ($p = 0.12$), but the vessel elements are 44% ($p = 0.006$) smaller than those of the non-galled proximal portion. The vessel elements in fusiform galls are 100% wider ($p = 0.003$) than those of the distal portions (Fig. 4). There is no significant statistical difference between the length of the vessel elements among the globoid, lenticular and fusiform galls ($p = 0.36$). However, the vessel elements are 59% ($p < 0.001$) longer in fusiform galls than in the globoid galls, and 65% ($p < 0.001$) longer than those of the lenticular galls (Fig. 5).

◂**Fig. 4** Fusiform gall induced by Cecidomyiidae (Diptera) on *Inga ingoides* (Fabaceae). **a**, **b** Transverse anatomical section of young galls stained with Astra blue and safranin. **a** Atypical vascular cambium activity. **b** Detail of cambium zone and nutritive tissue. **c**, **d** Longitudinal sections of mature galls stained with toluidine blue O with tissues differentiated from the typical activity of the vascular cambium. **c** Detail of tissue organization in the gall. **d** Continuity of the tissues under light microscopy. The red arrow indicates the galled tissues and the yellow arrow indicates the non-galled leaflet tissues. **e** Continuity of the tissues under epifluorescence microscopy, evidencing secondary xylem (yellow arrows). **f**, **h** Histochemical analyses. **f** IAA detected (yellow arrows) in transverse sections of galls. **f** Young gall. IAA detection in the phloem, cambium zone and vascular parenchyma. **g** Mature gall. IAA detection in the cells of cortical parenchyma and cambium zone. **h** Detection of transzeatin riboside (cytokinin) in the nucleus of cells in the cambium region (yellow arrows). *Fi* fibers, *CP* cortical parenchyma, *CZ* cambium zone, *LC* larval chamber, *OC* outer tissue compartment, *IC* inner tissue compartment, *Xy* xylem, *Ph* phloem, *NT* nutritive tissue. Section orientation:




The percentage of area occupied by the vascular tissues is higher in fusiform galls (39%; $p < 0.001$) than in globoid (8%; $p < 0.001$), and in lenticular galls (9%; $p < 0.001$). The area occupied by the vascular tissues is statistically similar ($p = 0.472$) between the globoid and lenticular extralaminar galls (Fig. 6).

### Relative water content

The relative water content (RWC) is similar in the proximal and distal portions of the globoid galls ($p = 0.240$). The RWC is higher in galls than in the proximal ($p < 0.001$) and distal portions ($p = 0.004$) of the non-galled tissues (Fig. 7).

## Discussion

### Influence of AIA and CKs over xylem development in galls

The histolocalization of auxins in the outer parenchyma cells of the extralaminar gall morphotypes associated to *I. ingoides* relates to sites of cell hypertrophy, as well as sites of the differentiation of xylem cells. This peculiar histolocalization of auxins seems to relate to cell redifferentiation mediated by phenolic compounds produced in response to redox imbalance, which end up blocking the AIA oxidases (Hori 1992; Bedetti et al. 2014, 2017), and consequently the concentration of auxins increase in gall developmental sites. The axial, polar, and continuous flow of auxins toward a specific group of cells results not only in sites of cell hypertrophy, but also in new vascular bundles, which connect to the bundles of the host organ (Sachs 2000; Aloni 2013, 2015). The cytological hypertrophying processes, and the formation of the vascular tissue network ensures the water supply needed for gall development, and for the favorable microenvironment that guarantees the survivorship of the galling herbivores.

Besides auxins, the cytokinins are fundamental for the redifferentiation of tracheary elements from mesophyll cells (Fukuda and Konamine 1980; Fukuda 1997; Milioni et al. 2001; Ye 2002; Schuetz et al. 2013). Cytokinins regulate vascular activity (Matsumoto-Kitano et al. 2008), and coordinate the intense hyperplasia in gall developmental sites (Ferreira et al. 2017b, 2019). The accumulation of this phytohormone in the nutritive tissues of the extralaminar galls on *I. ingoides* can be responsible for cell cycle progression and maintenance of the sink of photoassimilates to the region of high cytological metabolism, and intense feeding activity of the Cecidomyiidae. In the fusiform gall, i.e., the intralaminar morphotype, the accumulation of cytokinins seems to have positively regulated the activity of the vascular cambium, and have increased cambial cells sensitivity to auxin, promoting the new ontogenetic pattern peculiar of the gall. The cytokinins can stimulate the low transport of auxins in the vascular cambium (Aloni 2001), which ends up in the slow differentiation of vessel elements, and their larger expansion prior to secondary cell wall deposition (Aloni and Zimmermann 1983; Aloni 2015). Such balance on the speed of differentiation and cell expansion may explain the larger vessel elements width in the fusiform intralaminar galls on *I. ingoides*.

Auxins and cytokinins act synergistically toward the differentiation of new vascular bundles along the development of plant organs (Ursache et al. 2013), and may coordinate the difference in vessel elements width in extralaminar and intralaminar galls on *I. ingoides*. The narrow vessel elements of the outer tissue compartment of the extralaminar galls may be consequence of a fast differentiation, which relates to the intense accumulation of auxins (Aloni and Zimmermann 1983). On the other hand, the balance between the levels of auxins and cytokinins can result in the differentiation of wide vessel elements, as observed as a response of the cambium initials in the intralaminar fusiform galls. The different patterns of vessel elements in extralaminar and intralaminar galls on *I. ingoides* suggest that cytokinins and auxins vary within the galls toward peculiar compensatory mechanisms regarding water transport.

### Compensatory mechanisms of gall vascularization

The origin of vascular tissues in galls (parenchyma, procambium and vascular cambium) associated with local hormonal stimulation resulted in different structural responses toward water transportation in extralaminar

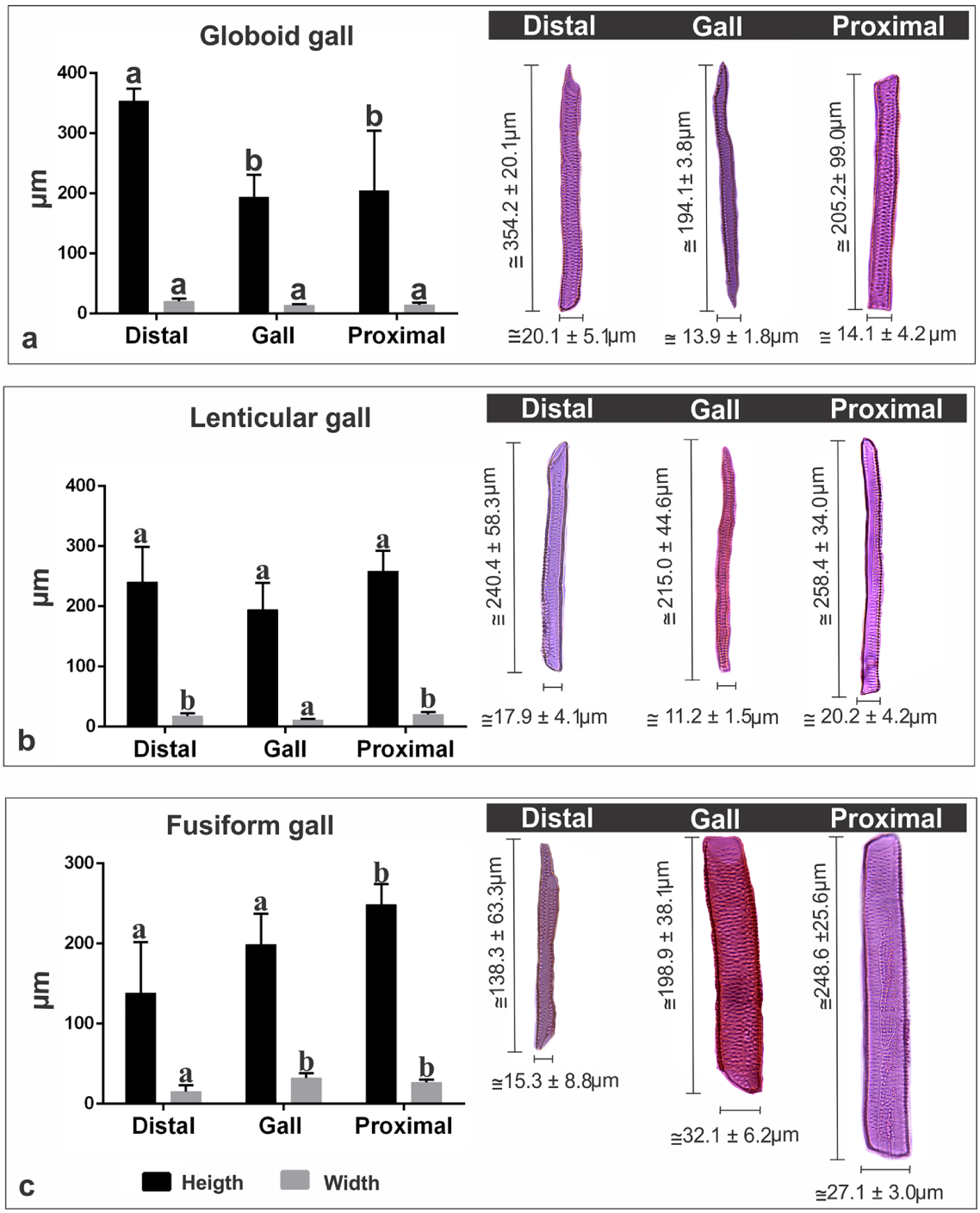



**Fig. 5** Width and height of vessel elements in distal portion, gall, and proximal portion of leaflets of *Inga ingoides* (mean ± standard deviation). **a** globoid gall, **b** lenticular gall, **c** fusiform gall

and intralaminar galls. Even though the need for vascular neoformation has been reported especially for extralaminar galls (Isaias et al. 2011, 2013, 2014; Carneiro et al. 2017; Guzika et al. 2017; Nogueira et al. 2018), the increment in vascular tissues has also been evidenced in the intralaminar gall on *I. ingoides*. Gall position on the host leaflets seems to be determinant not only for the increment of vascular cells, but also for the origin and width of the vessel elements. The differentiation of narrow vessels in extralaminar galls may rely on their ontogenetical origin, i.e., parenchyma cells or procambium initials, and results in low vascularization increment. However, the resultant

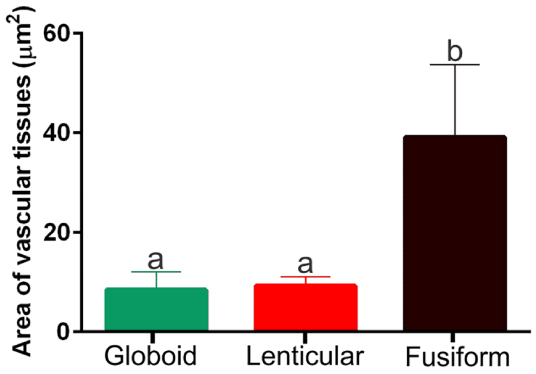



**Fig. 6** Area ($\mu m^2$) occupied by vascular tissues in the globoid, lenticular and fusiform galls induced by Cecidomyiidae (Diptera) on *Inga ingoides* (Fabaceae). Bars indicate the means and standard deviations. Same letters on bars indicate statistically equal values and different letters indicate significant differences ($p < 0.05$)

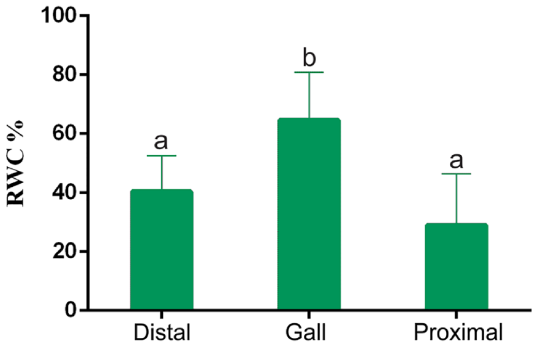



**Fig. 7** Comparison between the relative water content (%) in the non-galled portions of galled leaflets and globoid galls induced by Cecidomyiidae (Diptera) on *Inga ingoides* (Fabaceae). Bars indicate the means and standard deviations. Same letters on bars indicate statistically equal values and different letters indicate significant differences ($p < 0.05$)

vascular pattern may confer a reduction in hydraulic conductivity, but vessels with high pressure (Feild and Brodribb 2013), which implies in alternative mechanisms that guarantee cell turgidity and humid microenvironment. The water conduction both by the symplast or by the apoplast of parenchyma cells (Evert 2013; Buckley 2015) is indicated by the high index of relative water content in the extralaminar globoid galls on *I. ingoides*, and may work out as a compensatory mechanism for water flow.

We assume that galls that maintain a morphological continuum with their host plant organs, such as the intralaminar fusiform galls on *I. ingoides*, can transport water even after the galling herbivore exit. In this case, the largest investment in the differentiation of vascular cells results in efficient water transport even after gall senescence. In *I. ingoides*, this vascular increment is function both of the gall position and of the source for new vascular cells, i.e., the stimuli on cambium initials, which result in high the differentiation of large vessel elements, and high rates of increment in vascular tissues. The deviation of water to gall developmental site may be compensated by the peculiar pattern of large vessels.

### Vascularization of leaflet galls in light of the gall-constriction hypothesis

The gall-constriction hypothesis proposed by studies on the vascularization of stem galls induced by *Agrobacterium tumefaciens* (Aloni et al. 1995; Ullrich and Aloni 2000) predicts that vessel elements located above the galls are narrow due to hormonal signals that restrain their expansion. The width of the vessel elements of the extralaminar leaf galls on *I. ingoides* does not support this hypothesis, but the width of the vessel elements of the intralaminar gall morphotype does. Such differences refer to the maintenance of the host plant developmental patterns in distal and proximal portions of the leaflets in the extralaminar galls, which seems to develop like foliar appendages. The development of the extralaminar galls as adventitious organs, which are not in strict morphological continuum with their host leaflets, results in the differentiation of vessel elements of similar width along the gall. However, in the intralaminar fusiform galls, the galling herbivore disrupts the pattern of vascular development by stimulating alterations in cambium activity. Even though the differentiation of vascular tissues occurs in the apex-base direction (Sachs 1981; Aloni 1987; Fahn 1990), in the intralaminar galls on *I. ingoides*, the galling herbivore seems to disrupt this apex-based pattern, and stimulates the production of large vessels in gall portions proximal to the petiole. Alternatively, there may be a delay in the differentiation of vascular cells, and the vessel elements may enlarge prior to cell wall lignification (Aloni and Zimmermann 1983; Aloni 2015).

The vessel constriction observed in *I. ingoides* seems to be associated with the continuity between the tissues of the fusiform gall and of the host leaflet veins. The alterations in the pattern of xylem differentiation triggered by hormonal signals (Aloni et al. 1995; Ullrich and Aloni 2000) allow the water flow to the distal portions of the host leaflet veins, in parallel to what is predicted in the gall-constriction hypothesis for *Agrobacterium tumefaciens* galls (Aloni et al. 1995).

## Conclusions

The xylem morphogenesis involves the synergism between auxins and cytokinins in cells recruited to redifferentiate in vascular elements both in the extralaminar and intralaminar galls on *I. ingoides*. To ensure efficient hydraulic conductivity, three strategies are triggered: (1) a high rate of differentiation of parenchyma cells in extralaminar galls, (2) a high

differentiation of vessel elements in intralaminar gall, and (3) differences in the width of vessel elements in both gall morphotypes. The alterations in the width of vessel elements seem to be related to alterations in vascular differentiation in gall morphotypes with a morphological continuum with their host leaflets, i.e. the intralaminar galls. Extralaminar galls develop like appendages of adventitious origin, connected to the host organ by a peduncle analogous to a petiole, which theoretically imply in the ontogenetic rupture with the host leaf developmental pattern.

**Author contributions statement** GPPB and RMSI designed experiments. GPB and MSCF collected material and performed experiments. All authors analyzed the results, discussed data, wrote and reviewed the manuscript. RMSI coordinated the project.

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**Availability of data and materials** The data and material can be made available as needed.

## Compliance with ethical standards

**Conflict of interest** The authors declare that they have no conflict of interest.

## References

Aloni R (1987) Differentiation of vascular tissues. Ann Rev Plant Physiol 38:179–204

Aloni R (2001) Foliar and axial aspects of vascular differentiation-hypotheses and evidence. J Plant Growth Regul 20:22–34. https://doi.org/10.1007/s003440010001

Aloni R (2013) Role of hormones in controlling vascular differentiation and the mechanism of lateral root initiation. Planta 238:819–830. https://doi.org/10.1007/s00425-013-1927-8

Aloni R (2015) Ecophysiological implications of vascular differentiation and plant evolution. Trees 29:1–16. https://doi.org/10.1007/s00468-014-1070-6

Aloni R, Zimmermann MH (1983) The control of vessel size and density along the plant axis - a new hypothesis. Differentiation 24:203–208. https://doi.org/10.1111/j.1432-0436.1983.tb01320.x

Aloni R, Zimmermann MH (1984) Length, width, and pattern of regenerative vessels along strips of vascular tissue. Bot Gaz 145(1):50–54

Aloni R, Pradel KS, Uilrich CI (1995) The three-dimensional structure of vascular tissues in *Agrobacterium tumefaciens*-induced crown galls and in the host stems of *Ricinus communis* L. Planta 196:597–605. https://doi.org/10.1007/BF00203661

Aranda-Rickert A, Rothen C, Diez P, González AM, Marazzi B (2017) Sugary secretions of wasp galls: a want-to-be extrafloral nectar? Ann Bot 120(5):765–774. https://doi.org/10.1093/aob/mcx075

Arduin M, Kraus JE (2001) Anatomia de galhas de ambrosia em folhas de *Baccharis concinna* e *Baccharis dracunculifolia* (Asteraceae). Rev Bras Bot 24:63–72

Bedetti CS, Modolo LV, Isaias RMS (2014) The role of phenolics in the control of auxin in galls of *Piptadenia gonoacantha* (Mart) MacBr (Fabaceae: Mimosoideae). Biochem Syst Ecol 55:53–59. https://doi.org/10.1016/j.bse.2014.02.016

Bedetti CS, Bragança GP, Isaias RMS (2017) Influence of auxin and phenolic accumulation on the patterns of cell differentiation in distinct gall morphotypes on *Piptadenia gonoacantha* (Fabaceae). Aust J Bot 65:411–420. https://doi.org/10.1071/BT16257

Bedetti CS, Jorge NC, Trigueiro FCG, Bragança GP, Modolo LV, Isaias RMS (2018) Detection of cytokinins and auxin in plant tissues using histochemistry and immunocytochemistry. Biotech Histochem 93:149–154. https://doi.org/10.1080/10520295.2017.1417640

Buckley TN (2015) The contributions of apoplastic, symplastic and gas phase pathways for water transport outside the bundle sheath in leaves. Plant Cell Environ 38:7–22. https://doi.org/10.1111/pce.12372

Bukatsch F (1972) Bemerkungen zur Doppelfärbung Astrablau-Safranin. Mikrokosmos 61:255

Carneiro RGS, Castro AC, Isaias RMS (2014) Unique histochemical gradients in photosynthesis-deficient plant gall. South Afr J Bot South 92:94–104. https://doi.org/10.1016/j.sajb.2014.02.011

Carneiro RGS, Isaias RMS, Moreira ASFP, Oliveira DC (2017) Reacquisition of new meristematic sites determines the development of a new organ, the Cecidomyiidae gall on *Copaifera langsdorffii* Desf. (Fabaceae). Front Plant Sci 8:1622. https://doi.org/10.3389/fpls.2017.01622

Dias GG, Ferreira BG, Moreira GR, Isaias RMS (2013) Developmental pathway from leaves to galls induced by a sap-feeding insect on *Schinus polygamus* (Cav.) Cabrera (Anacardiaceae). An Acad Bras Cienc 85(1):187–200

Dietrich A, Wolf T, Eimert K, Schröder MB (2010) Activation of gene expression during hypersensitive response (HR) induced by auxin in the grapevine rootstock cultivar 'Börner'. Vits 49(1):15–21. https://doi.org/10.5073/vitis.2010.49.15-21

Dolzblasz A, Banasiak A, Vereecke D (2018) Neovascularization during leafy gall formation on *Arabidopsis thaliana* upon *Rhodococcus fascians* infection. Planta 247(1):215–228. https://doi.org/10.1007/s00425-017-2778-5

Evert RF (2013) Anatomia das plantas de Esau: meristemas, célula e tecidos do corpo da planta: sua estrutura e função e desenvolvimento. Blucher, São Paulo

Fahn A (1990) Plant anatomy. Pergamon Press, Oxford

Ferreira BG, Isaias RMS (2013) Developmental stem anatomy and tissue redifferentiation induced by a galling Lepidoptera on *Marcetia taxifolia* (Melastomataceae). Bot 91:752–760. https://doi.org/10.1139/cjb-2013-0125

Ferreira BG, Falcioni R, Guedes LM, Avritzer SC, Antunes WC, Souza LA, Isaias RM (2017a) Preventing false negatives for histochemical detection of phenolics and lignins in PEG-embedded plant tissues. J Hitochem Cytochem 65(2):105–116. https://doi.org/10.1369/2F0022155416677035

Ferreira BG, Avritzer SC, Isaias RMS (2017b) Totipotent nutritive cells and indeterminate growth in galls of *Ditylenchus gallaeformans*

(Nematoda) on reproductive apices of *Miconia*. Flora 227:36–44. https://doi.org/10.1016/j.flora.2016.12.008
Ferreira BG, Freitas MSC, Bragança GP, Moreira ASFP, Carneiro RGS, Isaias RMS (2019) Enzyme-mediated metabolism in nutritive tissues of galls induced by *Ditylenchus gallaeformans* (Nematoda: Anguinidae). Plant Biol 21(6):1052–1062. https://doi.org/10.1111/plb.13009
Feild TS, Brodribb TJ (2013) Hydraulic tuning of vein cell microstructure in the evolution of angiosperm venation networks. New Phytol 199:720–726. https://doi.org/10.1111/nph.12311
França MGC, Prados LMZ, Lemos-Filho JP, Ranieri BD, Vale FHA (2012) Morphophysiological differences in leaves of *Lavoisiera campos-portoana* (Melastomataceae) enhance higher drought tolerance in water shortage events. J Plant Res 125:85–92. https://doi.org/10.1007/s10265-011-0416-z
Fukuda H (1997) Tracheary element differentiation. Plant Cell 9:1147–1156
Fukuda H, Komamine A (1980) Direct evidence for cytodifferentiation to tracheary elements without intervening mitosis in a culture of single cells isolated from the mesophyll of *Zinnia elegans*. Plant Physiol 65:61–64. https://doi.org/10.1104/pp.65.1.61
Guzicka M, KarolewskiGiertych PMJ (2017) Structural modification of *Quercus petraea* leaf caused by *Cynips quercusfolii*—histological study of galls. J Plant Interact 12(1):7–13. https://doi.org/10.1080/17429145.2016.1269214
Hori K (1992) Insect secretion and their effect on plant growth, with special reference to hemipterans. In: Shorthouse JD, Rohfritsch O (eds) Biology of insect-induced galls. Oxford University Press, New York, pp 157–170
Isaias RMS, Oliveira DC, Carneiro RGS (2011) Role of *Euphalerus ostreoides* (Hemiptera: Psylloidea) in manipulating leaflet ontogenesis of *Lonchocarpus muehlbergianus* (Fabaceae). Bot 89:581–592. https://doi.org/10.1139/b11-048
Isaias RMS, Carneiro RGS, Oliveira DC, Santos JC (2013) Illustrated and annotated checklist of Brazilian gall morphotypes. Neotrop Entomol 42:230–239. https://doi.org/10.1007/s13744-013-0115-7
Isaias RMS, Oliveira DC, Carneiro RGS, Kraus JE (2014) Developmental anatomy of galls in the neotropics, arthropods stimuli versus host plant constraints. In: Fernandes GW, Santos JC (eds) Neotropical insect galls. Springer, Netherlands, pp 15–34
Jeffrey EC (1919) The structure of wood, 2nd edn. Adam and Charles Black, London
Johansen DA (1940) Plant microtechnique. McGraw-Hill Book Company, New York
Karnovsky MJ (1965) A formaldehyde-glutaraldehyde fixative of high osmolarity for use in electron microscopy. J Cell Sci 27:137–138
Kraus JE, Arduin M (1997) Manual básico de métodos em morfologia vegetal. EDUR, Seropédica
Kraus JE, Arduin M, Venturelli M (2002) Anatomy and ontogenesis of hymenopteran leaf galls of *Struthanthus vulgaris* Mart. (Loranthaceae). Rev Brasil Bot 25(4):449–458
Leopold AC, Plummer TH (1961) Auxin–phenol complexes. Plant Physiol 36:589–592
Matsumuoto-Kitano M, Kusumoto T, Tarkowski P, Kinoshita-Tsujimura VK, Miyawaki K, Kakimoto T (2008) Cytokinins are central regulators of cambial activity. PNAS 105(50):20027–20031. https://doi.org/10.1073/pnas.0805619105
Mazur E, Benková E, Friml J (2016) Vascular cambium regeneration and vessel formation in wounded inflorescence stems of *Arabidopsis*. Sci Rep 6:33754. https://doi.org/10.1038/srep33754
Milioni D, Sado PE, Stacey NJ, Domingo C, Roberts K, McCann MC (2001) Differential expression of cell-wall-related genes during the formation of tracheary elements in the Zinnia mesophyll cell system. In: Carpita NC, Campbell M, Tierney M (eds) Plant cell walls. Springer, Dordrecht
Nogueira RM, Costa EC, Silva JS, Isaias RMS (1908) Structural and histochemical profile of Lopesia sp. Rübsaamen 1908 pinnula galls on *Mimosa tenuiflora* (Willd.) Poir. in a Caatinga environment. Hoehnea 45(2):314–322. https://doi.org/10.1590/2236-8906-80/2017
O'Brien TP, McCully ME (1981) The study of plant structure principles and selected methods. Termarcarphi, Melbourne
Paiva JGA, Fank-de-Carvalho SM, Magalhães MP, Graciano-Ribeiro D (2006) Verniz vitral incolor 500®: uma alternativa de meio de montagem economicamente viável. Acta Bot Bras 20:257–264
Sachs T (1981) The control of patterned differentiation of vascular tissues. Adv Bot Res 9:151–262. https://doi.org/10.1016/S0065-2296(08)60351-1
Sachs T (2000) Integrating cellular and organismal aspects of vascular differentiation. Plant Cell Physiol 41:649–656. https://doi.org/10.1093/pcp/41.6.649
Schuetz M, Smith R, Ellis B (2013) Xylem tissue specification, patterning, and differentiation mechanism. J Exp Bot 64(1):11–31. https://doi.org/10.1093/jxb/ers287
Suzuki AYM, Bedetti CS, Isaias RMS (2015) Detection and distribution of cell growth regulators and cellulose microfibrils during the development of *Lopesia* sp. galls on *Lonchocarpus cultratus* (Fabaceae). Bot 93:435–444. https://doi.org/10.1139/cjb-2015-0012
Touchstone JC (1992) Practice of thin layer chromatography. Wiley, New York
Ullrich CL, Aloni R (2000) Vascularization is a general requirement for growth of plant and animal tumours. J Exp Bot 51(353):1951–1960. https://doi.org/10.1093/jexbot/51.353.1951
Ursache R, Nieminen K, Helariutta Y (2013) Genetic and hormonal regulation of cambial development. Physiol Plant 147:36–45. https://doi.org/10.1111/j.1399-3054.2012.01627.x
Wetmore R, Rier J (1963) Experimental induction of vascular tissues in callus of angiosperms. Am J Bot 50:418–430
Ye Z-H (2002) Vascular tissue differentiation and pattern formation in plants. Annu Rev Plant Biol 53:183–202. https://doi.org/10.1146/annurev.arplant.53.100301.135245

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# Capítulo III

# Hemicelluloses and associated compounds determine gall functional traits

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RESEARCH PAPER

# Hemicelluloses and associated compounds determine gall functional traits

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## ABSTRACT

- The intriguing questions concerning gall development refer to the processes of the remodelling of the host plant organ. Such processes involve the restructuring of cell walls and can be influenced by phenolics, indole-3-acetic acid (IAA) and reactive oxygen species (ROS). Alterations in cell walls demand the interference in the coupling of cellulose fibrils and hemicelluloses (xyloglucans) at specific stages of gall development. In addition to cell wall remodelling, hemicelluloses, such as the, xyloglucans and heteromannans can act as reserve carbohydrates, while xylans provide rigidity to the secondary cell walls.
- Developmental traits of the lenticular, fusiform and globoid galls on *Inga ingoides* (Fabaceae) were analysed using anatomical, cytometric, histochemical and immunocytochemical tools.
- Phenolics, IAA and ROS accumulated in similar gall tissue compartments, and may have influenced the restructuring of hemicelluloses and pectins. Contrary to expectations, cell wall flexibility regarding the dynamics of xyloglucans and cellulose fibrils does not relate to a temporal scale. The detection of xyloglucans in nutritive cell walls relate to carbohydrate nutritional resources to the galling insect, while xylans were associated to the lignified cell walls. Heteromanans were not detected, either in non-galled or galled tissues.
- The patterns of cell expansion during gall development relied on the relationship among phenolics, ROS and IAA with the hemicelluloses (xyloglucans and xylans) and cellulose fibrils. Although cell wall dynamics is specific to each gall morphotype in *I. ingoides*, the xyloglucans function as carbohydrate reserve to the gall inducers, which constitutes a functional trait common to the three morphotypes.

## INTRODUCTION

The morphogenesis of plant organs and of gall morphotypes involves refined cell dynamics (Baskin 2005; Crowell *et al.* 2010; Isaias *et al.* 2013), which may reflect alterations in cell wall components (Carneiro *et al.* 2014; Magalhães *et al.* 2014; Suzuki *et al.* 2015; Teixeira *et al.* 2018; Meidani *et al.* 2019; Ferreira *et al.* 2020). The development of gall morphotypes demands alterations in the physical properties of the primary cell walls, and consequently in the dynamics of cell expansion (Suzuki *et al.* 2015; Bedetti *et al.* 2017; Teixeira *et al.* 2018). These alterations affect the cellulose microfibrils, hemicelluloses, pectins and glycoproteins (Formiga *et al.* 2013; Oliveira *et al.* 2014; Suzuki *et al.* 2015).

Studies in galls of Neotropical regions revealed that pectin and protein composition influence the equilibrium between flexibility and rigidity and are related to new cellular functions in specific tissue compartments (Formiga *et al.* 2013; Carneiro *et al.* 2014; Oliveira *et al.* 2014; Carneiro *et al.* 2015; Teixeira *et al.* 2018). The establishment of gall tissue compartments implies the reorientation of cellulose microfibrils, as elegantly demonstrated during the development of galls on *Baccharis dracunculifolia* (Magalhães *et al.* 2014) and *Lonchocarpus cultratus* (Suzuki *et al.* 2015). The reorientation of cellulose microfibrils depends on the loosening of microfibril–hemicellulose–microfibril junctions (Cosgrove 2016; Voiniciuc *et al.* 2018), as well as on the dynamics of pectins (Micheli 2001). Hemicelluloses are a group of polysaccharides (xyloglucans, xylans and heteromannans) (Thompson 2005; Evert 2006; Moreira & Filho 2008), whose functions involve both carbohydrate reserves (Lieperman *et al.* 2007; Moreira & Filho 2008; Scheller & Ulvskov 2010) and the regulation of cell expansion at specific sites, the so-called 'biomechanical hotspots' (Park & Cosgrove 2015; Cosgrove 2016). The loosening of microfibril–xyloglucans–microfibril junctions facilitates cell elongation (Cosgrove 2016; Cosgrove 2018), which is required during cell restructuring stimulated by the galling activity.

In galls, the loosening of the 'biomechanical hotspots' may be related to the accumulation of growth regulators in specific cells and tissue layers (Suzuki *et al.* 2015; Bedetti *et al.* 2017). High phenolic concentration triggered by gall induction can inhibit indole-3-acetic acid (IAA) oxidase activity and increase the levels of IAA (Hori 1992; Zhao 2010; Bedetti *et al.* 2014). Such phenolic–IAA oxidase interaction stimulates acid growth and the production of reactive oxygen species (ROS) (Rayle & Cleland 1992; Rose 2003), which favour cell wall flexibility and expansion (Cosgrove 2000; Madja & Roberts 2018).



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Gall development can be characterized into four stages: induction, growth and development (young), maturation and dehiscence (Rohfritsch 1992). This last stage is also referred to as senescence (Isaias & Oliveira 2012; Isaias *et al.* 2014). The recognition between a galling herbivore and its host plant is initially mediated by chemical and structural signals of the interaction that occur at the time of gall induction (Isaias *et al.* 2014). The young gall stage is marked by intense hyperplasia and cellular hypertrophy, guaranteeing the increment in gall size (Oliveira & Isaias 2010; Carneiro *et al.* 2014). In the maturation stage, the final shape is established (Rohfritsch 1992; Carneiro *et al.* 2014), while senescence is marked by the end of the stimuli of the galling herbivore and its exit from the gall structure (Isaias & Oliveira 2012).

We consider that the best model to test the dynamics of hemicelluloses and cellulose fibrils involves diverse gall morphotypes that are associated with a superhost plant, such as *Inga ingoides* (Fabaceae). This plant hosts three Cecidomyiidae-induced galls, known as the globoid, lenticular and fusiform gall morphotypes. Fungal hyphae partially cover the inner surface of the nutritive cells in globoid galls, which characterize them as ambrosia galls (Rohfritsch 2008).

We hypothesize that in sites where ROS, IAA and phenolics accumulate, the dynamics of cell wall components will be altered. Due to the highest rates of cell expansion in the young stage, the hemicelluloses may be reallocated to the cytoplasm (Barnes & Anderson 2018), and as they will become decoupled from cellulose fibrils, they may be difficult to detect or be undetectable. In the maturation stage, the final shape of the gall is established, the reorientation of the cell expansion axes stops, and the cellulose–hemicelluloses relink. Two questions direct our discussion: (i) do the categories of hemicelluloses co-occur during gall developmental stages; and (ii) is the overlapping histolocalization of ROS, IAA and phenolics related to the cellulose fibrils–hemicelluloses relationship?

## MATERIAL AND METHODS

### Sampling

Immature (expanded young leaflets) and mature non-galled leaflets, and globoid, fusiform and lenticular galls in three stages of development – young, mature and senescent ($n \geq 10$ per stage) – were collected from seven individuals at the Parque Estadual Serra Verde (PESV) (19°47′, 22.47″ S, 43°57′32.35″ W), Belo Horizonte, Minas Gerais, Brazil. The young and mature stages were defined by colour, and the senescent stage by the absence of the galling herbivore. Globoid galls are light green in the young stage and dark green in the mature stage. Lenticular galls are green in the young stage and red in the mature and senescent stages. Fusiform galls are green in the young stage and brown in the mature and senescent stages.

### Anatomical analysis

Samples of non-galled leaflets, globoid, lenticular and fusiform galls ($n \geq 10$ per stage) were fixed in Karnovsky solution (2.5% glutaraldehyde, 4.5% formaldehyde in 0.1 mol·l$^{-1}$ phosphate buffer, pH 7.2) (Karnovsky 1965) for 48 h at room temperature. The samples were dehydrated in an *n*-butyl series (Johansen 1940) and embedded in Paraplast® (Kraus & Arduin 1997). Sections (12–14 μm) were obtained with a rotary microtome (Leica BIOCUT 2035), deparaffinized and stained with 9:1 (v/v, 0.5%) solution of Astra blue and safranin (Bukatsch 1972). The slides were mounted in colourless varnish Acrilex (Paiva *et al.* 2006). Samples of the fusiform galls ($n \geq 10$ per stage) were dehydrated in an ethanol series and embedded in Leica Historesin (Kraus & Arduin 1997), sectioned (5–7 μm) with rotary microtome (Leica BIOCUT 2035), and stained with 0.05% toluidine blue O in 1 mol·l$^{-1}$ phosphate buffer (PBS), pH 6.8 (O'Brien *et al.* 1964). The sections were photographed with a light microscope (Leica ICC50 HP; Leica, Wetzlar, Germany).

### Cytometric analysis

The origin of each galled tissue layer was determined in relation to its continuity in the tissues of the non-galled portion of the leaf lamina. Cytometric measurements were performed in transverse sections. Cell area, height and width of ten galls per developmental stage (four cells of each tissue in transverse section, 40 images per developmental stage; n = 160 cells per plant tissue) were obtained. The cortical parenchyma and vascular cells, fibres and vessel elements of immature leaflets, as well as the cells of the abaxial epidermis and spongy parenchyma of mature leaflets were measured. In the globoid and lenticular galls, cells of the dermal system, outer tissue compartment, sclerenchyma layers and nutritive tissue were measured. In the fusiform galls, cells of the outer tissue compartment, fibres, vessel elements and nutritive tissue were measured.

Statistical analysis was used to compare cell and tissue area, height and width between non-galled leaflets and young, mature and senescent galls. Parametric data were compared by one-way ANOVA, followed by Tukey's test. The non-parametric data were transformed to logarithmic scale to achieve normality and homoscedasticity. After transformation, the data were compared using Kruskall–Wallis test and Dunn's test with SigmaStat® (Systat, Slough, UK) and Graphprism 5.0 (Motulsky 1192-2009) software. The level of significance used in all tests was $P \leq 0.05$. The type of cell expansion and degree of anisotropy can be measured as the ratio between the average anticlinal and periclinal axes (Baskin 2005). Ratio values ranging between 0.9 and 1.10 indicate a tendency to isotropic expansion; when the ratio is <0.89, cell expansion is anisotropic with a tendency to a periclinal direction; when it is >1.1, cell expansion is considered anisotropic with a tendency to an anticlinal direction of expansion (Carneiro *et al.* 2015).

### Cellulose fibrils

To obtain samples of integral walls and cellulose fibrils, handmade sections of fresh samples ($n \geq 5$) of mature non-galled leaves and mature galls were treated with 1:1 (v/v) calcofluor white (Sigma-Aldrich) in distilled water for 30 min in darkness (Herth & Schnepf 1980). The sections were washed in distilled water, mounted in 50% glycerin and analysed in a fluorescence microscope (Leica DFC 7000T®) using a 4',6-diamidino-2-phenylindole filter (DAPI), with excitation wave-lenght of 320–380 nm and emission filter at 425 nm. The orientation of the cellulose fibrils was established by orientation of the pit-fields, as the fibrils surround pitfields and are parallel to their

larger axis (Hogetsu 1991; Sauter *et al.* 1993), which were visualized by the fluorescence of the calcofluor white.

### Immunocytochemistry

Sections of fixed samples of non-galled leaflets and young, mature and senescent galls (n = 3), embedded in Paraplast (Kraus & Arduin 1997), deparaffinized and treated with 10 $\mu g \cdot ml^{-1}$ pectate lyase (Sigma-Aldrich) in 2 mM $CaCl_2$ buffer, 50 mM 3-(cyclohexylamino)-1-propanesulfonic acid (CAPS) (Sigma-Aldrich), pH 10 (Marcus *et al.* 2008), for 2 h at room temperature. After enzyme treatment, the sections were incubated with the monoclonal antibodies LM10, LM15 and LM21, diluted 1:10 in milk/PBS for 1.5 h to label xylans, xyloglucans and heteromannans, respectively (Centre for Plant Sciences, University of Leeds, UK). For the control, the primary antibodies were suppressed. The treated sections were washed in PBS and subsequently incubated with the secondary antibody anti-rat IgG-FITC, 1:100 diluted in milk/PBS (Sigma-Aldrich) for 1.5 h. The slides were washed and mounted in 50% glycerin kept in darkness and analysed in a fluorescence microscope (Leica DFC 7000T), with excitation wave-lenght of 450–490 nm and emission filter of 515 nm.

### Histochemistry

Handmade sections of fresh samples ($n \geq 5$) of mature non-galled leaflets and young mature galls were used for detection of ROS, IAA and phenolics. The ROS were detected after staining with 0.5% 3.3′-diaminobenzidine (DAB) (Sigma-Aldrich) for 15–60 min, in the dark (Rosseti & Bonatti 2001). For detection of IAA, sections were immersed in 1% *p*-dimethylaminobenzaldehyde in 1 $mol \cdot l^{-1}$ HCl, w/v (Ehrlich reagent) for 5 min at room temperature (Leopold & Plummer 1961). Purple staining indicated a positive result with Erlich's reagent as sites of IAA accumulation (Bedetti *et al.* 2018). Phenolics were detected as a black precipitate formed after addition of 10% ferric chloride to sections for 5 min at room temperature (Johansen 1940). For staining of the fungi, globoid galls were included in resin, stained with 5% cotton blue in lactophenol for 20 min, and counter stained with 1% safranin for 10 s. The slides were washed three times in distilled water for 1 min (Marques *et al.* 2013) and fungal hyphae were stained blue. Blank sections were used as controls for all histochemical tests. The images were obtained using a digital camera (Leica ICC50HP) coupled to a light microscope (Leica DM500).

## RESULTS

### Anatomy of non-galled leaflets and galls

The non-galled leaflets of *Inga ingoides* have uniseriate epidermis and dorsiventral mesophyll (Figure S1A). The vascular system is bicollateral and involved by pericyclic fibres (Figure S1B).

The tissue compartments of the globoid galls originated from the epidermis, spongy and vascular parenchyma. At the young stage, the epidermis is uniseriate, the parenchyma of the outer tissue compartment is homogeneous, and the inner tissue compartment is constituted of nutritive cells (Figure S1C). In the maturation stage, parenchymatic cell walls of the outer tissue compartment lignify (Figure S1D) and fungal hyphae are interspersed within the nutritive tissue (Figure S1E and F). In the senescent stage, nutritive tissue is absent (Figure S1G).

The tissues of the lenticular galls originated from redifferentiation of the epidermis, spongy and vascular parenchyma. In the young stage, the outer tissue compartment is divided in two regions: upper and lower tissue compartments. The inner tissue compartment is formed by nutritive tissue (Figure S1H). During the maturation stage, the upper and the lower regions have lignified cell walls. Cells of the lower region and of the nutritive tissue divide periclinally and expand anticlinally, determining the gall lenticular shape (Figure S1I). In the senescent stage, the nutritive tissue has lignified cell walls (Figure S1J).

The tissues of the fusiform galls originated by redifferentiation of vascular parenchyma, and cambium and phellogen activity. The epidermis and cortical parenchyma developed toward gall structure. At the young stage, the outer tissue compartment is formed by cortical parenchyma cells, fibres and the vascular system (Figure S1K). The inner tissue compartment is formed by nutritive cells that originate from atypical vascular cambium activity (Figure S1L). At the stage of maturation (Figure S1M), typical secondary growth occurs, and the outer tissue compartment is formed by cortical parenchyma, fibres, secondary phloem, cambial zone and secondary xylem. The inner tissue compartment is formed by the nutritive tissue with isodiametric cells. In the senescent stage (Figure S1N), the outer tissue compartment maintains the features of the previous stage and nutritive tissue is absent.

### Cytometry and detection of cellulose fibrils, hemicelluloses and accumulation of phenolics, IAA and ROS

*Non-galled leaflets*

The mesophyll is dorsiventral (Fig. 1A), and the cells of the epidermis are periclinally elongated (Table S1) with cellulose fibrils perpendicular to the major axis of cell expansion (Fig. 1B). The cellulose fibrils are randomly arranged in the cell walls of the spongy parenchyma, whose cells tend to isotropic growth (Fig. 1C; Table S1). The epitopes of xyloglucans are recognized by LM15 in the cell walls of the palisade and spongy parenchyma (Fig. 1D), and epitopes of xylans are recognized by LM10 in lignified cell walls of the pericyclic fibres and xylem (Fig. 1E). The heteromannans are not recognized by LM21 in any cell walls of the immature or mature non-galled leaflets. The accumulation of phenolics and ROS is more intense in the palisade parenchyma of the non-galled leaflets (Fig. 1F and G), and IAA is detected in the epidermis (Fig. 1H).

In the midrib vein (Fig. 2A), cells of the cortical and vascular parenchyma, as well as fibres, are periclinally elongated and vessel elements are anisodiametric (Table S1). The cellulose fibrils are perpendicular to the major axis of cell expansion in cortical (Fig. 2B) and vascular parenchyma cells (Fig. 2C). The epitopes of xyloglucans are recognized by LM15 in cell walls of cortical and phloem cells (Fig. 2D), while epitopes of xylans are recognized by LM10 in cell walls of pericyclic fibres and xylem (Fig. 2E). The phenolics are not detected in any cell walls in the midrib region (Fig. 2F), ROS are detected in cell walls of the cortical parenchyma, xylem and phloem (Fig. 2G), and the IAA accumulates in epidermal and phloem cells (Fig. 2H).

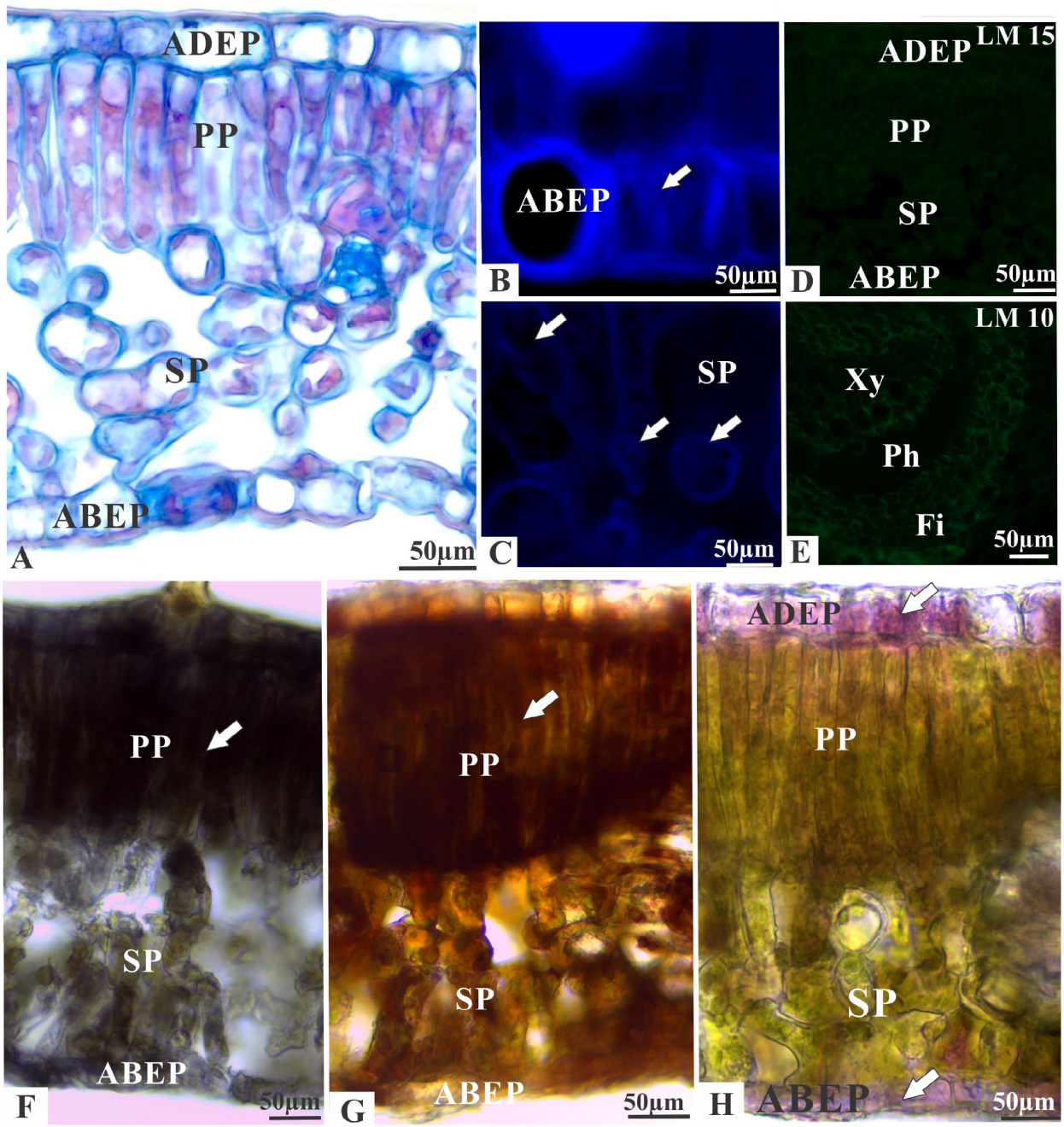



**Fig. 1.** Non-galled leaflets of *Inga ingoides* (Rich.) Willd. (*Fabaceae*). A: General aspects of the anatomical organization of mesophyll. B and C: Cellulose fibrils stained with calcofluor white, seen with fluorescence microscopy. B: Cells of abaxial epidermis with aligned fibrils (arrow). C: Cells of spongy parenchyma with randomly arranged fibrils (arrows). D and E: Immunocytochemistry of hemicelluloses in cell walls. D: Labelling of xyloglucans with LM15 in epidermis and mesophyll. E: Labelling of xylans with LM10 in xylem and fibres. F–H: Histochemistry. F: Phenolics (black colour) after ferric chloride staining in mesophyll (arrow). G: Reactive oxygen species (ROS; brown colour) detected with 3.3′-diaminobenzidine (DAB) in palisade and spongy parenchyma (arrow). H: Indole-3-acetic acid (IAA; pink colour) in epidermis (arrows). ABEP, abaxial epidermis; ADEP, adaxial epidermis; PP, palisade parenchyma; SP, spongy parenchyma.

*Globoid galls*

The direction of cell expansion is altered in hypertrophied cells of the outer tissue compartment (Fig. 3A), from isotropic to anisotropic on the periclinal axis (Tables S1 and S2), with cellulose fibrils aligned perpendicular to the major axis of cell expansion (Fig. 3B). The orientation of cellulose fibrils in the nutritive tissue is obscured due to the presence of fungal hyphae. The abaxial epidermis of globoid galls increased about 65%. Cells of the spongy parenchyma grew about 1,500% toward the outer tissue compartment, 501% toward the lignified cells near the larval chamber, and 142% toward the nutritive tissue (Table S2).

During the young stage of the globoid gall, epitopes of xyloglucans are recognized by LM15 in cell walls of the outer

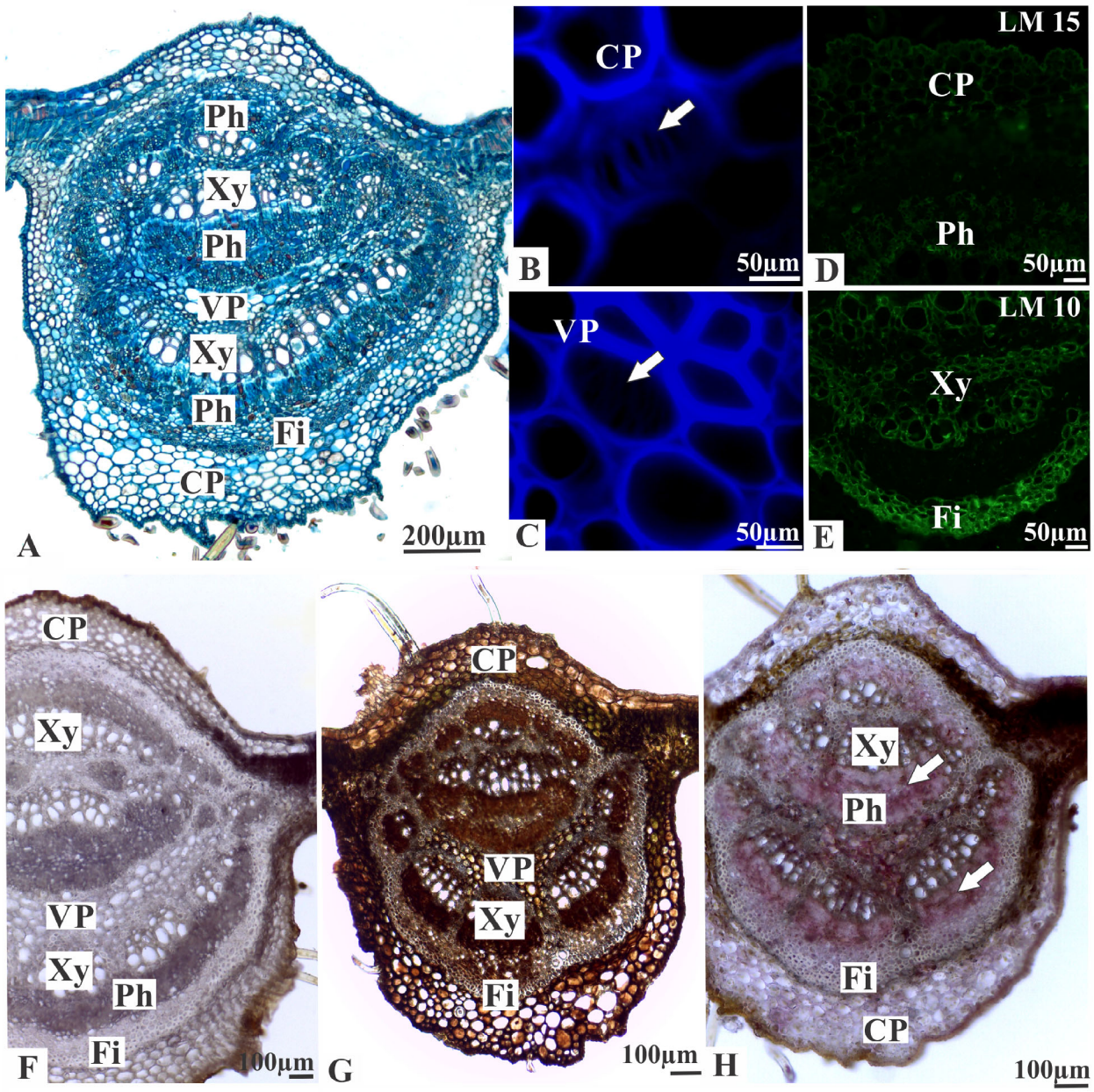



**Fig. 2.** Midrib vein of non-galled leaflets on *Inga ingoides* (Rich.) Willd. (*Fabaceae*). A: General anatomical organization of a midrib vein. B and C: Cellulose fibrils stained with calcofluor white seen with fluorescence microscopy. B: Cortical cells with aligned fibrils (arrow). C: Vascular parenchyma with aligned fibrils (arrow). D and E: Immunocytochemistry of hemicelluloses in cell walls. D: Labelling of xyloglucans with LM15 in cortical parenchyma and phloem. E: Labelling of xylans with LM10 in xylem and fibres. F: Midrib vein showing the absence of phenolics. G: ROS (brown colour) detected with 3.3′-diaminobenzidine (DAB) in all regions of the midrib vein. H: Detection of indole-3-acetic acid (IAA) (pink colour) with Erhlich reagent in phloem cells of a midrib vein (arrows). Fi, fibre; Ph, phloem; Xy, xylem, VP, vascular parenchyma.

tissue compartment (Fig. 3C) and of the nutritive tissue (Fig. 3D). These epitopes of xyloglucans are also recognized by LM15 in cell walls of the outer tissue compartment in the mature stage (Fig. 3E). In the senescent stage, epitopes of xyloglucans are not recognized by LM15. Epitopes of xylans are recognized by LM10 in the lignified cell walls of mature and senescent galls (Fig. 3F). At all stages of gall development, no heteromannans are recognized by LM21. Phenolics (Fig. 3G), ROS (Fig. 3H) and auxins (Fig. 3I) are detected in the vacuoles and cell walls of the cells of the outer tissue compartment, while ROS appear to have a centrifugal gradient from the inner tissue compartment toward the epidermis.

*Lenticular galls*

The cells of epidermis and upper outer tissue compartment tend to show periclinal expansion during all developmental stages of the gall. The lower outer tissue compartment has anticlinal anisotropy in the young, mature and senescent stages

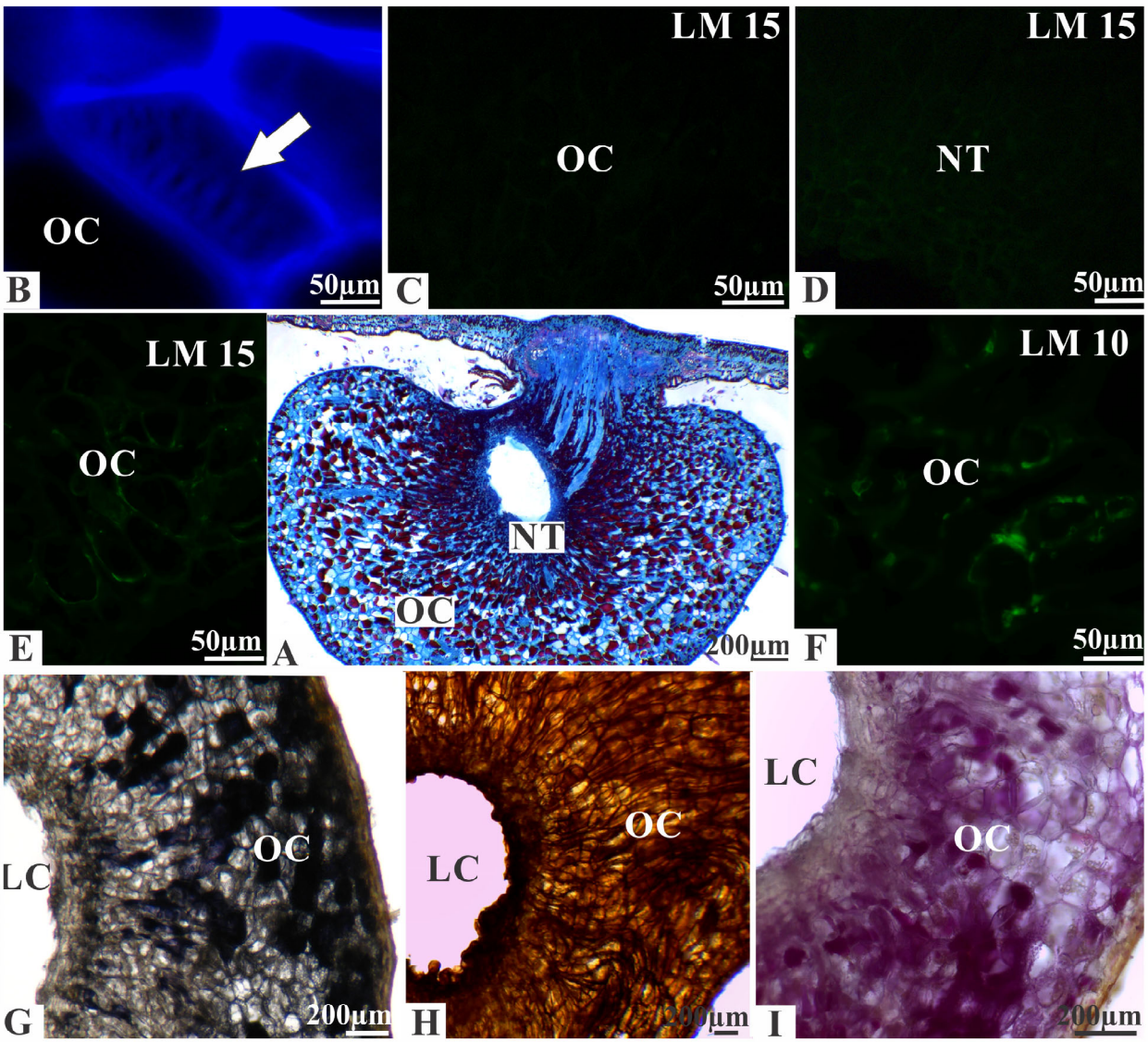



**Fig. 3.** Globoid galls induced by Cecidomyiidae on *Inga ingoides* (Rich.) Willd. (*Fabaceae*). A: General aspects of anatomical organization of a young gall. B: Cell of outer tissue compartment with aligned fibrils (arrow), stained with calcofluor white seen with fluorescence microscopy. C–F: Immunocytochemistry of hemicelluloses in cell walls. C–E: Labelling of xyloglucans with LM15 (C) in the outer tissue compartment in a young gall; (D) in nutritive tissue of a young gall; and (E) in outer tissue compartment of a mature gall. F: Labelling of xylans with LM10 in the outer tissue compartment in a senescent gall. G–I: Histochemistry. G: Phenolics (black colour) in vacuoles of parenchyma cells of the outer tissue compartment. H: ROS (brown colour) in cell walls and vacuoles of the nutritive tissue and parenchymatic cells of the outer tissue compartment. I: Detection of indole-3-acetic acid (IAA) (pink colour) in vacuoles of cells in the outer tissue compartment. LC, larval chamber; NT, nutritive tissue; OC, outer tissue compartment.

(Fig. 4A; Table S1). The cellulose fibrils of the lower outer tissue compartment (Fig. 4B) and nutritive cells (Fig. 3C) are oriented parallel to the major axis of cell elongation. Cells of the adaxial epidermis increased in size about 390%. Cells of the spongy parenchyma enlarged 1,150% toward the lower outer tissue compartment, 502% toward the nutritive tissue, and 292% toward the upper outer tissue compartment (Table S2).

The epitopes of xyloglucans are recognized by LM15 in cell walls of the lower outer tissue compartment of galls in the young stage (Fig. 4D). These epitopes of xyloglucans are recognized by LM15 in the nutritive tissue of young and mature galls (Fig. 4E and F). In the senescent stage, epitopes of xyloglucans are not recognized by LM15. The xylans are intensely recognized by LM10 in lignified cell walls of young galls (Fig. 3G), but poorly identified in lignified cell walls of mature and senescent galls (Fig. 4H and I). Heteromannans are not recognized by LM21 during any gall developmental stages. Accumulation of phenolics (Fig. 4J), ROS (Fig. 4K) and IAA (Fig. 4L) is detected in the vacuoles, while ROS are detected in cell walls and form a centrifugal gradient from the nutritive tissue toward the epidermis.

*Fusiform galls*

In fusiform galls (Fig. 5A), the cortical cells are isodiametric at the young stage and grow periclinally in the mature and senescent stages. Fibres are periclinally elongated during the young,

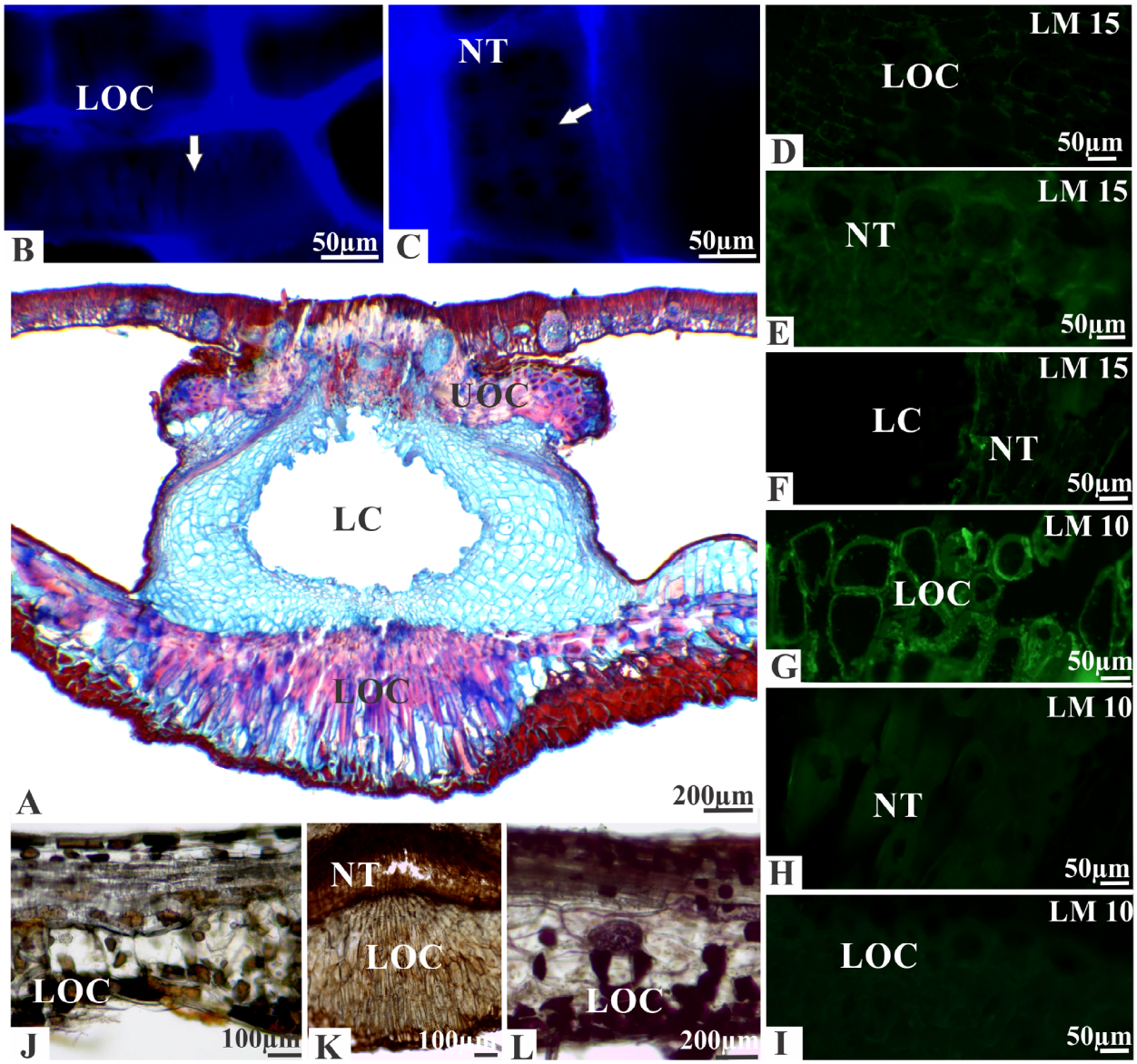



**Fig. 4.** Lenticular galls induced by Cecidomyiidae on *Inga ingoides* (Rich.) Willd. (*Fabaceae*). A: General anatomical organization of a mature gall. B and C; Cellulose fibrils stained with calcofluor white seen with fluorescence microscopy. B: Cells of lower outer tissue compartment with aligned fibrils (arrow). C: Cell of nutritive tissue with aligned fibrils (arrow). D–I: Immunocytochemistry of hemicelluloses in cell walls. D–F: Labelling of xyloglucans with LM15. D–F: Xyloglucans labelled with LM15 (D) in lower outer tissue compartment of a young gall; (E) in nutritive tissue of a young gall; and (F) in nutritive tissue of a mature gall. G–I: Labelling of xylans with LM10. G: Xylans in lower outer tissue compartment of a young gall; (H) in nutritive tissue of a senescent gall; (I) in lower outer tissue compartment of a senescent gall. J: Phenolics (black colour) in vacuoles of epidermis and parenchyma of lower outer tissue compartment. K: Detection of ROS (brown colour) in nutritive tissue lining the larval chamber. L: Indole-3-acetic acid (IAA) (pink colour) detected in vacuoles of parenchyma cells in lower outer tissue compartment. LC, larval chamber; LOC, lower outer tissue compartment; NT, nutritive tissue; UOC, upper outer tissue compartment.

mature and senescent stages. The vessel elements and nutritive cells are isodiametric throughout gall development (Table S1). The cellulose fibrils are perpendicular to the major axis of cell expansion in cortical cells (Fig. 5B) and randomly arranged in the isodiametric nutritive cells (Fig. 5C). The gall cortex enlarged 360% in relation to the cortex of young non-galled cortex. The fibres increased 1,372% during the formation of young galls, however, their area decreased about 53% toward the stage of maturation, and 17% from maturation toward senescent stage. The vessel elements increased 108% at the senescent stage, and the nutritive cells were 220% larger than the vascular parenchyma (Table S2).

In young fusiform galls, epitopes of xyloglucans are recognized by LM15 in cell walls of the secondary phloem (Fig. 5D). In the mature stage, xyloglucans are poorly recognized by LM15 in cell walls of the nutritive tissue (Fig. 5E). The senescent galls have epitopes of xyloglucans recognized by LM15 in cell walls of the cortical parenchyma, periderm (Fig. 5F) and secondary phloem (Fig. 5G). In the lignified cell walls of vascular tissues, epitopes of xylans are

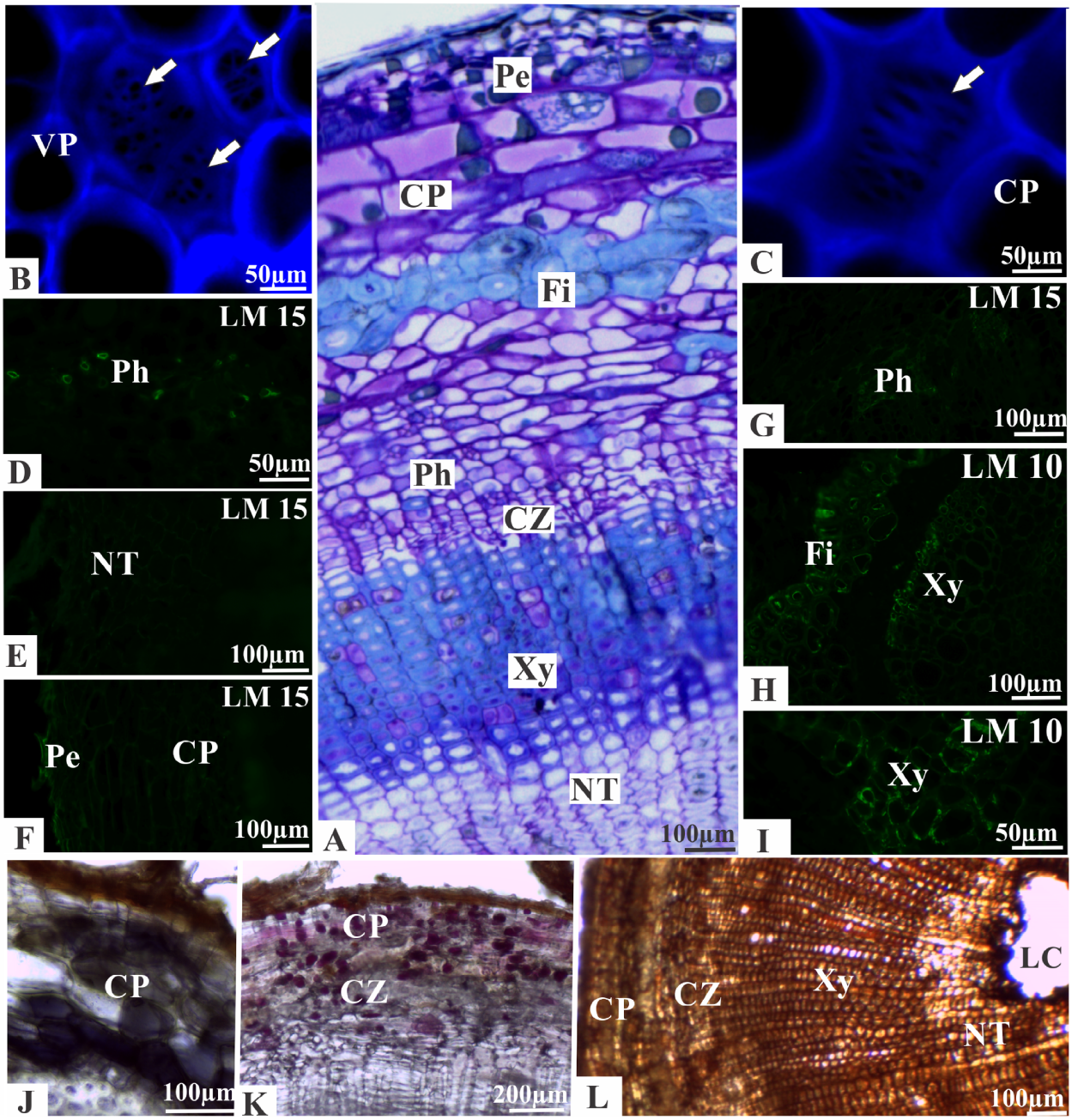



**Fig. 5.** Fusiform galls induced by Cecidomyiidae on *Inga ingoides* (Rich.) Willd. (*Fabaceae*). A: General anatomical organization of a mature gall. B and C: Cellulose fibrils stained with calcofluor white seen with fluorescence microscopy. B: Cell of outer tissue compartment with aligned fibrils (arrows). C: Cells of nutritive tissue with randomly arranged fibrils (arrow). D–I: Immunocytochemistry of hemicelluloses in cell walls. D–G: Labelling of xyloglucans with LM15; (D) in phloem of a young gall; (E) in nutritive tissue of a mature gall; (F) in periderm and cortical parenchyma in a senescent gall; and (G) in phloem of a senescent gall. H and I: Labelling of xylans with LM10; (H) in fibres and xylem of a young gall; and (I) in xylem of a senescent gall. J–L: Histochemistry. J: Phenolics (black colour) in vacuoles of cortical parenchyma cells. K: Detection of indole-3-acetic acid (IAA) (pink colour) in vacuoles of cortical parenchyma and cambial zone. L: ROS (brown colour) detected in cell walls and vacuoles of all tissues. CP, cortical parenchyma; CZ, cambium zone; Fi, fibre; NT, nutritive tissue; Pe, periderm; Ph, phloem; VP, vascular parenchyma; Xy, xylem.

recognized by LM10 in young (Fig. 5H), mature and senescent stages (Fig. 5I). At all stages of gall development, heteromannans were not labelled by LM21. Accumulation the phenolics (Fig. 5J) and IAA (Fig. 5K) occurs only in vacuoles of the outer tissue compartment cells, while ROS are strongly detected in cell walls and vacuoles of the nutritive cells and outer tissue compartment cells (Fig. 5L).

### Comparative analysis of the accumulation of metabolites and labelling by monoclonal antibodies

The accumulation of IAA, ROS and phenolics in the outer tissue compartment, and the accumulation of ROS only in nutritive tissue, were similar in all three gall morphotypes associated with *I. ingoides*; however, the hemicellulose

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labelling was distinct. In the outer tissue compartment, xyloglucans were similarly labelled in the globoid and young lenticular galls, but these hemicelluloses were only labelled in the phloem of the fusiform galls. The xylans are only labelled in lignified cell walls. The cell walls of nutritive tissues of all three gall morphotypes were xyloglucan-positive in young and mature stages but became negative at senescence. Peculiarly, in the senescent stage of the lenticular galls, the nutritive cell walls were lignified and contain xylans labelled by LM10. The dynamics of xyloglucans altered from accumulation to non-accumulation in different cell walls of the different tissue compartments, but the dynamics of xylans remained constant in lignified cell walls as soon as the sclerenchyma and xylem cells had differentiated (Fig. 6).

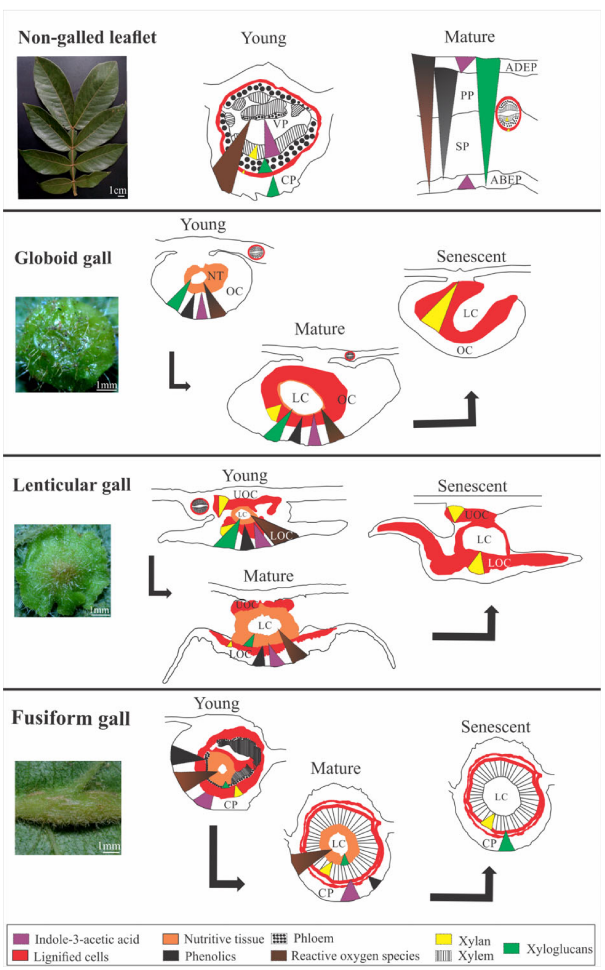



**Fig. 6.** Diagrams showing sites of accumulation of IAA, ROS and phenolics, and labelling of hemicelluloses using monoclonal antibodies (MAb) for three gall morphotypes induced on *Inga ingoides* (Rich.) Willd. (Fabaceae). Accumulation of IAA, phenolics and ROS represented in pink, black and brown, respectively. Detection of hemicelluloses, xyloglucans and xylans represented in green and yellow, respectively. ABEP, abaxial epidermis; ADEP, adaxial epidermis; CP, cortical parenchyma; LOC, lower outer tissue compartment; NT, nutritive tissue; OC, outer tissue compartment; PP, palisade parenchyma; SP, spongy parenchyma; UOC, upper outer tissue compartment; VP, vascular parenchyma.

## DISCUSSION

### Hemicelluloses and functional traits during gall development

The spatial detection of hemicelluloses by the monoclonal antibodies differed among gall morphotypes and their developmental stages. In the outer tissue compartment, the site of intense cell hypertrophy, xyloglucan detection was age-dependent, *i.e.* xyloglucans were detected in mature galls when the final gall shape had been established. Unexpectedly, xyloglucans were also detected at the young stage of the lenticular and globoid galls, when they may have prevented the microfibrils from slipping and thus limited cell expansion. In this case, theoretically, other factors may have influenced cell wall flexibility and consequent cell expansion. Due to auxin accumulation, the pH and ion flux are altered in cell walls (Madja & Roberts 2018), which may favour the random activity of pectin methylesterases (PME) and cell wall loosening (Micheli 2001; Yoneda *et al.* 2010). The flexibility of the cell walls mediated by pectins was observed in various host plant–galling insect systems (Carneiro *et al.* 2014; Oliveira *et al.* 2014; Carneiro *et al.* 2015; Teixeira *et al.* 2018) and may be also associated with the cell wall loosening in the young stage on *I. ingoides* galls.

At maturation stage, xyloglucans were detected only in the outer tissue compartment of the globoid galls, where they could influence cell wall rigidity (Cavalier *et al.* 2008; Cosgrove 2016). The lack of detection of xyloglucans in the mature stage of the lenticular galls may be compensated by the deposition of secondary walls, which confer rigidity to the cell walls.

In fusiform galls, xyloglucans were detected only in the senescent stage, which, as expected, indicates the end of cell elongation. The mechanical resistance acquired during senescence is a consequence of the end of the stimuli produced by the galling herbivores, and the permanence of the intralaminar gall tissues immersed in the host organ. The lack of detection of xyloglucans in the globoid and lenticular galls may be a consequence of their remobilization to other sites in the plant tissue. The remobilization process involves degradation of cell wall components (Matile 1974; Chen *et al.* 2018), which is particularly important from the extra-laminar galls toward the host leaf lamina, as these galls will be abscised from the host plant through senescence.

In addition to the imbalance in flexibility and resistance of the cell walls, the xyloglucans can also provide nutritional resources to the galling insect. The larvae of Cecidomyiidae induce cell disruption using their prothoracic spatula. Cellular fluids are extracorporeally digested and then sucked by the larvae (Rohfritsch 1992; Harris 1994; Ferreira *et al.* 2019). Xyloglucans can be made available during the degradation of cell walls, intense oxidative stress and the action of enzymes that can cleave xyloglucans and release their constituent sugars (Barnes & Anderson 2018). These sugars can be internalized and made available for the feeding of the galling insect.

The labelling of xyloglucans with LM15 in the nutritive tissue of the lenticular, fusiform and globoid galls suggests a trait similar to that in seeds of *Fabaceae* species (Buckeridge *et al.* 2000; Santos *et al.* 2004). This denotes a potential of the host plant cells to redirect xyloglucans to the gall developmental site, and reinforces the possible role of xyloglucans as reserves directed to the galling insects associated with *I. ingoides*.

The differential detection of xylans in the three gall morphotypes associated with *I. ingoides* revealed the unique

characteristics of each galling insect. For all three gall morphotypes, xylans were associated with lignified cell walls throughout the developmental stages, as expected for secondary cell wall development (Evert 2006; Rennie & Scheller 2014).

The third investigated hemicelluloses, the heteromannans, despite acting as carbohydrate reserves in seed vacuoles, vegetative tissues and endosperm walls (Lieperman *et al.* 2007; Moreira & Filho 2008), were not detected in tissues of non-galled leaflets or galls on *I. ingoides*.

### Hemicelluloses and the accumulation of other compounds

The dynamics of hemicelluloses and fibrils was related to the accumulation of ROS, IAA and phenolics in specific tissue compartments of the three gall morphotypes. The histolocalized overlap of these compounds may have contributed to flexibility of the cell walls. In the outer tissue compartment, auxins accumulated because of inhibition of IAA-oxidases by phenolics (Hori 1992), which will favour cell wall flexibility through acid growth (Madja & Roberts 2018). The alterations in the dynamics of cellulose fibrils–hemicelluloses in the inner tissue compartment may have been mediated by ROS signal transmission (Isaias *et al.* 2015), as $H_2O_2$ was the only molecule detected in this gall tissue site. Although phenolic compounds, ROS and IAA were crucial for cell wall flexibility during the development of the gall morphotypes (Suzuki *et al.* 2015; Bedetti *et al.* 2017), each galling herbivore orchestrated cell wall flexibility to generate its own specific gall morphotype through changes in the direction of cell enlargement. In addition to the association with auxin, allowing flexibility to cell walls, phenolic compounds also contribute to cell wall rigidity. Such rigidity is conferred by the deposition of lignins, a class of phenolics derived from the phenylpropanoid pathway (Liu *et al.* 2018; Vanholme *et al.* 2019), which are deposited in the secondary cell walls, forming a mechanically rigid structure (Liu *et al.* 2018). In gall developmental sites, lignification represents a method for the control of oxidative stress to maintain redox homeostasis (Isaias *et al.* 2015) and the protection of the galling herbivore (Mani 1964). The galling insects seem to use different strategies to guarantee the dynamics between rigidity and flexibility of cell walls during gall development. These strategies are associated with the refined control of hemicelluloses, microfibrils, pectins and lignins.

## FINAL CONSIDERATIONS

The alterations in the axes of cell expansion and patterns of elongation depended on the relationship of ROS and IAA with the hemicelluloses (xyloglucans and xylans) and are related to cellulose fibril reorientation. Xyloglucan detection is independent of the developmental stage of the three gall morphotypes but their labelling with LM15 in nutritive cells of the three gall morphotypes revealed a new functional trait related to the nutrition of the gall inducers.

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## AUTHOR CONTRIBUTIONS

GPPB and RMSI designed experiments. GPPB, CFA and MSCF collected material and performed experiments. All authors analysed the results, discussed data, wrote and reviewed the manuscript. RMSI coordinated the project.

## SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

**Table S1.** Degree of isotropy and anisotropy during the development of leaflets and galls in *Inga ingoides* (Fabaceae).

**Table S2.** Dynamics of cell expansion during development of the leaflets and galls in *Inga ingoides* (Fabaceae) (mean cell areas, $\mu m^2 \pm$ SD).

**Figure S1.** Anatomy of non-galled leaflets and galls of *Inga ingoides* (Rich.) Willd. (Fabaceae).

## REFERENCES

Barnes W.J., Anderson C.T. (2018) Release, recycle, rebuild: cell-wall remodeling, autodegradation, and sugar salvage for new wall biosynthesis during plant development. *Molecular Plant*, **8**, 31–46.

Baskin T.I. (2005) Anisotropic expansion of the plant cell wall. *Annual Review of Cell and Developmental Biology*, **21**, 203–222.

Bedetti C.S., Modolo L.V., Isaias R.M.S. (2014) The role of phenolics in the control of auxin in galls of *Piptadenia gonoacantha* (Mart) MacBr (Fabaceae: Mimosoideae). *Biochemical Systematics and Ecology*, **55**, 53–59.

Bedetti C.S., Bragança G.P., Isaias R.M.S. (2017) Influence of auxin and phenolic accumulation on the patterns of cell differentiation in distinct gall morphotypes on *Piptadenia gonoacantha* (Fabaceae). *Australian Journal of Botany*, **65**, 411–420.

Bedetti C.S., Jorge N.C., Trigueiro F.C.G., Bragança G.P., Modolo L.V., Isaias R.M.S. (2018) Detection of cytokinins and auxin in plant tissues using histochemistry and immunocytochemistry. *Biotechnic & Histochemistry*, **9**, 149–154.

Buckeridge M.S., Santos H.P., Tiné M.A.S. (2000) Mobilization of storage cell wall polysaccharides in seeds. *Plant Physiology and Biochemistry*, **38**, 141–156.

Bukatsch F. (1972) Bermerkungenzur Doppelfärbung Astrablau-Safranin. *Mikrokosmos*, **61**, 255.

Carneiro R.G.S., Oliveira D.C., Isaias R.M.S. (2014) Developmental anatomy and immunocytochemistry reveal the neo-ontogenesis of the leaf tissues of *Psidium myrtoides* (Myrtaceae) towards the globoid galls of *Nothotrioza myrtoidis* (Triozidae). *Plant Cell Reports*, **33**, 2093–2106.

Carneiro R.G., Pacheco P., Isaias R.M.S. (2015) Could the extended phenotype extend to the cellular and subcellular levels in insect-induced galls? *PLoS ONE*, **8**, 01–20.

Cavalier D.M., Oliver L., Neumetzler L., Yamauchi K., Reineck A., Freschour G., Zanbotina O.A., Hanh M.G., Burgest I., Pauly M., Raikhel N.V., Keegstra K. (2008) Disrupting two *Arabidopsis thaliana* xylosyltransferase genes results in plants deficient in xyloglucan, a major primary cell wall component. *The Plant Cell*, **20**, 1519–1537.

Chen C., Zeng L., Ye Q. (2018) Proteomic and biochemical changes during senescence of *Phalaenopsis* 'Red dragon" petals. *International Journal of Molecular Sciences*, **19**, 1317.

Cosgrove D.J. (2000) Expansive growth of plant cell walls. *Plant Physiology and Biochemistry*, **38**, 109–124.

Cosgrove D.J. (2016) Plant cell wall extensibility: connecting plant cell growth with cell wall structure, mechanics, and the action of wall modifying enzymes. *Journal of Experimental Botany* **67**, 463–476.

Cosgrove D.J. (2018) Diffuse growth of plant cell walls. *Plant Physiology*, **176**, 16–27.

Crowell E.F., Gonneau M., Vernhettes S., Höfte H. (2010) Regulation of anisotropic cell expansion in higher plants. *Comptes Rendus Biologies*, **333**, 320–324.

Evert R.F. (2006) *Esau's plant anatomy: meristems, cells, and tissues of the plant body: their structure, function and development*, 3rd edn. Wiley, São Paulo, Brazil.

Ferreira B.G., Alvarez R., Bragança G.P., Alvarenga D.R., Perez-Hidalgo N. (2019) Feeding and other gall facets: patterns and determinants in gall structure. *The Botanical Review*, **85**, 78–106.

Ferreira B.G., Bragança G.P., Isaias R.M.S. (2020) Cytological attributes of storage tissues in nematode and eriophyid galls: pectin and hemicellulose functional insights. *Protoplasma*, **257**, 229–244.

Formiga A.T., Oliveira D.C., Ferreira B.G., Magalhães T.A., Castro A.C., Fernandes G.W., Isaias R.M.S. (2013) The role of pectic composition of cells walls in the determination of the new shape-functional design of *Baccharis reticularia* (Asteraceae). *Protoplasma*, **250**, 899–908.

Harris K.M. (1994) Gall midges (Cecidomyiidae): classification and biology. In: Williams M. A. J. (Ed), *Plant galls: organisms, interactions, populations*. The Systematics Association, Vol. Special 49, Clarendon Press, Oxford, UK, pp 201–211.

Herth W., Schnepf E. (1980) The fluorochrome, calcofluor white, binds oriented to structural polysaccharide fibrils. *Protoplasma*, **105**, 129–133.

Hogetsu T. (1991) Mechanism for formation of the secondary wall thickening in tracheary elements: microtubules and microfibrils of tracheary elements of *Pisum sativum* L. and *Commelina communis* L. and the effects of amiprophosmethyl. *Planta*, **185**, 190–200.

Hori K. (1992) Insect secretions and their effect on plant growth, with special reference to hemipterans. In: Shorthouse J. D., Rohfritsch O. (Eds), *Biology of insect-induced galls*. Oxford University Press, Oxford, UK, pp 157–170.

Isaias R.M.S., Oliveira D.C. (2012) Gall phenotypes – product of plant cells defensive responses to the inducers attack. In: Mérillon J. M., Ramawat K. G. (Eds), *Plant defense: biological control*. Springer, Berlin, Germany, pp 273–290.

Isaias R.M.S., Carneiro R.G.S., Oliveira D.C., Santos J.C. (2013) Illustrated and annotated checklist of Brazilian gall morphotypes. *Neotropical Entomology*, **42**, 230–239.

Isaias R.M.S., Oliveira D.C., Carneiro R.G.S., Kraus J.E. (2014) Developmental anatomy of galls in the neotropics: arthropods stimuli versus host plant constraints. In: Fernandes G. W., Santos J. C. (Eds), *Neotropical insect galls*. Springer, Berlin, Germany, pp 15–34.

Isaias R.M.S., Oliveira D.C., Moreira A.S.F.P., Soares G.L.G., Carneiro R.G.S. (2015) The imbalance of redox homeostasis in arthropod-induced plant galls: mechanisms of stress generation and dissipation. *Biochimica et Biophysica Acta*, **1850**, 1509–1517.

Johansen D.A. (1940) *Plant microtechnique*. McGraw-Hill, New York, USA.

Karnovsky M.J. (1965) A formaldehyde-glutaraldehyde fixative of high osmolarity for use in electron microscopy. *Journal of Cell Science*, **27**, 137–138.

Kraus J.E., Arduin M. (1997) *Manual básico de métodos em morfologia vegetal*. Editora da Universidade Federal Rural do Rio de Janeiro, Seropédica, Brazil.

Leopold A.C., Plummer T.H. (1961) Auxin–phenol complexes. *Plant Physiology*, **36**, 589–592.

Lieperman A.H., Naim C.J., Willats G.T., Sørensen A.W.R., Keegstra K. (2007) Functional genomics analysis supports conservation of function among cellulose synthase-like a gene family members and suggests diverse roles of mannans in plants. *Plant Physiology*, **143**, 1881–1189.

Liu Q., Luo E., Zheng L. (2018) Lignins: biosynthesis and biological functions in plants. *International Journal of Molecular Science*, **19**, 335.

Madja M., Robert S. (2018) The role of auxin in cell wall expansion. *International Journal of Molecular Science*, **19**, 951.

Magalhães T.A., Oliveira D.C., Suzuki A.Y.M., Isaias R.M.S. (2014) Patterns of cell elongation in the determination of the final shape of *Baccharopelma dracunculifoliae* (Psyllidae) on *Baccharis dracunculifolia* DC. *Protoplasma*, **251**, 747–753.

Mani M.S. (1964) *Ecology of plant galls*. Dr. W. Junk, The Hague, the Netherlands.

Marcus S.E., Verhertbruggen Y., Hervé C., Ordaz-Ortiz J.J., Farkas V., Pedersen H.J., Willats W.G.T., Knox J.P. (2008) Pectic homogalacturonan masks abundant sets of xyloglucan epitopes in plant cell walls. *BMC Plant Biology*, **8**, 60–71.

Marques J.P.R., Soares M.K.M., Appezzato-da-Glória B. (2013) New staining technique for fungal-infected plant tissues. *Turkish Journal of Botany*, **37**, 784–787.

Matile P.H. (1974) Cell wall degradation in senescing tobacco leaf discs. *Cellular and Molecular Life Sciences*, **30**, 98–99.

Meidani C., Ntalli N.G., Giannoutsou E., Adamakis I.D.S. (2019) Cell wall modifications in giant cells induced by the plant parasitic nematode *Meloidogyne* incognita in wild-type (Col-0) and the *fra2 Arabidopsis thaliana* Katanin mutant. *International Journal of Molecular Science*, **20**, 5465.

Micheli F. (2001) Pectin methylesterases: cell wall enzymes with important roles plant physiology. *Trends in Plant Science*, **6**, 414–417.

Moreira L.R.S., Filho E.X.F. (2008) An overview of mannan structure and mannan-degrading enzyme systems. *Applied Microbiology and Biotechnology*, **79**, 165–178.

O'Brien T.P., Feder, N., McCully M.E. (1964) *Polychromatic staining of plant cell walls by toluidine blue O. Protoplasma*. **59**, 368–373.

Oliveira D.C., Isaias R.M.S. (2010) Cytological and histochemical gradients induced by a sucking insect in galls of *Aspidosperma australe* Arg. Muell (Apocynaceae). *Plant Science*, **178**, 350–358.

Oliveira D.C., Magalhães T.A., Ferreira B.G., Teixeira C.T., Formiga A.T., Fernandes G.W., Isaias R.M.S. (2014) Variation in the degree of pectin methylesterification during the development of *Baccharis dracunculifolia* kidney-shaped gall. *PLoS ONE*, **9**, e94588.

Paiva J.G.A., Fank-de-Carvalho S.M., Magalhães M.P., Graciano-Ribeiro D. (2006) Verniz vitral incolor 500®: uma alternativa de meio de montagem economicamente viável. *Acta Botanica Brasilica*, **20**, 257–264.

Park Y.B., Cosgrove D.J. (2015) Xyloglucan and its interactions with other components of the growing cell wall. *Plant & Cell Physiology*, **56**, 180–194.

Rayle D.L., Cleland R.E. (1992) The acid growth theory of auxin-induced cell elongation is alive and well. *Plant Physiology*, **99**, 1271–1274.

Rennie E.A., Scheller H.V. (2014) Xylan biosynthesis. *Current Opinion in Biotechnology*, **26**, 100–107.

Rohfritsch O. (1992) Patterns in gall development. In: Shorthouse J. D., Rohfritsch O. (Eds), *Biology of insect-induced galls*. Oxford University Press, New York, USA, pp 60–86.

Rohfritsch O. (2008) Plants, gall midges, and fungi: a three-component system. *Entomologia Experimentalis et Applicata*, **128**, 208–216.

Rose J.C. (2003) *The plant cell wall*. Blackwell, Oxford, UK.

Rosseti S., Bonatti P.M. (2001) In situ histochemical monitoring of ozone and TMV induced reactive oxygen species in tobacco leaves. *Plant Physiology and Biochemistry*, **39**, 433–442.

Santos H.P., Purgatto E., Mercier H., Buckeridge M.S. (2004) The control of storage xyloglucan mobilization in cotyledons of *Hymenaea courbaril*. *Plant Physiology*, **135**, 287–299.

Sauter M.S., Seagull R.W., Kende H. (1993) Internodal elongation and orientation of cellulose microfibrils and microtubules in deepwater rice. *Planta*, **190**, 354–362.

Scheller H.V., Ulvskov P. (2010) Hemicelluloses. *Annual Review of Plant Biology*, **61**, 263–289.

Suzuki A.Y.M., Bedetti C.S., Isaias R.M.S. (2015) Detection and distribution of cell growth regulators and cellulose microfibrils during the development of *Lopesia* sp. galls on *Lonchocarpus cultratus* (Fabaceae). *Botany-Botanique*, **93**, 435–444.

Teixeira C.T., Oliveira D.C., Kuster V.C., Isaias R.M.S. (2018) Immunocytochemical demonstration of cell wall components related to tissue compartments in the globoid galls induced by *Clinodiplosis* sp. (Cecidomyiidae) on *Croton floribundus* Spreng. (Euphorbiaceae). *Botany-Botanique*, **96**, 9–18.

Thompson D.S. (2005) How do cell walls regulate plant growth? *Journal of Experimental Botany*, **56**, 2275–2285.

Vanholme R., De Meester B., Ralph J., Boerjan W. (2019) Lignin biosynthesis and its integration into metabolism. *Current Opinion in Biotechnology*, **56**, 230–239.

Voiniciuc C., Pauly M., Usadel B. (2018) Monitoring polysaccharide dynamics in the plant cell wall. *Plant Physiology*, **176**, 2590–2600.

Yoneda A., Takuya I., Takumi H., Natsumaro K., Tamio S., Takeshi I., Hiroyuki O., Seiichiro H., Minami M., Taku D. (2010) Cobtorin target analysis reveals that pectin functions in the deposition of cellulose microfibrils in parallel with cortical microtubules. *The Plant Journal*, **64**, 657–667.

Zhao Y. (2010) Auxin biosynthesis and its role in plant development. *Annual Review of Plant Biology*, **61**, 49–64.

**Supporting information**

**TABLE S1:** Degree of isotropy and anisotropy during the development of leaflets and galls in *Inga ingoides* (Fabaceae).

| | **Tissue** | **Developmental stage** | | |
|---|---|---|---|---|
| | | Immature | Mature | Senescent |
| **Non-galled leaflet** | Abaxial epidermis | - | 0.70 | - |
| | Spongy parenchyma | - | 1.09 | - |
| | Cortex | 0.73 | - | - |
| | Fibers | 0.76 | - | - |
| | Vessel element | 1.11 | - | - |
| | Vascular parenchyma | 0.62 | - | - |
| | **Tissue** | Young | Mature | Senescent |
| **Globoid gall** | Dermal system | 0.79 | 0.69 | 0.62 |
| | Outer tissue compartment | 0.75 | 0.67 | 0.72 |
| | Inner tissue compartment | - | 0.63 | 0.71 |
| | Nutritive tissue | 0.81 | 0.77 | - |
| **Lenticular gall** | Dermal system | 0.68 | 0.62 | 0.45 |
| | Lower outer tissue compartment | 1.76 | 1.91 | 2.35 |
| | Nutritive tissue | 0.95 | 1.32 | 1.63 |
| | Upper outer tissue compartment | - | 0.68 | 0.62 |
| **Fusiform gall** | Cortex | 1.03 | 0.48 | 0.49 |
| | Fiber | 0.87 | 0.76 | 0.80 |
| | Vessel element | 0.95 | 1.09 | 0.99 |
| | Nutritive tissue | 0.95 | 1.06 | - |

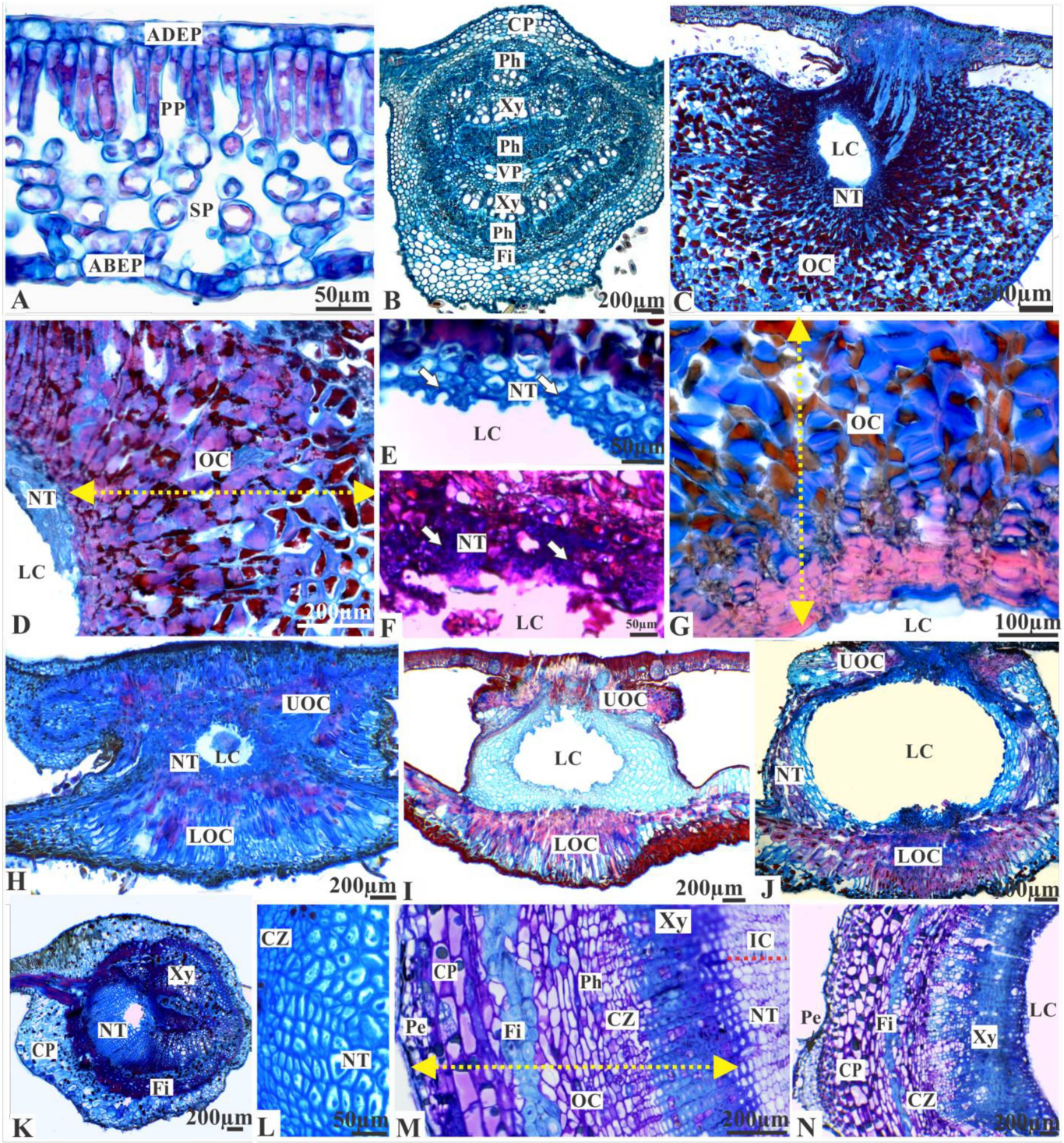
Notes: Values lower than 0.89 indicate tendency to periclinal direction expansion; values between 0.9 - 1.10 indicate a tendency to isotropic expansion, values higher than 1.11 indicate tendency to anticlinal expansion direction.

**TABLE S2** Dynamics of cell expansion during development of the leaflets and galls in *Inga ingoides* (Fabaceae) (mean cell areas-µm$^2$ ± standard deviations).

| **Non-galled tissue leaflet** | **Non galled leaflet** | |
|---|---|---|
| | Young | Mature |
| ABEP | - | 165.0±44.5 a |
| SP | - | 249.5±55.1 a |
| CP | 412.6±99.6 a | - |
| Fi | 98.4±12.6 a | - |
| VE | 472.2±47.7 a | - |
| VP | 159.5±64.6 a | - |
| **Non-galled tissue/Gall** | **Globoid gall** | |
| | Young | Mature | Senescent |
| ABEP /DS | 306.1±62.4 b | 342.7±79.6 b | 273.0±70.2 b |
| SP/OC | 1295.1±337.2 b | 3833.9±823.3 c | 4000.9±575.1 c |
| SP/IC | - | 940.8±225.3 b | 1027.3±236.2 b |
| SP /NT | 384.1±75.4 b | 604.9±162.1 c | - |
| **Non-galled tissue/Gall** | **Lenticular gall** | |
| | Young | Mature | Senescent |
| ABEP/DS | 442.9±87.3 b | 804.7±282.6 c | 808.8±137.2 c |
| SP/LOC | 1381.6±365.8 b | 2717.7±796.7 c | 3114.2±934.8 c |
| SP /NT | 468.3±178.3 ab | 1528.6±680.0 c | 1501.4±526.4 bc |
| SP/UOC | - | 923.3±225.8 c | 928.7±116.0 c |
| **Non-galled tissue/Gall** | **Fusiform gall** | |
| | Young | Mature | Senescent |
| CP/CP | 2621.5±683.4 b | 2356.4±804.3 b | 1900.1±815.5 b |
| Fi/Fi | 1450.1±339.2 b | 683.2±195.3 c | 566.8±132.8 c |
| VE/VE | 863.3±392.4 b | 1091.6±393.1 b | 983.2±485.1 b |
| VP/NT | 626.5±55.0 b | 511.6±117.3 c | - |

Notes: Data correspond to the statistical analysis of ANOVA. Values followed by different letters between the tissues of the non-galled leaflet and the stages of development of globoid, lenticular and fusiform galls indicate statistical differences in the cell areas between groups. abep, abaxial epidermis; cp, cortical parenchyma; ds, dermal system; fi, fibers; ic, inner tissue compartment; laoc, lateral outer tissue compartment; loc, lower outer tissue compartment; nt, nutritive tissue; oc, outer tissue compartment; sp, spongy parenchyma; uoc, upper outer tissue compartment; ve, vessel elements; vp, vascular parenchyma.

**Figure S1. Anatomy of non-galled leaflets and galls of *Inga ingoides* (Rich.) Willd. (Fabaceae).** (A) Leaflet evidencing uniseriate epidermis and dorsiventral mesophyll. (B) Midrib vein with bicollateral vascular system. (C-G) Globoid gall. (C) Young stage. (D-F) Mature stage. (D) Cell wall lignification next to the nutritive tissue. (E) Detail of nutritive tissue. (F) Fungi stained by cotton blue in lactophenol (white arrows) in the nutritive tissue of a mature gall. (G) Senescent stage evidencing the outer tissue compartment and absence of nutritive tissue. (H-J) Lenticular gall. (H) Young stage with the beginning of lignification process in the upper and lower outer tissue compartment and inner tissue compartment with nutritive cells. (I) Mature stage evidencing the lignification of cell walls of lower and upper outer tissue compartment. (J) Senescent stage evidencing the lignification of cell walls of nutritive tissue. (K-N) Fusiform gall. (K) General anatomical aspect of a gall in young stage. (L) Cambium zone and the nutritive tissue of a young gall. (M) Mature stage with periderm, cortical parenchyma, fibers, cambium zone, secondary phloem and xylem, and nutritive tissue. (N) Senescent stage evidencing the absence of the nutritive tissue. ABEP, abaxial epidermis; ADEP, adaxial epidermis; CP, cortical parenchyma; CZ, cambium zone; Fi, fibers; IC, inner tissue compartment; LC, larval chamber; LOC, lower outer tissue compartment; NT, nutritive tissue; OC, outer tissue compartment; PE, periderm; Ph, phloem; PP, palisade parenchyma; SP, spongy parenchyma; UOC, upper outer tissue compartment; VP, vascular parenchyma; Xy, xylem.


ADEP
PP
SP
ABEP
A
50µm
CP
Ph
Xy
Ph
VP
Xy
Ph
Fi
B
200µm
LC
NT
OC
C
OC
NT
LC
D
NT
LC
E
50µm
NT
LC
F
50µm
OC
LC
G
100µm
UOC
NT
LC
LOC
H
200µm
UOC
LC
LOC
I
200µm
UOC
LC
NT
LOC
J
Xy
NT
CP
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200µm
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50µm
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Ph
Pe
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200µm
Pe
Fi
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LC
CP
CZ
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200µm

# Capítulo IV

# Distinct cytological mechanisms for food availability in three *Inga ingoides* (Fabaceae) – Cecidomyiidae gall systems

**Manuscrito submetido ao periódico científico Protoplasma**

Distinct cytological mechanisms for food availability in three *Inga ingoides* (Fabaceae) – Cecidomyiidae gall systems

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**Declarations**

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***Ethics approval***: Not applicable

***Consent to participate***: Not applicable

***Consent for publication***: Not applicable

***Availability of data and material:*** The data and material can be made available as needed.

***Code availability:*** Not applicable

***Authors' contributions:*** GPPB, RMSI, and BGF designed the experiments. GPB collected material and performed the experiments. All authors analyzed the results, discussed data, wrote, and reviewed the manuscript. RMSI coordinated the project.

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**Abstract**

Gall cytological, metabolic, and structural traits are established due to the feeding habits of the associated galling herbivores, and sometimes are influences by of other organisms involved in the interaction. We tested this assumption on three gall morphotypes, the globoid, the lenticular and the fusiform, induced by Cecidomyiidae on leaflets of *Inga ingoides* (Rich.) Willd. (Fabaceae: Caesalpinioideae. Taking for granted that the three Cecidomyiidae galls are induced on the same host plant and organ, we assume that the cytological and histochemical traits of their nutritive cells may be similar, but under the fungi influence, the ambrosia gall cytological profile may be peculiar, and reflect on the accumulation of primary metabolites. The globoid galls are ambrosia galls involving three organisms (host plant, gall inducer, and fungi), while the fusiform and the lenticular galls have two organisms (host plant and gall inducer). The accumulation of primary metabolites is similar among the three gall morphotypes, except for the non-detection of reducing sugars in the fusiform galls. The fungi presence in the ambrosia globoid gall impacts its cytological profile but does not imply in an exclusive cytological profile regarding the fusiform and the lenticular galls. In fact, the cytological traits have revealed three different cytological mechanisms for food resources availability to the three galling Cecidomyiidae: a) cell wall dissolution by fungal intermediation in the ambrosia globoid galls, b) necrosis-type cell death in the fusiform galls, and c) maintenance of continuous metabolic activity in the lenticular galls.

**Keywords:** ambrosia gall, cell wall, gall anatomy, histochemistry, nutritive cells

**Introduction**

Galling insects alter the structural, cytological, and metabolic patterns of plant tissues, regulating the specific patterns of their galls (Rohfritsch 1992, Bronner 1992). Gall development results in new histological arrangement associated with different ecophysiological traits (Stone and Schönrogge 2003, Bragança et al. 2017, Miller and Raman 2018), and cytological patterns (Bronner 1992, Carneiro and Isaias 2015, Ferreira et al. 2015, 2020). In the cecidomyiid galls, the outermost tissue layers have more vacuolated and less cytologically active cells (Oliveira et al. 2011). The inner tissue compartment has nutritive cells with dense cytoplasm, abundant organelles, evident nucleus, and nucleolus, which indicate their intense metabolic activity (Bronner et al. 1992, Carneiro and Isaias 2015, Ferreira et al. 2015, 2020). Nutritive cells accumulate primary metabolites, such as proteins, lipids, or reducing sugars directed to the galling insect nutrition

(Bragança et al. 2017, Ferreira et al. 2017, Isaias et al. 2018). To access food resources, the gall inducer may use its enzymatic apparatus (Gagné 1994), trigger the mediation of host plant enzymes (Oliveira et al. 2010, Oliveira et al. 2011), or induce the death of the nutritive cells (Bronner 1992, Harris et al. 2006). In addition, gall nutritive metabolism can have the influence of different feeding guilds (Weis 1982, Fernandez et al. 1999, Luz et al. 2019), as is true for the ambrosia fungi (Rohfritsch 2008), the parasitoids, cecidophages, and inquilines (Luz et al. 2019). The influence of these organisms in gall systems may result in new cytological and histochemical profiles (Rezende et al. 2019). In ambrosia galls, the adult cecidomyiid female inoculates the fungi spores inside plant tissues during the oviposition, and a mix of fungal mycelia and nutritive-like cells develop adjacent to the larval chamber (Bronner 1992, Yukawa and Rohfritsch 2005, Rohfritsch 2008). In the symbiotic relationship established in ambrosia galls, the fungi facilitate the gall inducer access to plant cell protoplast (Rohfritsch 2008, Pan et al. 2015, Raman and Suryanarayanan 2017).

We hypothesize that the cytological traits of nutritive tissues differentiate under the influence of the gall inducer feeding activity, but other associated organisms impact the gall cytological profiles, which reflect on the accumulation of primary metabolites. Such hypothesis is elegantly tested on a superhost plant associated with different gall systems, *Inga ingoides* (Rich.) Willd. (Fabaceae: Caesalpinioideae), where we comparatively analyzed a host plant-Cecidomyiidae-fungi gall system, an ambrosia gall, with two host plant-Cecidomyiidae gall systems. The following questions guided the discussion: (1) which histochemical pattern of the nutritive tissue of the three gall morphotypes? And (2) how do the cytological profiles of the nutritive cells relate to the availability of primary metabolites and to the associated organisms?

**Material and methods**

*Sampling.* Samples of *Inga ingoides* leaflets with the three Cecydomyiidae-induced gall morphotypes (globoid, fusiform and lenticular) in mature stage (Figure 1) (n = 7 per gall morphotype) were collected at the Parque Estadual Serra Verde (PESV) (19°47’22.47"S, 43°57'32.35"W) and at the Estação Ecológica (EEco) of the Universidade Federal de Minas Gerais (UFMG) (19°52’29” S; 43°58’21” W), Belo Horizonte, Minas Gerais, Brazil. The mature stage was used for it represents the final phase of development of gall tissues, and the main trophic phase of the gall inducer (Rohfritsch 1992).

*Anatomical analyses.* The tissue compartments of the galls were defined by their histological profiles in transverse sections. Gall fragments (2 $mm^2$) were fixed in 2.5% glutaraldehyde and 4.5% formaldehyde (Karnovsky 1965, modified to 0.1 mol $L^{-1}$ pH 7.2 phosphate buffer) for 24h. The fragments were dehydrated in an *n*-butyl series (Johansen 1940), embedded in Paraplast (Sigma®) (Kraus and Arduin 1997), and

sectioned in a rotatory microtome (12-14 μm) (Leica® Biocut 2035). The histological sections were deparaffinized, hydrated, and stained with Astra blue and safranin (9:1, v/v) (Bukatsch 1972, modified to 0.5%), and mounted with colorless varnish Acrilex® (Paiva et al. 2006). A second set of fragments were dehydrated in an ethanol series, embedded in glycolmethacrylate Leica® Historesin, sectioned in rotatory microtome (5-7 μm), and stained with toluidine blue O in 0.1 mol.$L^{-1}$ acetate buffer (O'Brien and McCully 1964, modified to pH 4.7). The histological sections were observed and photographed with a digital camera Leica ICC50 HD coupled to a light microscope Leica DM 500 (Leica, Wetzlar, Germany).

*Histochemical analyses.* Fresh fragments of the three gall morphotypes were handmade transversely sectioned with razor blades and submitted to specific reactions for the detection of primary metabolites. Lipids was red-stained with a saturated solution of Sudan red B in ethanol 70% for 5 min (Brundett et al. 1991). Starch was stained as blackish blue coloration with 1% potassium iodine–iodide solution (Lugol's reagent) for 5 min (Johansen 1940). Proteins were stained in blue with 0.1% bromophenol blue in a saturated solution of 10% magnesium chloride in ethanol for 15 min, washed in 0.5% HCl for 20 min, and washed in water for 3 min (Mazia et al. 1953). Reducing sugars form reddish-brown precipitates with 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 (Sass 1951). Blank sections were used as control. The sections were mounted on slides with water, and immediately analyzed under a light microscope (Leica DM500®) (Leica, Wetzlar, Germany) and photographed with a coupled digital camera Leica ICC50 HP®.

*Cytological analyses.* Fixed fragments of the nutritive tissues of the three gall morphotypes were post-fixed in 1% osmium tetroxide ($OsO_4$) in 0.1 mol.$L^{-1}$ PBS, dehydrated in an ethanol series (O'Brien and McCully 1981), and embedded in Spurr® resin (Sigma-Aldrich) (Lufth 1961). The samples were sectioned in an ultramicrotome Reichert-Jung Ultracut (Leica, Wetzlar, Germany), contrasted with uranyl acetate and lead citrate (Reynolds 1963), and analyzed under a transmission electron microscope Tecnai™ G2-12 Spirit BioTwin (FEI, Hillsboro, USA; 120KV) at the Centro de Microscopia of the Universidade Federal de Minas Gerais (CM-UFMG).

**Results**

*General aspects*

The three gall morphotypes (Figure 1a-c) are induced on the leaflets. The globoid galls (Figure 1a) are extralaminar, isolated, and induced on the adaxial or the abaxial surface of the leaflet lamina. The fusiform

galls (Figure 1b) are intralaminar, isolated or coalescent, and induced on the midrib or secondary veins. The lenticular galls are extralaminar (Figure 1c), isolated, and induced on the abaxial surface of the leaflet lamina.

*Globoid galls*

The globoid galls have two distinct tissue compartments: an outer tissue compartment and an nutritive-like tissue (Figure 2a). The inner tissue compartment has 2-4 layers of nutritive cells associated with fungal hyphae, forming a nutritive-like tissue (Figure 2b). The nutritive cells accumulate proteins (Figure 2c), lipids (Figure 2d) and reducing sugars (Figure 2e), and have pyknotic nucleus, dense cytoplasm, several mitochondria, and vesicles. The cell walls have sites of dissolution and rupturing (Figure 2f). The cell walls facing the lignified zone have several plasmodesmata, mitochondria, prolamellar bodies, and chloroplasts with disorganized thylakoids and many vesicles (Figure 2g). The hyphae have cells with sinuous walls and cytoplasm rich in vesicles (Figure 2h); some cell wall portions are in process of dissolution (Figure 2i) (Table 1).

*Fusiform galls*

The outer and inner tissue compartments of the fusiform galls develop by secondary growth process (Figure 3a). The inner tissue compartment is formed by the typical nutritive tissue, which is divided into two regions. The outer region has 3-4 cell layers with unevenly thickened cell walls (Figure 3b), and the inner 6-8 layers in direct contact with the gall chamber have polyhedral cells with thin non-lignified and sinuous walls (Figure 3c-d). The whole typical nutritive tissue accumulates lipophilic substances (Figure 3e) and proteins (Figure 3f). The cell wall dissolution and rupturing process (Figure 3g-h) occurs in centripetally concomitant to cytoplasm compartmentalization (Figure 3h-i) (Table 1).

*Lenticular galls*

Lenticular galls (Table 1) have two tissue compartments: an outer tissue compartment and an inner tissue compartment (Figure 4a). The inner compartment is adjacent to the larval chamber with 7-12 layers of typical nutritive cells, which accumulate proteins (Figure 4c), lipids (Figure 4d), and reducing sugars (Figure 4e). The nutritive cells have thin walls, and multivesicular bodies associated with the plasma membrane. The cytoplasm is dense with numerous mitochondria (Figure 4f), many vesicles, ribosomes, smooth and rough endoplasmic reticulum, and Golgi apparatus (Figure 4g-h).

**Discussion**

Three distinct pathways are activated in the three gall morphotypes toward providing lipids, carbohydrates, and proteins to the nutrition of the associated galling herbivores. In the ambrosia globoid galls, nutrients availability occurs through cell wall degradation, mediated by the fungi activity. In the fusiform galls, the galling insect accesses the nutrients overcoming the thickened cell walls, due to the triggering of host plant hypersensitive responses, dissolution of cell walls, and cell death. In the lenticular galls, high cellular metabolism maintenance results in the accumulation of nutrients directed to the galling herbivore feeding.

In the ambrosia globoid galls, the fungal hyphae do not inhibit the formation of a nutritive-like tissue, which is defined as the indirect feeding site of the galling herbivore. Nutritive like-cells have evident and granular cytoplasm, prominent nucleus and accumulate proteins, lipids, and sugars (Ferreira et al. 2017). The fungi alters the cytological features of the nutritive like-tissue on *I. ingoides* and contribute to the loss of cell integrity via degradation of the host plant cell walls, which together with the pyknotic nuclei indicate cell death process (Oliveira et al. 2010, 2011, Van Door et al. 2011). Cell wall degradation is mediated by the enzymes produced by the fungi during its penetration into plant organs (Sillo et al. 2016, Heller 2019). By such process, the fungi hyphae obtain substrate for their growth (Isaac 1991), facilitate the access of the Cecidomyiidae to the protoplast and to the carbohydrates, lipids and proteins of its host cells, and serve as food to the galling larva.

In a relationship with two organisms, cell death processes can also be present (Oliveira et al. 2010), although it is not so common (Vechhi et al. 2005; Isaias et al. 2018; Ferreira et al. 2020). Both the fusiform and the lenticular galls involve just two organisms, but in the fusiform galls, the galling herbivore feeding activity results in loss of cell wall integrity and shrinkage of the cytoplasm, which are symptoms of necrosis-type cell death, activated as a hypersensitive response (Pennel and Lamb, 1997; van Doorn and Woltering 2005; van Doorn et al. 2011). This cytological process permits the galling insect to access the lipids and proteins accumulated in nutritive cells, as well as cellulose and hemicelluloses (Bragança et al. 2020), as additional carbohydrate reserves for its nutrition.

The maintenance of continuous metabolic activity (Bronner 1992) is observed in the typical nutritive tissue of the lenticular galls. The conspicuous endoplasmic reticulum, Golgi apparatus, and many vesicles reveal a system for recycling and remodeling of cell wall components (Barnes and Anderson 2018). In *I. ingoides*, the dynamics of cell wall components indicates the support for intense cell restructuring, and the accumulation of xyloglucans works out as a carbohydrate source to the galling insects (Bragança et al. 2020).

The mitochondria and ribosomes relate to a high cell respiratory activity and protein synthesis, which are crucial for cell division and growth (Fahn 1988, Evert 2006) along gall developmental processes. The endoplasmic reticulum actively synthesizes proteins and the trans-Golgi network relates to the granulocrine or endocytic pathways of the proteins and also of the lipophilic substances (Fahn 2000, Cui et al. 2016, 2018) through plasmodesmata toward the nutritive cells, as also observed in galls induced by *Leptocybe invasa* (Hymenoptera: Eulophidae) on *Eucalyptus camaldulensis* (Myrtaceae) (Isaias et al. 2018). The multivesicular bodies work out in mechanisms of repairing cell membranes in response to intense oxidative stress (Carneiro and Isaias 2015; Ferreira et al. 2015), estimated by the intense accumulation of ROS in the nutritive tissues of galls on *I. ingoides* (Bragança et al. 2020). Also, the multivesicular bodies can be associated with the granulocrine or endocytic pathways (Fahn 2000; Cui et al. 2016, 2018), which assist in the transport of lipids and proteins in the well-developed nutritive tissue The endoplasmic reticulum, trans-Golgi network restructuring, and the multivesicular bodies support the viability of the nutritive tissue of the lenticular galls on *I. ingoide*s, which does not have symptoms of necrosis-type cell death. The maintenance of the active metabolism due to the energy demand increase the sink strength toward nutritive cells, and, in consequence, to the accumulation of carbohydrates, lipids and proteins related to the galling herbivore maintenance (Oliveira et al. 2011, Castro et al. 2012).

**Final considerations**

The cytological traits of the three gall morphotypes on *I. ingoides* relate to the associated organisms and to distinct pathways triggered toward the mobilization of primary metabolites in nutritive tissues. The presence of a third organism in the ambrosia globoid gall impacts its cytological profile, but does not imply in an exclusive cytological profile regarding the fusiform and the lenticular galls. Three different strategies for making food resources available to the galling Cecydomyiidae are triggered: a) cell wall dissolution by fungal intermediation; b) necrosis-type cell death; and c) maintenance of continuous metabolic activity.

**References**

Barnes WJ, Anderson CT (2018) Release, recycle, rebuild: cell-Wall remodeling, autodegradation, and sugar salvage for new wall biosynthesis during plant development. Mol Plant 11: 31–46. https://doi.org/10.1016/ j.molp.2017.08.011

Bragança GP, Oliveira DC, Isaias RMS (2017) Compartmentalization of metabolites and enzymatic mediation in nutritive cells of Cecidomyiidae galls on *Piper arboreum* Aubl. (Piperaceae). J Plant Stud 6:11. https://doi.org/10.5539/jps.v6n1p11

Bragança GP, Alencar CF, Freitas, MSC, Isaias RMS (2020) The dynamics of hemicelluloses-cellulose microfibrils and cell elongation axes determine gall morphotypes on *Inga ingoides* (Fabaceae). Plant Biology. https://doi.org/10.1111/plb.13151

Bronner R (1992) The role of nutritive cells in the nutrition of Cynipids and Cecidomyiids. In: Shorthouse JD, Rohfritsch O (eds) Biology of insect-induced galls. Oxford University Press, New York, pp 118–140

Brundett MC, Kendrick B, Peterson CA (1991) Efficient lipid staining in plant material with sudan red 7B or fluorol yellow 088 in polyethylene glycol-glycerol. Biotech Histochem 66: 111–116. https://doi.org/10.3109/10520299109110562

Bukatsch F (1972) Bermerkungenzur Doppelfärbung Astrablau-Safranin. Mikrokosmos 61:255

Carneiro RG, Isaias, RMS (2015) Cytological cycles and fates in *Psidium myrtoides* are altered towards new cell metabolism and functionalities by the galling activity of *Nothotrioza myrtoidis.* Protoplasma 252:637–646. https://doi.org/10.1007/s00709-014-0709-x

Castro AC, Oliveira DC, Moreira ASFP, Lemos-Filho JP, Isaias RMS (2012) Source– sink relationship and photosynthesis in the horn-shaped gall and its host plant *Copaifera langsdorffii* Desf. (Fabaceae). S Afr J Bot 83:121–126. https://doi.org/10.1016/j.sajb.2012.08.007

Cui Y, Shen J, Gao C, Zhuang X, Wang J, Jiang L (2016). Biogenesis of plant prevacuolar multivesicular bodies. Mol Plant. https://doi.org/10.1016/j.molp.2016.01.011

Cui Y, He Y, Cao W, Gao J, Jiang L. (2018). The multivesicular body and autophagosome pathways in plants. Front Plant Sci 9: 1837. https://doi.org/10.3389/fpls.2018.01837

Evert RF (2006) Esau's plant anatomy: meristems, cells, and tissues of the plant body: their structure, function and development, 3rd edn. John Wiley & Sons Inc, Hoboken

Fanh A (1988) Secretory tissues in vascular plants. New physiology 3: 229–257. https://doi.org/10.1111/j.1469-8137.1988.tb04159.x

Fahn A (2000) Structure and Function of Secretory Cells. In: Hallahan DL, Gray JC, Callow JA (eds) Advances in botanical research, incorporating advances in plant pathology. Academic Press, London, pp 37-66

Fernandes GW, Fagundes M, Woodman RL, Price PW (1999) Ant effects on three-trophic level interactions: plant, galls, and parasitoids. Ecol Entomol 24: 411–415

Ferreira BG, Carneiro RGS, Isaias RMS (2015) Multivesicular bodies differentiate exclusively in nutritive fast-dividing cells in *Marcetia taxifolia* galls. Protoplasma 252:1275–1283. https://doi.org/10.1007/s00709-015-0759-8

Ferreira BG, Álvarez R, Avritzer SC, Isaias RMS (2017) Revisiting the histological patterns of storage tissues: Beyond the limits of gall-inducing taxa. Bot 95: 173–184. https://doi.org/10.1139/cjb-2016-0189

Ferreira BG, Bragança GP, Isaias RMS (2020) Cytological attributes of storage tissues in nematode and eriophyid galls: pectin and hemicellulose functional insights. Protoplasma 257: 229–244. https://doi.org/10.1007/s00709-019-01431-w

Gagné RJ (1994) The Gall Midges of the Neotropical Region. Cornell Univ. Press, Ithaca.

Heller A (2019) Host-parasite interaction during subepidermal sporulation and pustule opening in rust fungi (Pucciniales). Protoplasma 257: 783–792. https://doi.org/10.1007/s00709-019-01461-4

Harris MO, Freeman TP, Rohfritsch O, Anderson KG, Payne SA (2006) Virulent Hessian Fly (Diptera: Cecidomyiidae) larvae induce a nutritive tissue during compatible interaction with wheat. Ann Entomol Soc Am 99: 305–316. https://doi.org/10.1603/0013-8746(2006)099[0305:VHFDCL]2.0.CO;2

Isaac S (1991) Fungal – Plant interactions. Chapman and Hall. London, UK

Isaias RMS, Ferreira BG, Alvarenga DR, Barbosa LR, Salminen J, Steinbauer MJ (2018) Functional compartmentalization of nutrients and phenolics in the tissues of galls induced by *Leptocybe invasa* (Hymenoptera: Eulophidae) on *Eucalyptus camaldulensis* (Myrtaceae). Aust Entomol 57:238–246. https://doi.org/10.1111/aen.12336

Johansen D A (1940). Plant microtechnique. McGraw-Hill, New York

Karnovsky MJ (1965) A formaldehyde-glutaraldehyde fixative of high osmolarity for use in electron microscopy. J Cell Sci 27:137–138

Kraus JE, Arduin M (1997) Manual básico de métodos em morfologia vegetal. Editora da Universidade Federal Rural do Rio de Janeiro, Seropédica

Luft JH (1961) Improvements in epoxy resin embedding methods. J Biophys Biochem Cytol 9: 404–414. https://doi.org/10.1083/jcb.9.2.409

Luz FA, Mendonça Júnior MS (2019) Guilds in Insect Galls: Who is Who. BioOne (1): 207–210. https://doi.org/10.1653/024.102.0133

Mazia D, Brewer PA, Alfert M (1953) The cytochemistry staining and measurement of protein with mercuric bromophenol blue. Biol Bull 104: 57–67. https://doi.org/10.2307/1538691

Miller DG, Raman A (2018) Host–plant relations of gall-inducing insects. Ann Entomol Soc Am 112:1–19. https://doi.org/10.1093/aesa/say034

O'Brien TP, McCully ME (1981) The study of plant structure: principles and selected methods. Termacarphi Pty Ltd, Melbourne

Oliveira DC, Magalhães, TA, Carneiro RGS, Alvin MN, Isaias RMS (2010) Do Cecidomyiidae galls of *Aspidosperma spruceanum* (Apocynaceae) fit the pre-established cytological and histochemical patterns? Protoplasma 242: 81–93. https://doi.org/10.1007/s00709-010-0128-6

Oliveira DC, Carneiro RGS, Magalhães TA, Isaias RMS (2011) Cytological and histochemical gradients on two *Copaifera langsdorffii* Desf (Fabaceae) Cecidomyiidae gall systems. Protoplasma 248: 829–837. https://doi.org/10.1007/s00709-010-0258-x

Paiva JGA, Fank-de-Carvalho SM, Magalhães MP, Graciano-Ribeiro D (2006) Verniz vitral incolor 500®: uma alternativa de meio de montagem economicamente viável. Acta Bot Bras 20:257–264. http://dx.doi.org/10.1590/S0102-33062006000200002

Pan LY, Chen WN, Chiu ST, Raman A, Chiang TC, Yang MM (2015) Is a gall an extended phenotype of the inducing insect? A comparative study of selected morphological and physiological traits of leaf and stem galls on *Machilus thunbergii* (Lauraceae) induced by five species of *Daphnephila* (Diptera: Cecidomyiidae) in Northeastern Taiwan. Zool Sci 32: 314–321. https://doi.org/10.2108 / zs140244

Pennel R, Lamb C (1997) Plant cell death in plants. The plant cell 9:1157–1168

Raman A, Suryanarayanan TS (2017) Fungus-plant interaction influences plant-feeding insects. Fungal Ecol 29: 123–132. https://doi.org/10.1016/j.funeco.2017.06.004

Reynolds ES (1963) The use of lead citrate at high pH as an electron-opaque stain in electron microscopy. J Cell Biol 17: 208–212. https://doi.org/10.1083/jcb.17.1.208

Rezende UC, Custódio JF, Kuster VC, Gonçalves LA, Oliveira DC (2019) How theactivity of natural enemies changes the structure and metabolism of the nutritive tissue in galls? Evidence from the *Palaeomystella oligophaga* (Lepidoptera)–*Macairea radula* (Metastomataceae) system. Protoplasma 256: 669–667. https://doi.org/10.1007/s00709-018-1321-2

Rohfritsch O (1992) Patterns in gall development. In: Shorthouse JD, Rohfritsch O (eds) Biology of insect-induced galls. Oxford University Press, New York, pp 60–86

Rohfritsch O (2008) Plants, gall midges, and fungi: A three-component system. Entom Exp Appl 128: 208–216. https://doi.org/10.1111/j.1570-7458.2008.00726.x

Sass JE (1951) *Botanical microtechnique*. Iowa State College Press, Ames

Sillo F, Fangel JU, Henrissat B, Faccio A, Bonfante P, Martins F, Willats WGT, Balestrini R (2016) Understanding plant cell-wall remodelling during the symbiotic interaction between *Tuber melanosporum* and *Corylus avellana* using a carbohydrate microarray. Planta 244: 347–359. https://doi.org/10.1007/s00425-016-2507-5

Stone GN, Schonrögge K (2003) The adaptive significance of insect gall morphology. Trends Ecol Evol 18: 512–522. https://doi.org/10.1016/S0169-5347(03)00247-7

Van Doorn WG, Woltering EJ (2005) Many ways to exit? Cell death categories in plants. Trends Plant Sci 10: 117–122. https://doi.org/10.1016/j.tplants.2005.01.006

Van Doorn W, Beers E, Dangl J, Franklin-Tong V, Gallois P, HaraNishimura I, Jones A, Kawai-Yamada M, Lam E, Mundy J (2011) Morphological classification of plant cell deaths. Cell Death Differ 18: 1241–1246. https://doi.org/10.1038/cdd.2011.36

Vecchi C, Menezes NL, Oliveira DC, Ferreira BG, Isaias RMS (2013) The redifferentiation of nutritive cells in galls induced by Lepidoptera on *Tibouchina pulchra* (Cham.) Cogn. reveals predefined patterns of plant development. Protoplasma 250: 1363–1368. https://10.1007 / s00709-013-0519-6

Weis AE (1982) Resource utilization patterns in a community of gall-attacking parasitoids. Environmental Entomology 11: 809–815. https://doi.org/10.1093/ee/11.4.809

Yukawa J, Rohfritsch O (2005) Biology and ecology of gall inducing Cecidomyiidae (Diptera). In: Raman A, Schaefer CW, Withers TW (eds) Biology, ecology, and evolution of gall-inducing Arthropods. Science Publishers, Enfield, pp 273–304

**Figure captions**

**Fig 1 Galls on *Inga ingoides* leaflets (Fabaceae: Caesalpinioideae): a)** Ambrosia globoid gall **b)** Fusiform gall. **c)** Lenticular gall

**Fig 2 Cytological and histochemical features of the globoid galls on *Inga ingoides* (Rich.) Willd. (Fabaceae). a)** Diagram of transverse section of mature gall, showing the outer tissue compartment (OC), nutritive like-tissue (NLT) in a mature gall, and larval chamber (LC). **b)** Transverse anatomical section of the nutritive-like tissue (NLT). **c-e)** Histochemical tests of the nutritive-like tissue evidencing: **c)** proteins; **d)**

lipids; **e)** reducing sugars. **f-i)** Cytological features of nutritive like-tissue. **f-g)** Gall cells. **f)** Cell wall rupture (red arrow) and degradation of the nuclei (Nu), many vesicles (white arrow), and mitochondria (Mi). **g)** Cells close to the lignified layers with vesicle (white arrow) and chloroplast (Ch). Communication between cells with several plasmodesmata (red arrow), mitochondria (Mi) and prolamellar bodies (Pb). **h-i)** Fungi cells. **h)** Cells with many vesicles (white arrows), and mitochondria (Mi). **i)** Dissolution of cell wall (red square) and multivesicular bodies (white arrows)

**Fig 3 Cytological and histochemical features of the fusiform galls on *Inga ingoides* (Rich.) Willd. (Fabaceae). a)** Diagram of transverse section of mature gall, showing the outer tissue compartment (OC), fibers (Fi), Ph (phloem), cambium zone (CZ), and xylem (Xy), typical nutritive tissue (TNT) and larval chamber (LC) in a mature gall. **b-d)** Transverse anatomical section of typical nutritive tissue (TNT). **e-f)** Histochemical tests of the typical nutritive tissue evidencing: **e)** lipids; and **f)** proteins. **g-i)** Cytological features of typical nutritive tissue. **g)** Cell with irregular thickened walls (CW) with oleosomes (Ol). **h)** Region closest to the larval chamber with cell wall dissolution (black arrows), and ruptured cell walls (red arrow), shrunk cytoplasm accumulating lipophilic substances. **i)** Detail of shrunk cytoplasm with oleosomes (ol) and vesicles (arrow)

**Fig 4 Cytological and histochemical features of the lenticular galls *Inga ingoides* (Rich.) Willd. (Fabaceae). a)** Diagram of transverse section of mature gall, showing the outer tissue compartment subdivided in lower tissue compartment (LOC) and upper tissue compartment (UOC) and typical nutritive tissue (TNT) and larval chamber (LC). **b-d)** Transverse anatomical section of typical nutritive tissue (TNT). **c-e)** Histochemical tests of typical nutritive tissue evidencing: **c)** proteins; **d)** lipids; and **e)** reducing sugars. **f-h)** Cytological features of the typical nutritive tissue. **f)** Cells with multivesicular bodies (red arrow), many vesicles (yellow arrows), and mitochondria (Mi). **g-h)** Rough endoplasmic reticulum (RER) and the Golgi apparatus (red dotted circle) with many vesicles (yellow arrows)

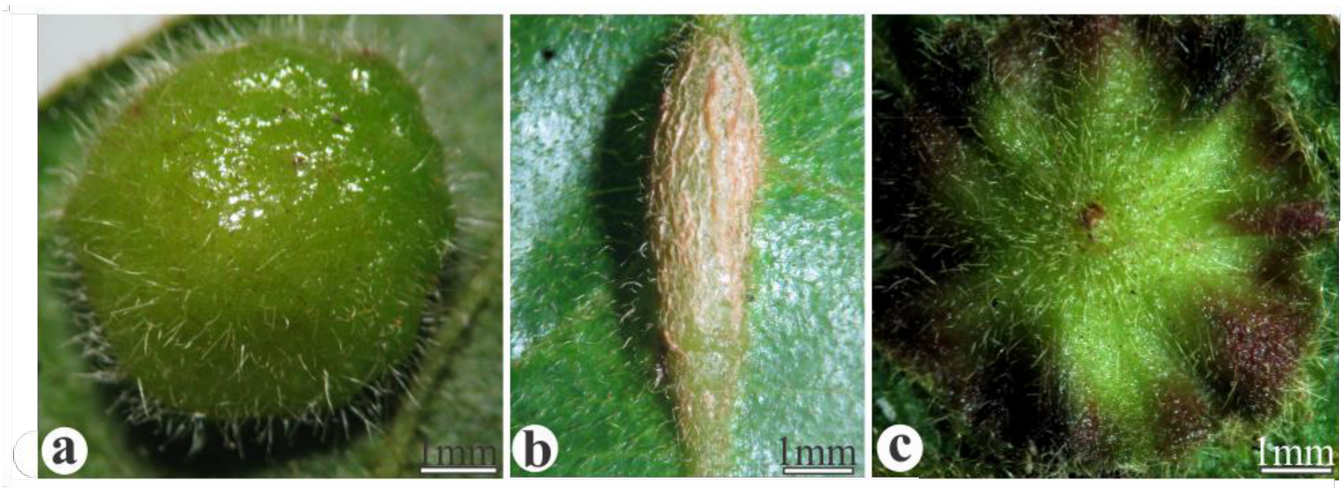



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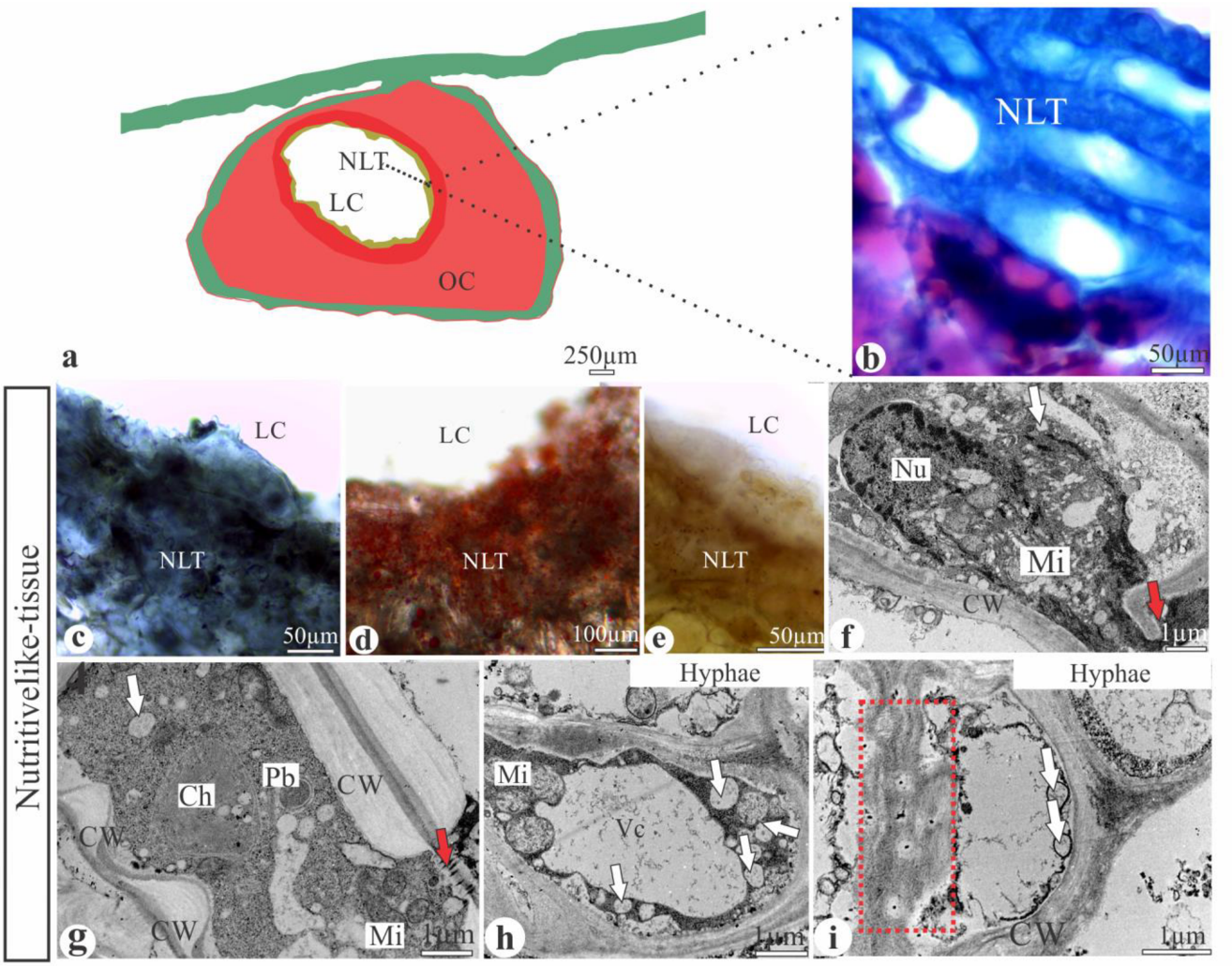



**Fig. 2 Cytological and histochemical features of the globoid galls on *Inga ingoides* (Rich.) Willd. (Fabaceae). a)** Diagram of transverse section of mature gall, showing the outer tissue compartment (OC), nutritive like-tissue (NLT) in a mature gall, and larval chamber (LC). **b)** Transverse anatomical section of the nutritive-like tissue (NLT). **c-e)** Histochemical tests of the nutritive-like tissue evidencing: **c)** proteins; **d**) lipids; **e)** reducing sugars. **f-i)** Cytological features of nutritive like-tissue. **f-g)** Gall cells. **f)** Cell wall rupture (red arrow) and degradation of the nuclei (Nu), many vesicles (white arrow), and mitochondria (Mi). **g)** Cells close to the lignified layers with vesicle (white arrow) and chloroplast (Ch). Communication between cells with several plasmodesmata (red arrow), mitochondria (Mi) and prolamellar bodies (Pb). **h-i)** Fungi cells. **h)** Cells with many vesicles (white arrows), and mitochondria (Mi). **i)** Dissolution of cell wall (red square) and multivesicular bodies (white arrows)

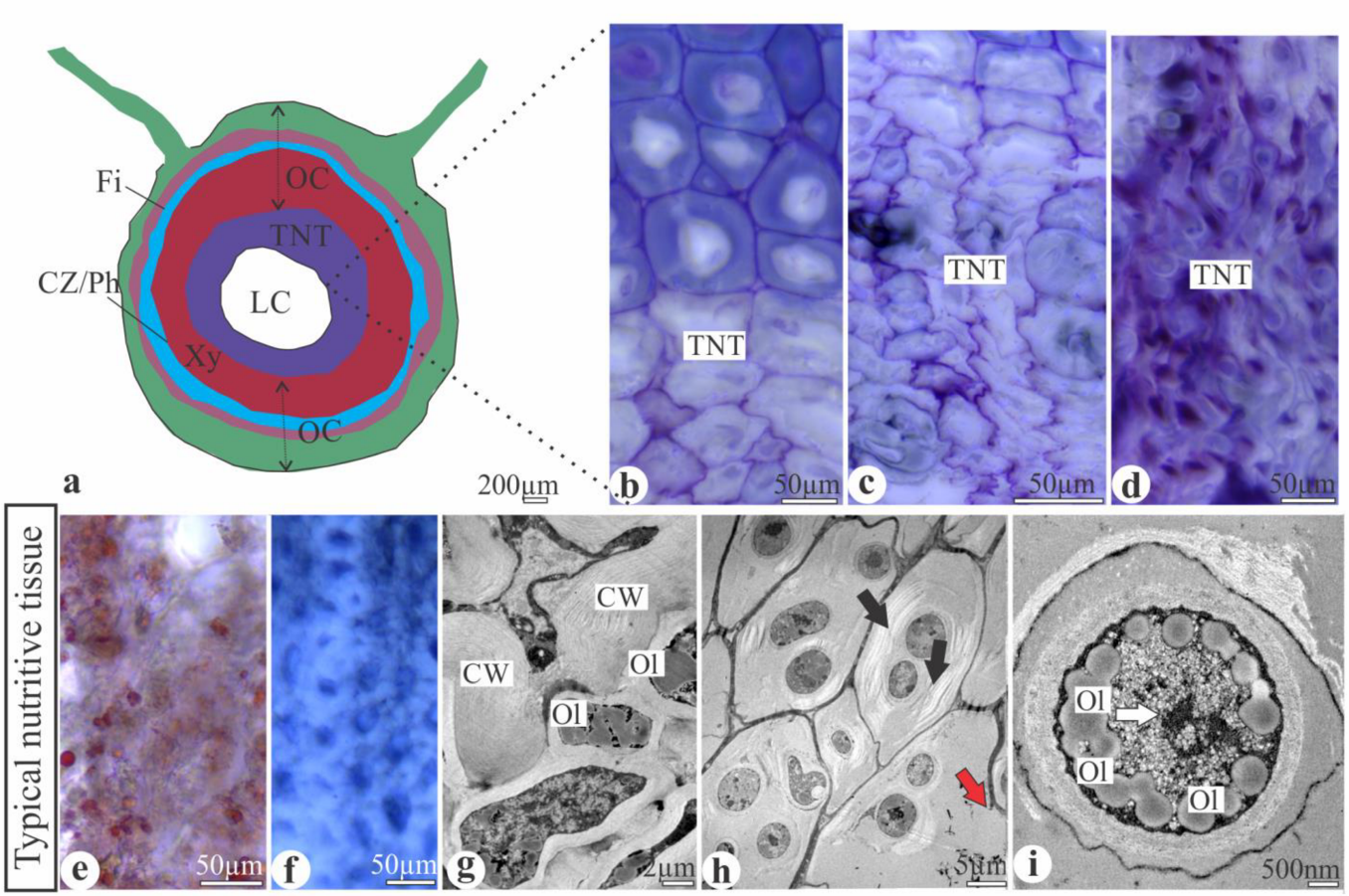



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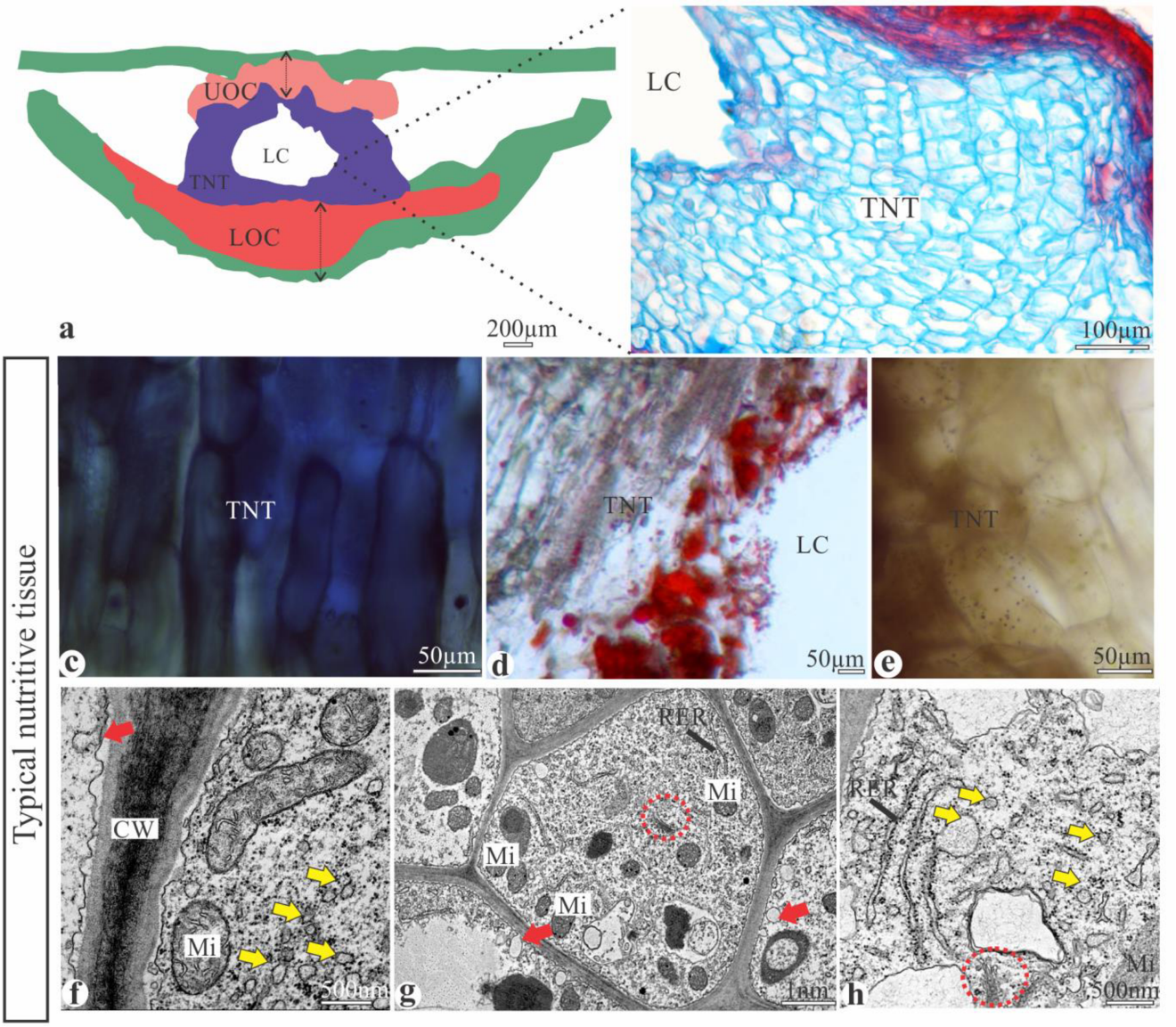



**Fig. 4 Cytological and histochemical features of the lenticular galls *Inga ingoides* (Rich.) Willd. (Fabaceae). a)** Diagram of transverse section of mature gall, showing the outer tissue compartment subdivided in lower tissue compartment (LOC) and upper tissue compartment (UOC) and typical nutritive tissue (TNT) and larval chamber (LC). **b-d)** Transverse anatomical section of typical nutritive tissue (TNT). **c-e)** Histochemical tests of typical nutritive tissue evidencing: **c)** proteins; **d)** lipids; and **e)** reducing sugars. **f-h)** Cytological features of the typical nutritive tissue. **f)** Cells with multivesicular bodies (red arrow), many vesicles (yellow arrows), and mitochondria (Mi). **g-h)** Rough endoplasmic reticulum (RER) and the Golgi apparatus (red dotted circle) with many vesicles (yellow arrows)

**Table 1:** Metabolites accumulation and cytological features in galls on *I. Ingoides* (Rich.) Willd. (Fabaceae)

| | Globoid gall | Fusiform gall | Lenticular gall |
|---|---|---|---|
| **Prymary metabolites** | | | |
| Lipids | + | + | + |
| Proteins | + | + | + |
| Reducing sugar | + | - | + |
| Starch | + | + | + |
| | | | |
| **Cytological features** | | | |
| Alteration in cell wall | + | + | - |
| Chloroplasts | + | - | + |
| Cytoplasm granulation | - | - | + |
| Endoplasmic reticulum | - | - | + |
| Golgi apparatus | - | - | + |
| Large vacuole | + | - | + |
| Mitochondria | + | - | + |
| Multivesicular bodies | + | - | + |
| Nuclei degradation | + | + | - |
| Polylamellated cell walls | + | + | + |
| Proeminent nucleous and nucleoli | - | - | + |
| Prolamellar bodies | + | - | - |
| Oleosomes | - | + | - |
| Shrunk cytoplasm | - | + | - |
| Vesicles | + | + | + |

**Note:** Histochemistry results (Reserves) and cytological features: –, absent; +, present

## CONSIDERAÇÕES FINAIS

As galhas globoides, fusiformes e lenticulares, produto dos sistemas *I. ingoides*-Cecidomyiidae, são estruturalmente complexas, segundo os atributos descritos por Ferreira et al. (2019), sendo que as galhas globoides apresentam, comparativamente, um nível de complexidade maior do que as galhas fusiformes e lenticulares (Figura 01). A diferença de complexidade dos três morfotipos de galhas corresponde ao microsítio de indução nos folíolos, ou seja, a região internervural ou as nervuras, ao potencial morfogenético das células vegetais que originam os compartimentos teciduais das galhas e aos organismos envolvidos em cada morfotipo. As peculiaridades teciduais resultam em mecanismos compensatórios desenvolvidos no sistema vascular de modo a garantir a condutividade hidráulica. A variação dos padrões de expansão celular e a dinâmica das fibrilas de celulose-hemiceluloses no sistema fundamental corresponde ao desenvolvimento de cada um dos três morfotipos. Por fim, vias citológicas peculiares do tecido nutritivo de cada galha garantem a liberação de recursos nutricionais para os galhadores por três vias distintas.

As estratégias de cada galha para garantir a condutividade hidráulica está relacionada à posição da galha intra- ou extralaminar, à continuidade de seus tecidos com aqueles do órgão hospedeiro e à origem dos tecidos vasculares, que ocorre pela rediferenciação de células parenquimáticas, ou por diferenciação de células procambiais ou cambiais. A garantia da condutividade hidráulica nas galhas tem papel relevante para a manutenção do aporte hídrico e da pressão de turgor necessários para as modificações nos padrões de expansão celular em cada um dos compartimentos teciduais das galhas. A mudança nos eixos de alongamento celular e as variações na composição da parede celular, especialmente relativas à dinâmica das hemiceluloses foram peculiares durante o desenvolvimento de cada um dos três morfotipos de galhas, exceto

para o acúmulo de xiloglucanos nas paredes das células nutritivas, revelado como um novo atributo funcional comum entre as galhas em *I. ingoides*. Os componentes nutritivos usuais acumulados no citoplasma das células nutritivas, ou seja, lipídios, proteínas e carboidratos, somados aos xiloglucanos das paredes celulares foram acessados pelos galhadores por meio de estratégicas citológicas distintas que envolvem a intermediação fúngica nas galhas globóides de ambrosia, a morte celular nas galhas fusiformes e a manutenção do metabolismo celular, nas galhas lenticulares. O estudo dos três sistemas *I. ingoides*-Cecidomyiidae corrobora a hipótese proposta de que em uma superhospedeira de herbívoros galhadores, a origem dos tecidos galhados e a presença de um terceiro organismo são fatores determinantes para a complexidade estrutural de galhas induzidas pelo mesmo taxa galhador. O estudo da vascularização com abordagem histométrica e citométrica foi realizado pela primeira vez em galhas de insetos na região neotropical e conclui que a vasoconstrição em galhas de insetos está associada à forma da galha e ao seu posicionamento no órgão hospedeiro.

Devido ao fechamento dos laboratórios da UFMG, face à necessidade de isolamento social para conter a pandemia da COVID-19, algumas análises não puderam ser incluídas nesta tese, alguns objetivos foram reformulados e parte do texto reescrito. Apesar deste contratempo, os manuscritos/artigos que compõem esta tese versando sobre alterações estruturais que geram os três morfotipos de galhas, peculiaridades do sistema vascular, a parede celular como um novo sítio de acúmulo de nutrientes e vias citológicas ligadas a disponibilização de metabolitos no tecido nutritivo contribuem para o avanço do estado da arte e para manter o Brasil como um polo de produção de conhecimento sobre galhas no mundo contemporâneo.

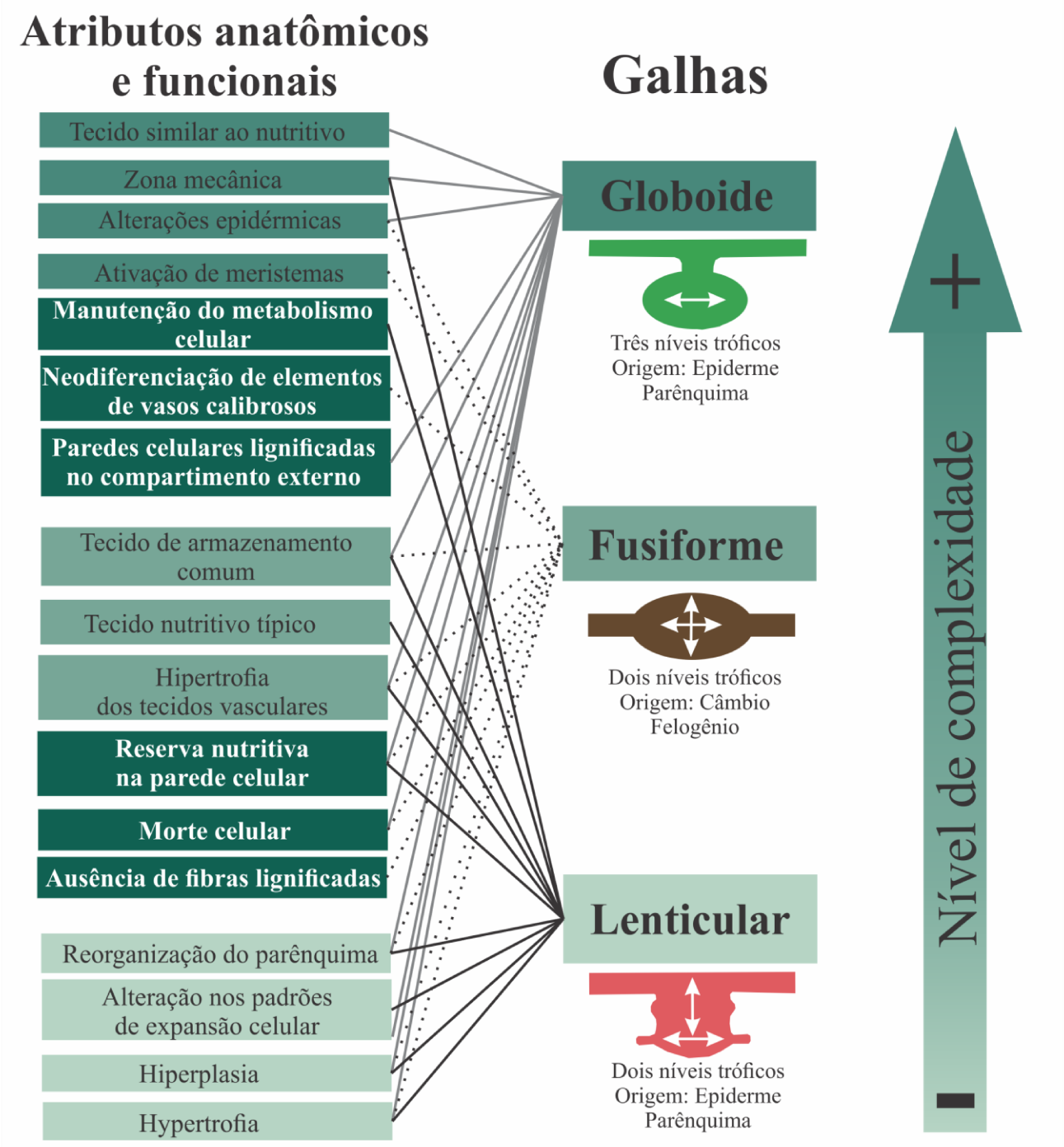



**Figura 01: Atributos anatômicos e funcionais associados a complexidade de galhas em *Inga ingoides* (Fabaceae).** O gradiente de coloração (escuro – claro) indica os atributos estruturais propostos por Ferreira et al. (2019). As caixas verde escuro com texto branco contém os atributos citológicos e vasculares adicionados. Linhas cinzas, tracejadas e contínuas representam respectivamente os atributos anatômicos e funcionais das galhas globoides, fusiformes e lenticulares. A direção da setas brancas indica o eixo de alongamento predominante em cada morfotipo (periclinal, anticlinal ou isodiamétrico) e a seta com gradiente de coloração verde indica o nível crescente de complexidade das galhas.